Catalysis of DNA cleavage and nucleoside triphosphate synthesis by NM23-H2/NDP kinase share an active site that implies a DNA repair function

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NM23/NDP kinases play an important role in development and cancer but their biological function is unknown, despite an intriguing collection of biochemical properties including nucleoside-diphosphate kinase (NDP kinase), DNA binding and transcription, a mutator function, and cleavage of unusually structured DNA by means of a covalent enzyme–DNA complex. To assess the role of the nuclease in human NM23-H2, we sought to identify the amino acid responsible for covalent catalysis. By sequencing a DNA-linked peptide and by site-directed mutagenesis, we identified lysine-12, a phylogenetically conserved residue, as the amino acid forming the covalent complex with DNA. In particular, the ε-amino group acts as the critical nucleophile, because substitution with glutamine but not arginine completely abrogated covalent adduct formation and DNA cleavage, whereas the DNA-binding properties remained intact. These findings and chemical modification data suggest that phosphodiester-bond cleavage occurs by a DNA glycosylase/lyase-like mechanism known as the signature of base excision DNA repair nucleases. Involvement of NM23/NDP kinase in a DNA repair pathway would be consistent with its role in normal and tumor cell development. Additionally, lysine-12, which is known in the x-ray crystallographic structure to lie in the catalytic pocket involved in the NDP kinase phosphorylation reaction, was found essential also for the NDP kinase activity of NM23-H2, suggesting that the two catalytic activities of NM23-H2 are fundamentally connected.

NM23 is a large family of structurally and functionally conserved proteins consisting of four to six identical subunits of 16 to 20 kDa each, known also as NDP kinases (nucleoside diphosphate kinase; EC 2.7.4.6; refs. 1–4). In *Escherichia coli*, the gene for NDP kinase functions as a mutator gene (5), whereas the homologue in *Drosophila*, known as *altered wing discs* (*AWD*), is required for development (6). Multiple NM23/NDP kinase genes exist in vertebrates, including six in humans (*NM23-H1* to *NM23-H8*; ref. 7), where they also play a role in development (8). And, *NM23-H1* and *NM23-H2* have both been implicated in the metastasis (9, 10) and pathogenesis (11, 12) of tumors.

NDP kinases catalyze phosphoryl-group transfer between nucleoside di- and triphosphates through a conserved histidine as the phosphorylated intermediate (1–3), and they play a role in maintaining intracellular nucleotide concentrations. NDP kinases have broad substrate specificities and well understood catalytic mechanisms (13), and several crystal structures, both with and without bound nucleotide substrates, have been solved, including two of human NM23-H2/NDP kinase B (14, 15).

It has generally been assumed, however, that NM23/NDP kinases are far more than housekeeping enzymes, because experiments *in vivo* have indicated the existence of additional mechanisms (16, 17). In 1993, our laboratory reported that NM23-H2/NDP kinase B is a DNA-binding protein identical to the c-*MYC* transcription factor PuF, suggesting that NM23-H2/PuF may regulate gene expression through its DNA-binding activity (18, 19). Site-directed mutational studies subsequently demonstrated that sequence-specific DNA binding and phosphoryl transfer catalyzed by NDP kinase are governed by independent functional domains of the NM23-H2 protein (20, 21).

The c-*MYC* target of NM23-H2/PuF is the −160/101 region of the promoter, a nuclease-hypersensitive polypurine/polypyrimidine sequence termed NHE (22). The NHE consists of several directly repeated motifs and palindromes with the potential to form unusual, non-B-like DNA structures, namely, "slipped" DNA consisting of loops, mismatched bases, and hypercoiled regions (22, 23). We suggested, therefore, that NM23/PuF functions in transcription through recognition of the structural aspects of this target DNA sequence (18, 19, 22, 23). It is now known that in addition to the c-*MYC* gene, NM23/PuF recognizes and activates other target genes containing similar polypurine/polypyrimidine promoter sequences (23).

We have recently shown that the reaction of NM23/PuF with DNA results in a reversible site-specific cleavage of the phosphodiester bond through a covalent protein-DNA intermediate (24). This finding suggested that the biological function of NM23/PuF is recognition, cleavage, and subsequent structural alterations of the target DNA sequence (24). To gain insight into this previously unknown nuclease function, we have sought to identify the critical amino acid that cleaves the phosphodiester bond by transiently forming a covalent bond with DNA. Here we present evidence that the catalytic nucleophile of NM23/PuF is lysine-12. Mutational analysis and chemical modification experiments suggest, moreover, that the DNA-cleaving mechanism entails a nucleophilic attack by the amine of lysine-12 through a Schiff base intermediate, known as the signature mechanism of DNA glycosylase/lyases in the base-excision DNA repair pathway. This finding, in common with much that is known about the biology of NM23/NDP kinase, is consistent with a role for NM23 in DNA repair processes. Because lysine-12 is equally critical for the NDP kinase reaction, NM23 seems to use a single active site both for the cleavage of the DNA phosphodiester backbone and for the phosphorylation of nucleotides.

