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The hSNM1 protein is a DNA 5′-exonuclease

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ABSTRACT

The human SNM1 protein is a member of a highly conserved group of proteins catalyzing the hydrolysis of nucleic acid substrates. Although overproduction is unstable in mammalian cells, we have overproduced a recombinant hSNM1 protein in an insect cell system. The protein is a single-strand 5′-exonuclease, like its yeast homolog. The enzyme utilizes either DNA or RNA substrates, requires a 5′-phosphate moiety, shows very little activity on double-strand substrates, and functions at a size consistent with a monomer. The exonuclease activity requires the conserved β-lactamase domain; site-directed mutagenesis of a conserved aspartate inactivates the exonuclease.

INTRODUCTION

The SNM1 protein belongs to the metallo-β-lactamase family of proteins, characterized by conserved sequences for binding Zn^{2+} and cleaving substrates with ester linkages. The β-CASP subfamily (CPSF73, Artemis/SNM1 (or Pso2), and Elac1 and 2, further have conserved domains, A, B and C, in addition to the hydrolytic lactamase domain (1,2). SNM1 is the product of a gene first described in *Saccharomyces cerevisiae* as *PSO2*, a non-essential gene required for normal survival after exposure to psoralen or other DNA interstrand cross-linking (ICL) agents such as nitrogen mustard or cisplatin (3) (4). The *SNM1* gene is assigned to the nucleotide excision repair (NER) pathway (*RAD3* epistasis group), despite the fact that cells deficient in SNM1 protein in yeast show no increase in sensitivity to other DNA-damaging agents such as UV or radiation. A defect in SNM1 in yeast is additive to a defect in the *RAD52* pathway (recombination) or the *RAD6* pathway (post-replication) (5), showing that the three DNA repair epistasis groups in yeast are defined in ICL repair as well. For mutants defective in all three pathways, a single ICL is lethal (5).

ICL repair produces double-strand breaks (DSB) and *snm1* yeast mutants incise DNA with ICLs normally (6–8), showing normal DSB formation. However, intact DNA is not reformed at normal rates (8) in *snm1* mutants, but HO-induced mating type switching is processed normally (8), demonstrating that there are specific features of ICL-induced DSBs which require SNM1 function and that *snm1* mutants process some DSBs normally. Disruption of the β-lactamase domain of SNM1 prevents function in ICL repair *in vivo*. The yeast SNM1 enzyme is known to be a DNA 5′-exonuclease (9), and disruption of the β-lactamase domain destroys the exonuclease activity, linking this function to ICL repair. Thus it seems that the exonuclease must act in processing a specific structural intermediate in ICL repair.

The SNM1 family is well conserved in mammals and contains SNM1B/Apollo, SNM1C/Artemis, ELAC2 and CPSF73 (1). The CPSF subunit hydrolyzes mRNA (10). The mammalian orthologs all have the β-lactamase domain preserved, suggesting hydrolytic function. SNM1B/Apollo possesses a 5′-exonuclease activity and may play a role in telomere maintenance (11). Artemis has been shown to be a 5′-exonuclease interacting with DNA-PK_{cs}, which phosphorylates it, to gain a specific endonucleolytic function cleaving DNA hairpin structures, such as formed by the RAG1/RAG2 functions (12). That Artemis plays a role in V(D)J recombination and immune competency was demonstrated by the finding that some patients with radio-sensitive severe combined immune deficiency (RS-SCID) are defective in Artemis function (13–15).

No such functional role has been demonstrated for the mammalian *SNM1* gene. Disruption in mice produces remarkably little phenotype (16). Embryonic stem (ES) cells from mice with an *Snm1* disruption show increased sensitivity to MMC, but not to other DNA cross-linking agents, including cisplatin or psoralen (16). Another report found that SNM1 disruption alone or in combination with a p53 null mutation resulted in accelerated tumorigenesis, as well as decreased survival of *snm1*−/− mice (17). Murine embryonic fibroblasts (MEFs) from these mice show loss of a mitotic checkpoint and increased sensitivity to spindle poisons (18). Thus the multiple orthologs in mammals, along with the mild phenotype, cloud the role of SNM1 protein in genome stability and DNA repair. While human fibroblasts depleted for SNM1

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manifest a striking chromosomal instability (unpublished data), without a defined group of patients, the function of SNM1 is unknown.

We have cloned the *hSNM1* gene into an insect cell system and overexpressed the recombinant protein. It possesses a 5′-exonucleolytic activity on single-stranded DNA or RNA. The activity is reduced at least 97% on double-stranded DNA or hairpin structures. The protein functions as a monomer of ~115 kDa. Mutagenesis of the conserved aspartate residue at position 736 to an alanine inactivates the nucleolytic activity, indicating the β-lactamase domain encodes the nuclease function.

MATERIALS AND METHODS

Cell culture and plasmid construction

Kc 167 insect cell line (*Drosophila melanogaster*) was purchased from Drosophila Genomics Resource Center, Indiana University, Indiana, and was grown in Schneider's Drosophila Medium (SDM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA).

A full-length hSNM1 cDNA clone was isolated from a lymphoblast cDNA library (a gift from Dr Manuel Buchwald), and sub-cloned into the expression vector pAc5.1a (Invitrogen, Carlsbad, CA, USA) by PCR amplification of the coding region with primers tagged with a Kozak sequence and compatible restriction sites (MfeI and SmaI) (pAc5.1a-EcoRI and BstB1 + Klenow). To make the hSNM1-D736A mutant construct, the Quick-Change site-directed mutagenesis method was followed as recommended (Stratagene, La Jolla, CA, USA). Briefly, mutant sense and antisense primers (5′-CACACATTTTCATTCTGCTCATTATGCTGGATTGTC-3′, and 5′-GACAATCCAGCATAATGAGCAGAATGAAAATGTGTG-3′, respectively) were used in PCR amplification, with pAC5.1-hSNM1 as template and Pfu-Turbo DNA polymerase (Stratagene, La Jolla, CA, USA). The PCR product was digested with DpnI, and transformed into chemically competent *E. coli* XL1-Blue MRF′ cells (Stratagene, La Jolla, CA, USA). Colony PCR followed by BclI digestion (the D736A mutation eliminates a BclI restriction site) was performed to identify the mutant clones. The selected clones were then confirmed by sequencing.

Protein expression and purification

Transfection of plasmids into Kc 167 cells was performed using Cellfectin Transfection Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, 10 μg plasmid (pAc5.1-hSNM1 or pAc5.1a control) was mixed with 50 μl Cellfectin, and incubated at room temperature for 30 min. The mixture was then added to 4×10^7 Kc 167 cells, and incubated for 2 h at room temperature. After the incubation, the transfected cells were grown in SDM containing 5% FBS for 3 days before the harvest.

The following steps were performed at 4°C. The cells were harvested by centrifugation, washed twice with PBS, and resuspended in 500 μl Buffer A (50 mM $NaPO_4$, pH 7.1, 300 mM NaCl, 5 mM imidazole, 1% IGEPAL CA 630, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 mM sodium pyrophosphate, plus 1/100 volume Complete EDTA-free protease inhibitor cocktail (Roche, Nutley, NJ, USA), rocked gently for 1 min, then incubated on ice for 10 min. A cleared lysate was obtained by centrifugation at $800 \times g$ for 10 min at 4°C. The cleared lysate supernatant was then mixed with an equal volume of 50% v/v slurry of TALON beads (Clontech, Mountain View, CA, USA), pre-equilibrated in Buffer B (50 mM $NaPO_4$, pH 7.1, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF), and incubated at 4°C for 2 h with gentle rotation. The bound beads were then poured into a Poly-Prep chromatography column (Bio-Rad, Hercules, CA, USA), and unbound proteins were allowed to flow through by gravity. The column was then washed with 20 bed volumes of Buffer B, followed by 10 bed volumes of Buffer C (50 mM $NaPO_4$, pH 7.1, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF). Elution of hSNM1 was performed by adding 4 bed volumes of Buffer D (50 mM $NaPO_4$, pH 7.1, 300 mM NaCl, 150 mM imidazole, 10% glycerol) and collecting 250 μl fractions. All the fractions were brought to a final concentration of 5 mM DTT, frozen in liquid nitrogen, and stored at –80°C. Protein concentrations of the samples were determined with protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA). Purification fractions were analyzed by SDS-PAGE followed by Coomassie Blue, silver staining or western blotting.