Materials and Methods

Proteins, Site-Directed Mutagenesis, and DNA Substrates. Proteins were purified by overexpressing plasmids in BL21 (DE3) *E. coli* cells as described (21). Site-directed mutants were generated with the unique site elimination method as before (20, 21). The

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Abbreviation: WT, wild type.

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mutagenic primers were 5′-GCCAACCTGGAGCGCGTCT-TCATCGCCATCAAGCCG for T7V, 5′-CGCACCT-TCATCGCCATCCAGCCGGACGGCGTGGAGCGC for K12Q, and 5′-CGCACCTTCATCGCCATCAGGCCGGACG-GCGTGGAGCGC for K12R, with the mismatches underlined. The entire NM23/PuF amino acid coding region was sequenced to ensure the presence of only the desired mutations. DNA substrates were the pUC19MYC plasmid containing a 57-bp c-*MYC* NHE sequence and a ^{32}P-end-labeled 45-bp duplex NHE oligonucleotide 5′-AGTCTCCTCCCCACCTTCCCCAC-CCTCCCCACCCTCCCCATAAGC, prepared and labeled as described (24).

Analysis of Covalent Protein–DNA Complexes. *With uniformly ^{32}P-labeled plasmid DNA.* Uniformly labeled pUC19MYC plasmid (50 ng, specific activity 1–2 $\times$ 10^8 cpm/μg) was mixed with 4 μg of purified NM23-H2, and incubated in reaction buffer [50 mM Tris·HCl, pH 7.9/100 mM KCl/1.5 mM $MgCl_2$/50 μg/ml BSA/2% (vol/vol) Glycerol] for 30 min at room temperature. The reaction was terminated by heating for 10 min at 80°C, the plasmid DNA was digested with DNase I (Roche) and exonuclease III (Roche Molecular Biochemicals) and treated with EDTA and SDS, and the products were precipitated with 10% (vol/vol) trichloroacetic acid at 0°C. After being rinsed with ethanol, the samples were resuspended in SDS/PAGE sample buffer, boiled for 5 min, and resolved on 4–15% SDS/PAGE gels (24). The gels were stained with Coomassie brilliant blue for 1 h, destained overnight in 20% (vol/vol) acetic acid and 10% (vol/vol) methanol and vacuum dried before photography and exposure to x-ray film.

With 5′-end ^{32}P labeled 45-bp duplex oligonucleotide. Reaction mixtures were assembled in 15 μl of reaction buffer containing 5 ng of radiolabeled DNA and 2.5 μg of NM23/PuF protein (at a molar ratio of 1:50), and were incubated at 0°C for 20 min before termination by heating to 80°C and loading onto 4–15% SDS/PAGE gels. The gels were run and processed as described above.

$NaBH_3CN$ Trapping Analysis. Samples were prepared as described above in 15 μl of reaction buffer containing 5 ng of radiolabeled oligonucleotide and 2.5 μg of NM23/PuF protein (at a molar ratio of 1:50). Reaction mixtures were incubated for 20 min at 0°C, and terminated by heating for 10 min at 80°C. Samples were further treated with 50 mM $NaBH_3CN$ (Aldrich) for 1 h at 37°C, then boiled in SDS/sample buffer and loaded onto 4–15% SDS/PAGE gels.

DNA Cleavage Analysis. *Cleavage of supercoiled plasmid DNA.* Reaction mixtures (10 μl) were assembled in reaction buffer with a negatively supercoiled pUC19MYC plasmid and NM23/PuF protein as indicated in the legend in Fig. 5. After incubation for 30 min at 30°C, the reactions were terminated with 2% (vol/vol) Sarkosyl and 10 mM EDTA, and the mixtures were treated further with proteinase K (200 μg/ml) for 1 h at 55°C before resolving on 1% agarose gels (24).

Cleavage of 5′-end ^{32}P-labeled oligodeoxyribonucleotide duplex DNA. Reaction mixtures were assembled in 14 μl of reaction buffer with the indicated amounts of radiolabeled oligonucleotide and NM23/PuF protein, incubated at 0°C for 20 min, and analyzed by electrophoretic mobility-shift assay (24).