Approximately 100–125 μg of TALON-purified hSNM1 and hSNM1-D736A were further purified by gel filtration on Superose-6 or Superose-12 FPLC columns (GE Healthcare, Piscataway, NJ, USA) equilibrated in Buffer E (50 mM $NaPO_4.H_2O$ pH 7.1, 5 mM DTT, 300 mM NaCl, 5% glycerol). After a void volume of 8 ml, a total of 40 0.5 ml fractions were collected and analyzed by SDS-PAGE followed by silver staining and immunoblotting.

Immunoblotting

Approximately 20 μl of each protein fraction was separated by 8% SDS-PAGE, and transferred to Immobilon-P PVDF membrane (Millipore, Billerica, MA, USA). After blocking in PBST (PBS + 0.1% Tween-20) with 5% non-fat dry milk, the membrane was probed with anti-V5 monoclonal antibody (Invitrogen, Carlsbad, CA, USA) diluted 1:5000 in PBST with 5% non-fat dry milk. The blot was then incubated with goat anti-mouse IgG horseradish peroxidase conjugated secondary antibody (Calbiochem, San Diego, CA, USA) diluted 1:10 000 in PBST with 5% non-fat dry milk. Detection was by Immobilon-Western Chemiluminescent HRP reagent (Millipore, Billerica, MA, USA).

Substrates

Custom oligonucleotides (Integrated DNA Technologies Inc., Coralville, IA, USA) were 20-dT (5′-TTTTTTTTTTTTTTTTTTTT); S-1 (5′-GACCAAGGGGTACCAG);

S-2 (5′-CCAACCTACACACCTATATCCATTTGCTG GTACCCCTTGGTC). Shorter oligo-dT substrates (15-dT, 10-dT and 5-dT) were synthesized by the OHSU Molecular Microbiology and Immunology Research Core Facility. To make a 5′ labeled ssDNA, 25 pmol of 20-dT was labeled with [γ-^{32}P]-ATP, using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA). To make a 3′ labeled ssDNA substrate 25 pmol of 20-dT was labeled with [α-^{32}P]-ATP-dideoxyadenosine triphosphate using terminal deoxynucleotide transferase (New England Biolabs, Ipswich, MA, USA). The 3′-end-labeled substrate was then phosphorylated with cold ATP and T4 polynucleotide kinase or left unphosphorylated at the 5′-end. The internally labeled dsDNA (42 bp) was made by 5′ phosphorylating S-1 with cold ATP and T4 polynucleotide kinase, then annealing to 15 pmol S-2. The duplex was then extended with Klenow polymerase with dATP, dTTP, dGTP and [α-^{32}P]-dCTP. Unincorporated radionucleotides were removed by ProbeQuant G-50 micro columns (Amersham Biosciences, Piscataway, NJ, USA). A pseudoflap substrate was constructed by annealing 5′-end-labeled cru-13 (5′-CTCCATCCAAGGAGTTGGC GATCACGAAGCTTTAATCTGGAGCCA) with cru-14 5′-TCCAGATTAAAGCTTCGTGATCGACC GAGGTCCAAGAACTC). Hairpin and hairpin-loop substrates were generated with oligonucleotides HP-1 (5′-CCACTGCGTCTAAGGTGGAGGTGAACGTTCA CCTCCACCTTAGACG) and HP-loop (5′-CCACTGCG TCTAAGGTGGAGGTGAACTTTTTTTTTTGTTCAC CTCCACCTTAGACG), respectively, and labeling with [α-^{32}P]-dCTP and Klenow polymerase. This strategy incorporated a single 3′-[^{32}P]-C, and left the 5′-end unphosphorylated, thus protecting the 5′-end from exonucleolytic attack during the assay. A single-strand RNA substrate (5′-UGCCUUCCACCUCCACCAATT) was labeled at the 5′-end with polynucleotide kinase and [γ-^{32}P]-ATP, as for the 20-dT substrate.