Two-Dimensional SDS/PAGE Purification of a Peptide–DNA Complex. A reaction (as described above in *Cleavage of supercoiled plasmid DNA*) was scaled up to 20 μg of wild-type (WT) NM23/PuF and 2 μg of uniformly ^{32}P-labeled plasmid DNA. After heat treatment, DNase I digestion, and trichloroacetic acid precipitation, the ^{32}P-labeled NM23/PuF complex was separated on a 5–10% SDS/PAGE gel (24) as shown in Fig. 1. Next, the radioactive

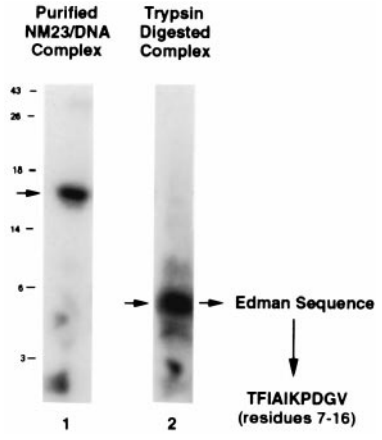

Fig. 1. Purification of a DNA-linked peptide by two-dimensional SDS/PAGE and determination of its amino acid sequence. The figure shows an autoradiogram of the Western blot after purification in the first dimension (lane 1), and after trypsin digestion and electrophoresis in the second dimension (lane 2). The sequence of the radioactive peptide (lane 2) was determined directly from the membrane by Edman degradation.

complex was excised from the gel, and treated according to the process described by Williams and Stone (25) with cold 95% (vol/vol) acetone. The dried gel slices were resuspended in 0.1 M NH_4HCO_3 and extracted for 20 h at 20°C with three changes of the eluting buffer. The eluates were pooled, precipitated with acetone, and resuspended in 0.1 M NH_4HCO_3. After reduction and alkylation, the sample was digested with 2 μg of trypsin (Promega) for 24 h, freeze dried, resuspended in sample buffer, and loaded onto a 10 $\times$ 15-cm high-resolution three component (4%/10%/16.5%) SDS/PAGE/Tricine gel (26). The resolved peptides were transferred onto polyvinylidene difluoride membrane and exposed to XAR film (Kodak), and the radioactive spot was excised and subjected to Edman amino acid sequencing on a model 47A Applied Biosystems automated sequencer in the Princeton University Molecular Biology Core Facility.

NDP Kinase Assay. NDP kinase activity was measured in a coupled-enzyme assay with ATP as the phosphate donor and dTDP as the acceptor nucleotide, by using the pyruvate kinase–lactate dehydrogenase method that measures ADP formation from ATP (20). The specific activity of WT protein (average of three independent enzyme preparations) was 620 units/mg.

Results

Isolation of a DNA-Bound Peptide After Two-Dimensional SDS/PAGE and Determination of Its Amino Acid Sequence. To identify the amino acid covalently bound to DNA, we isolated a DNA-bound tryptic peptide by two-dimensional SDS/PAGE and subjected it to Edman amino acid sequencing (Fig. 1). Ten cycles provided the readily assignable major sequence TFIAIKPDGV, corresponding to the second tryptic peptide from amino acid positions 7–16 of the full-length 152-amino acid NM23-H2/PuF sequence. The sequence continued past lysine-12 in the middle of the peptide, presumably because trypsin cleaves inefficiently between lysine and proline residues (27). There was no "blank" at any of the amino acid positions that might have signified a covalently attached residue, suggesting that although heat-stable, acid precipitable, and resistant to boiling in SDS, the amino acid–DNA linkage was nevertheless sensitive to the extreme pH conditions of the sequencing reactions. Because the identical peptide was obtained by an entirely different procedure (purification of a protein–DNA complex by electrophoresis in native acrylamide gels followed by SDS treatment, trypsin

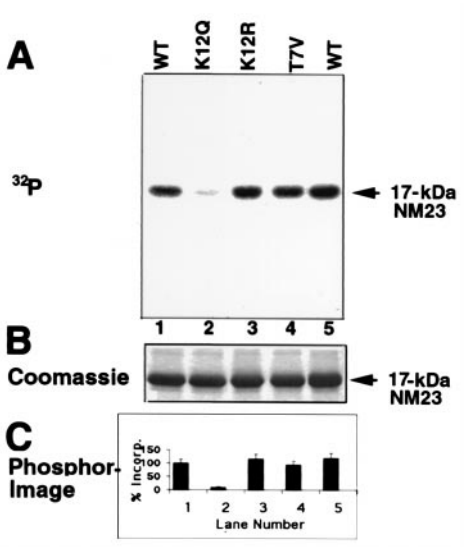

Fig. 2. SDS/PAGE analysis of covalent protein–DNA complexes formed between uniformly ^{32}P-labeled plasmid DNA containing the c-*MYC* NHE (PUC19MYC), and site-directed mutants of NM23/PuF. (*A*) Autoradiogram of dried down gel showing ^{32}P label stably associated with the 17-kDa NM23/PuF peptides. The positions of the 17-kDa NM23/PuF peptides are indicated on the right side. (*B*) Photograph of the same gel stained with Coomassie brilliant blue. (*C*) PhosphorImager (Molecular Dynamics) quantification of incorporated ^{32}P products expressed as percentage relative to WT in lane 1, after scanning and data analysis by using IMAGE QUANT software. The lanes are identical to those of *A*.

digestion, and separation of the peptides by reverse-phase HPLC; data not shown), we concluded that the peptide TFIAIKPDGV was covalently attached to DNA.

Site-Directed Mutagenesis of NM23/PuF Identifies Lysine-12 as the Nucleophile Forming the Covalent Protein–DNA Complex. To identify the amino acid on the DNA-linked peptide that is involved in the covalent binding, we constructed site-directed mutants of residues that have the potential to serve as nucleophiles and form covalent bonds with DNA. We thus mutated threonine-7 (to valine, T7V), because of the possible involvement of the threonine hydroxyl in the nucleophilic attack, and lysine-12, because the ε-amino group is known as a highly reactive nucleophile also capable of forming a covalent adduct. Thus, lysine-12 was replaced conservatively by arginine (K12R), thereby preserving a basic group, and nonconservatively by glutamine (K12Q), which has an unreactive amide.

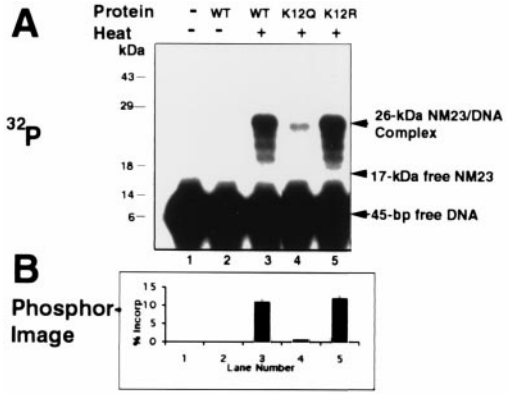

Fig. 3. SDS/PAGE analysis of covalent protein–DNA complexes formed between ^{32}P-end-labeled 45-bp duplex oligonucleotide and site-directed mutants of NM23/PuF. (*A*) Autoradiogram of dried gel. Positions of the free 45-bp oligonucleotide substrate, containing the c-*MYC* NHE element, the 17-kDa free, and 26-kDa complexed NM23/PuF peptides are indicated on the right side. The left side shows migration of molecular mass standards. (*B*) PhosphorImager quantification of ^{32}P products expressed as percentage of incorporated radioactivity in the sample, after scanning and data analysis using IMAGE QUANT software. The lanes are identical to those of *A*.

The ability of the mutant proteins to bind DNA irreversibly was assessed by denaturation with heat (80°C) or with SDS, a treatment that traps the enzyme and the DNA in a "dead-end" irreversibly bound complex (24). We have shown previously that the WT protein can retain a significant fraction of the radioactivity from a reaction with uniformly ^{32}P-radiolabeled supercoiled substrate DNA containing the c-*MYC* NHE (pUC19MYC) in an acid-precipitable form, after denaturation and DNase treatment of the protein–DNA complex. Stability of such complexes after SDS/PAGE can be taken as evidence for covalent-bond formation (Fig. 1). As shown again here, the WT protein (Fig. 2*A*, lanes 1 and 5) readily formed the stable complex, as did the T7V mutant (lane 4), whereas the glutamine-substituted lysine-12 (K12Q) retained only a fraction of the radioactivity (lane 2). This result suggests that lysine-12 is the residue on this peptide that forms the covalent contact with DNA. Replacement of lysine-12 with arginine (K12R) had no effect on complex formation (lane 3). Because the K12Q mutant lacks the ε-amino group of lysine-12 and is unable to form a complex, this amine is therefore presumed to be critical in the nucleophilic attack.

A heat-stable complex also formed between WT NM23/PuF and a ^{32}P-end-labeled duplex DNA oligonucleotide containing the c-*MYC* NHE. Complex formation resulted in an increase in the apparent molecular mass of the protein in SDS/PAGE gels from 17 kDa (monomeric NM23/PuF alone) to ≈26 kDa (mass of the shifted complex containing DNA; Fig. 3*A*). Again, lysine-12 could be replaced by arginine (K12R) with full retention of the complex-forming ability (lane 5), whereas replacement with glutamine (K12Q) greatly impaired formation of the heat-trapped complex (lane 4).

Cleavage of DNA involving the amino group of a lysine side chain or the α-amino group at the N terminus of the protein in a transiently covalent reaction is the signature mechanism of DNA glycosylase/lyases in the base excision DNA repair pathway (28–30). The reaction entails an attack of the glycosylic bond of deoxyribose and formation of a transiently covalent imino enzyme–DNA complex (a covalent Schiff base intermediate) between the ε-NH_2 group of a lysine and the C1′ of the ribose, followed by phosphodiester bond cleavage by β-elimination of the phosphate. Because of the clear requirement for the ε-amino group of lysine-12 in the formation of the covalent complex between NM23 and DNA, the intermediate formed in this case presumably is a Schiff base also. A Schiff base would still form between DNA and an arginine-replaced mutant (K12R), because arginine retains a nitrogen atom with an unshared electron pair, whereas with the neutral glutamine side chain (K12Q), the reaction would not be chemically feasible.

Sodium Cyanoborohydride Reduction as a Mechanistic Probe for Imino Enzyme-Substrate Intermediates. We used sodium cyanoborohydride ($NaBH_3CN$) reduction to confirm Schiff-base formation as an imino enzyme-substrate intermediate. "Borohydride trapping," as it is known, is considered a definitive test for enzymes using a lysine for the formation of a covalent Schiff-base intermediate, and as evidence, therefore, for a DNA glycosylase/lyase activity (29). Because the reaction with borohydride is irreversible, it results in a DNA–enzyme crosslinked product that can be detected in SDS/PAGE gels as a retarded band.

Clearly, the reaction of a basic nitrogen atom at position 12 does occur with borohydride, because both the WT and the

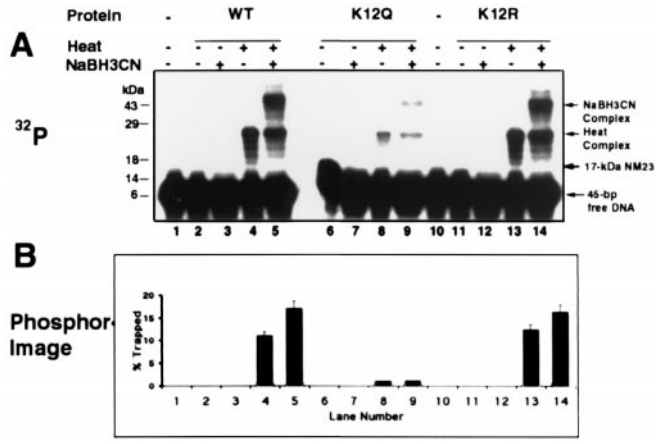

Fig. 4. Borohydride-trapping assay for stable complex formation by a Schiff-base intermediate between ^{32}P-end-labeled 45-bp duplex DNA and NM23/PuF proteins. (*A*) Autoradiogram of dried gel. Positions of the 45-bp free DNA substrate, the 17-kDa free NM23/PuF proteins, and the 26- and 52-kDa complexes are indicated on the right side. The left side shows migration of molecular weight standards. (*B*) PhosphorImager quantification of the percentage of borohydride-trapped ^{32}P products after scanning and data analysis using IMAGE QUANT software. The lanes are identical to those of *A*.

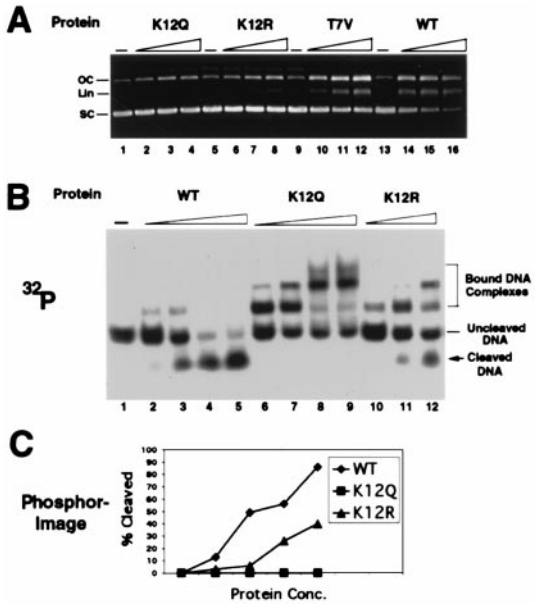

Fig. 5. Analysis of DNA cleavage by NM23/PuF mutants. (*A*) Cleavage of negatively supercoiled plasmid DNA by WT and mutants as monitored by agarose gel electrophoresis (24). Reaction mixtures contained plasmid DNA (615 ng), and, as indicated, increasing (from left to right by 2-fold) from 31 to 250 ng of each protein. The left side indicates plasmid states SC, supercoiled; Lin, linear; and OC, open circle/nicked circular (24). (*B*) DNA cleavage and binding to ^{32}P-end-labeled 45-bp duplex DNA substrate by WT and mutant NM23/PuF analyzed by electrophoretic mobility-shift assay. Reaction mixtures contained 5 ng of duplex DNA, and, as indicated, increasing (from left to right by 2-fold) from 125 to 1000 ng of each protein, representing DNA to protein (hexameric) ratios of 1:6 to 1:50. The figure is an autoradiogram of a dried down gel. The positions of free, complexed, and cleaved DNAs are indicated on the right. (*C*) The extent of cleavage is represented in histograms as the percentage of cleaved DNA after quantification by using a PhosphorImager with IMAGE QUANT software.

K12R proteins formed a previously unidentified high-molecular-weight stable complex with DNA (Fig. 4, lanes 5 and 14), as did the T7V mutant (not shown). Thus, arginine can replace lysine for Schiff-base formation, whereas the K12Q mutant cannot form this complex efficiently (Fig. 4, lane 9). A similar difference in the borohydride-dependent complex was observed with the K → Q and K → R mutations with the human DNA glycosylase/lyase hNTH1 endonuclease III (31). However, because of the incompatibility of the reaction temperatures in our experiments between DNA cleavage (0°C) and borohydride trapping (37°C), borohydride was added only after the DNA-cleaving reaction was terminated by heat. Under these conditions, both the 52-kDa borohydride-dependent complex, and the 26-kDa heat-trapped complexes were seen (Fig. 4, lanes 5, 9, and 14).

Interestingly, the oligomeric species crosslinked to DNA by borohydride seems to be primarily dimeric, as judged by the apparent molecular mass of the complex formed after the reduction (≈52 kDa), a mass twice that of the protein–oligonucleotide monomeric complex (≈26 kDa) formed by heat treatment alone. In other experiments (not shown), we observed, albeit in lesser quantities, formation of even slower migrating complexes after the borohydride treatment. We do not fully understand why the borohydride-crosslinked complex is oligomeric NM23, because, although the native subunit composition of NM23/PuF is hexameric, the samples were reduced and boiled in SDS before electrophoresis in SDS/polyacrylamide gels. Given the hexameric conformation of NM23 and the multiplicity of binding sites on the oligonucleotide substrate (22), the slow migrating species could have resulted from crosslinking two or more protein subunits to a single oligonucleotide. In earlier crosslinking experiments with glutaraldehyde, the predominant oligomeric species of NM23/PuF bound to DNA was also the dimer, which we interpreted to mean that DNA-binding by NM23/PuF was dimeric and combinatorial (21). Nevertheless, the results in Fig. 4 prove that irreversible crosslinking is exclusively dependent on the formation of a specific enzyme-substrate intermediate complex. Additional proof is provided by the fact that the crosslinking efficiency of the K12Q protein is reduced to background levels as this mutant cannot form the imino substrate with DNA necessary for the reduction step (Fig. 4, lane 9). Thus, lysine-12 of NM23/PuF may have a role similar to that of the catalytic lysines of DNA glycosylase/lyases endonucleases III/HtH (31, 32) and MutM/OOG1 (29, 33) that carry out sequential N-glycosylase and abasic lyase reactions involving a covalent, Schiff-base enzyme–DNA intermediate.

The residual radioactivity retained by the K12Q protein in Figs. 2–4 may be attributed to a covalent interaction between DNA and the N-terminal α-amino group, because both the α-amino group of a protein and the ε-amino group of lysine can participate in the same chemical reaction (34). Although most DNA glycosylase/lyases use the ε-amino group of an internal lysine as the catalytic nucleophile, T4 endonuclease V (35) and *E. coli* MutM (36) use the N-terminal α-amino group of the polypeptide. This possibility is suggested further by the presence of a minor peptide in the DNA-linked sample (Fig. 1) with the sequence ANLERTFNAI, matching the N-terminal sequence of NM23/PuF from positions 2–11. That other lysines on the protein surface may contribute to residual binding is an additional possibility.

Analysis of DNA Cleavage by NM23/PuF Mutants. DNA cleavage by the mutants was first assessed by the conversion of negatively supercoiled plasmid DNA to nicked circular and linear substrates (24). Whereas the WT and the T7V mutant exhibited rigorous cleaving (linear products) and nicking (open circles) activities (Fig. 5*A*, lanes 9–16), cleavage by the K12R (lanes 6–8) and K12Q (lanes 2–4) mutants was reduced or absent. We confirmed these results with the more specific electrophoretic mobility-shift assay analysis using duplex oligonucleotide substrates consisting entirely of the c-*MYC* NHE sequence. These

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Table 1. Nuclease and NDP kinase activities of NM23-H2/PuF mutants

Protein	DNA binding	% trapped covalent complex		DNA cleavage, %	NDP kinase, % specific activity
		Heat	$NaBH_3CN$		
WT		100	100	100	100
K12Q	WT	9.6	13.3	5.0	2.8
K12R	WT	112	108	29.5	14.6
T7V	WT	94	96	90	74

NDP kinase activity was measured in a coupled-enzyme assay as described (20). The specific activity of each mutant is expressed as a percentage of WT. Each category of assays was performed with several independent protein preparations, and the results were averaged.

data also demonstrate that the K12Q protein is an inactive nuclease (Fig. 5*B*, lanes 6–9), whereas the K12R mutant retains some activity (lanes 10–12). This result was expected because, although arginine does have a basic nitrogen atom and can form the covalent intermediate complex (Figs. 2–4), its proximity to the DNA substrate is altered. Indeed, similar levels of reduction in the nuclease activities of the repair nucleases Endo III and hOOG1 were found with the K → Q and K → R substitutions of their respective catalytic lysines (32, 37). The T7V mutant appeared normal in both DNA-cleavage assays (Table 1). These results therefore identify lysine-12 as the catalytic residue of NM23/PuF responsible for the DNA cleavage.

Measurements of the DNA-binding properties with respect to several different duplex oligonucleotide substrates (45-mer shown in Fig. 4*B*) strongly suggested that the K12R and K12Q mutant proteins, with partially or completely impaired catalytic properties, retained their ability to bind DNA (Fig. 5*B*, lanes 6–12). The apparently reduced DNA binding by the WT protein (lanes 2–5) occurs because of the excessive cleavage by this protein under these conditions (24). Overall, we surmise that although the presence of lysine-12 is critical for the cleaving, it is not required for sequence-specific DNA binding. This finding suggests that the defined sequence-specific DNA-binding surface consisting of residues arginine-34, asparagine-69, and lysine-135 (21), and the active site residue of the nuclease, lysine-12, located in the NDP kinase catalytic pocket, are independent DNA-binding entities of NM23/PuF.

NDP Kinase Activity of Mutants. On the basis of both the x-ray crystallographic data on NM23-H2/NDP kinase B (14, 15) and a previously analyzed mutant (K16A) of the equivalent residue in *Dictyostelium* NDP kinase (38), we expected that lysine-12 of NM23/PuF would be required for the kinase activity. Indeed, the results in Table 1 establish that the K12Q protein is a catalytically inactive NDP kinase. The NDP kinase activity of the K12R protein was intermediate, as was its nuclease activity. These data establish that the ε-amino group of lysine-12 is also the critical functional group for the NDP kinase activity. However, valine-7 is not involved in either of the catalytic functions, because the T7V mutation did not abolish covalent complex formation, and its nuclease and NDP kinase activities are also within normal range.

Discussion

The experiments described in this paper lead to three important findings. The first is that the active site amino acid of NM23/PuF nuclease is lysine-12, an evolutionarily conserved amino acid. This result also identifies, in addition to the sequence-specific DNA-binding surface described (21), a second DNA-binding region on NM23, namely, the catalytic site. Because the cleavage of DNA initially requires sequence-specific DNA binding (24), we presume that this region, located on the equatorial surface of the protein (21), is involved in the positioning of DNA into the active site for cleaving.

The second finding is that the ε-amino group of lysine-12 is the critical nucleophile in the DNA-cleaving reaction, and based on borohydride-crosslinking experiments, the reaction involves the formation of a Schiff-base intermediate (29, 37). Lysine-12 of NM23/PuF may thus have a similar role to that played by an essential amino group in the active site of DNA glycosylase/lyase repair proteins. A DNA repair-like activity for NM23 would be consistent with its involvement in the DNA damage response pathways of BRCA1 (39) and TIP30 (40). It would also explain the sensitivity of NM23 transfected tumor cell lines to the mutagen cisplatin (41), because increased mismatch repair activity is known as a contributing factor to cisplatin sensitivity (42). The induction of NDP kinase in plants after UV exposure (43) also suggests a DNA repair activity, as does the mutator phenotype of *E. coli* NDP kinase knockouts (5). Indeed, a mutator phenotype generally indicates a lesion in DNA base excision repair (44).

A direct correlation between increased mutation rates and cancer has been attributed to lesions in the DNA repair system. Although mutations are rare, NM23 is abnormally expressed in many tumors (9–11, 16). In colorectal carcinomas, for example, *NM23-H2* is among the top 20 transcripts overexpressed (12). The well established link between DNA mismatch repair and colon cancer (45, 46) might be attributed, at least in part, to a malfunction caused by overexpression of NM23. Database searches, however, do not reveal significant amino acid sequence similarities between NM23 and known DNA repair enzymes. This result is not really surprising, because, in general, DNA glycosylases show very little sequence similarity to one another (47). Nor have we found significant structural alignments with the signature HhH/D loop motifs shared by glycosylase/lyases that use lysine nucleophiles for catalysis (29, 47). Still, five of the residues surrounding and including the catalytic lysines are identical in the HhH motifs and NM23. It thus seems possible that NM23/NDP kinase represents a previously unrecognized class of DNA repair enzymes. Therefore, we have taken steps to verify a DNA repair activity for NM23/PuF by using the established techniques.

NM23 functioning in both transcription and repair would not be unusual, because many of the enzymes in the DNA repair pathways have multiple roles and participate in repair, transcription, and recombination (48). The underlying biochemical mechanism of NM23/PuF in these processes would be the same: binding to DNA, with subsequent catalysis of chemical reactions consistent with a nuclease action, by a mechanism that uses lysine-based covalent chemistry. A model we proposed to explain the transcriptional activity of NM23/PuF entails the catalysis of rearrangements in the phosphodiester backbone of DNA. We envisioned that sequence-specific distortions in DNA, such as those caused by loops and mismatched bases in slipped DNA structures in the c-*MYC* promoter, would have to be removed or restored to the B-form to facilitate transcription (23, 24). Such a mechanism may underlie both a general DNA repair and a more sophisticated recombinational function.

The third finding is that lysine-12 is also essential for the NDP kinase activity of NM23/PuF. This result was expected on the basis of the known crystal structure (14), and on the basis of mutational analysis of *Dictyostelium* NDP kinase (38). The sharing of an active site and the suggestion of a functional relationship between the nuclease and NDP kinase activities of NM23/PuF, however, is a significant development.

Why would a nuclease, catalyzing sequence-specific cleavage of the DNA backbone, and an NDP kinase that synthesizes dNTPs, share an active site for catalysis? One hint may be that

although the substrate is DNA in one case and nucleotides in the other, lysine-12 contacts the sugar moiety of a nucleotide in both instances. In the x-ray crystallographic structure of the complex with GDP, the base is on the outside of the protein, whereas the ribose is buried in the active site pocket, with the ε-amino group of lysine-12 contacting the O2′ and O3′ carbonyls (14). In the DNA glycosylase/lyase mechanism, the ε-amino group makes the nucleophilic attack on the C 1′ carbonyl of deoxyribose (28–30). In a DNA repair context, a single active site would allow dNTPs to be formed near their sites of use for repair synthesis. Another possibility is that the state of phosphorylation of the catalytic histidine-118 negatively regulates the DNA-binding and -cleaving activity, the latter of which, if constitutive, would be lethal to the cell. Nucleotide occupancy of the active site may act as a switch between a catalytically active nuclease and a catalytically active NDP kinase. Our previous observation that ATP is inhibitory to the DNA-cleavage reaction (24) supports this notion. The third possibility is that the relationship between lysine-12 and histidine-118 is indirect. Although these two residues occupy the same catalytic pocket, they are separated by at least 5 Å and do not seem to interact directly (14, 15). Consistent with an indirect role is an arguable relationship between the NDP kinase activity and the various other known properties of NM23. For example, in early transfection studies with *NM23-H1*, no correlation was found between elevated *NM23-H1* mRNA levels in transfected cells, and NDP kinase enzyme levels and specific activity in extracts made from these cells (16). Moreover, the role of histidine-118 for DNA-binding and *in vitro* transcription with partially purified protein extracts seems to be dispensable also (20). On the other hand, transfection experiments in *Drosophila* indicated that the NDP kinase catalytic histidine is necessary, but not sufficient, for the biological activity of AWD protein (17). It would be important to ascertain whether this "other" activity in AWD is related to the nuclease function.

Understanding of the enzyme mechanisms of the nuclease/NDP kinase will require identification of other catalytically important residues, as well as a three-dimensional structural analysis of enzyme–DNA complexes of substrates and products. The availability of an active site mutant for the nuclease should also open new avenues for examining the biological role of NM23.

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