In vitro nuclease assay

The assay was similar to an assay for yeast SNM1 (9) Briefly, 0.5 pmol of radiolabeled substrate was combined with indicated amounts of purified protein (see the figure legends) in 15 µl of 1× Buffer F (50 mM Tris-acetate pH 7.2, 10 mM Mg acetate, 75 mM Potassium acetate, 1 mM DTT) supplemented with 100 µg/ml BSA, and incubated at 37°C for 20 min. For control reactions, 10 units of Rec-J_f or λ-exonuclease (for double-stranded substrate) (New England Biolabs, Ipswich, MA, USA) were used as recommended by the supplier. The reactions were stopped by adding an equal volume of formamide gel loading buffer (95% formamide, 20 mM EDTA pH 8.0, 0.04% xylene cyanol, 0.04% bromophenol blue) and heating at 65°C for 20 min. DNA was resolved on a 12% denaturing polyacrylamide gel. Greater resolution was obtained with 16% acrylamide gels, as indicated in the figure legends. The gel was wrapped in Saran wrap and analyzed by autoradiography. One unit of enzymatic activity is defined as the release of 1 pmol of ^{32}P as mononucleotide in 10 min at 37°C.

RESULTS

Overproduction of hSNM1 is not stable in human fibroblasts

In order to isolate and purify hSNM1, the cDNA for *SNM1* (GenBank D42045) was synthesized by PCR and cloned into a pIRES vector system with a 3′-V5 epitope and 6xHis tag (Figure 1A). This construct was transfected into human fibroblasts. Production of the SNM1 protein was verified by immunoblot analysis for the V5 epitope (Figure 1B), and the cells were continued on selection with puromycin. After fourteen days the cells remained puromycin resistant, but the expressed protein was absent by immunoblot (Figure 1B). Comparison of over 12 independent transfections, with subsequent culture, showed that hSNM1 expression was lost in each case by two weeks. The overall transfection efficiency of the SNM1 construct was poor, suggesting it had a deleterious effect on cell growth. Thus, apparently the SNM1 protein is not tolerated at an increased or unregulated level in human fibroblasts. Our finding is in agreement with the observations of others, suggesting that SNM1 is toxic (16,19). Intolerance of overexpression of SNM1 is in contrast to lack of the protein, which is well tolerated.

Overproduction of hSNM1 in an insect cell system

In light of the finding that hSNM1 cannot be stably overproduced in human cells, we turned to a different expression system. The hSNM1 coding sequence was cloned into pAc5.1 behind an insect cell promoter, Ac5 (Figure 1A). The 3′-epitopes allowed tracking the protein purification by immunoblot (Figure 1C). The column purification (immobilized metal ion affinity chromatography, IMAC) was done by passing a cleared cell lysate over the matrix in low imidazole (5 to 10 mM), followed by washes with 20 mM imidazole and elution with buffer containing 150 mM imidazole. The peak of the activity and epitope tag typically eluted in the second and third fractions, indicating a uniform binding and elution pattern for the 6xHis tag; there was slight batch-to-batch variation in the relative amount of SNM1 protein in peak elution fractions, most likely due to the small scale of the purifications and the manual elution and collection of the fractions.

hSNM1 has single-strand 5′-exonuclease activity requiring a phosphate

With a single-stranded substrate 20 bases in length, with a 5′-^{32}P label (9) the hSNM1 protein manifests 5′-exonuclease activity, similar to that reported for yeast SNM1 and Artemis (12) (Figure 1D). The RecJ_f single-strand 5′-exonuclease of *E. coli* served as a control (20). Since the assay measures only the removal of the 5′-nucleotide containing the label, and since the product migrates at the position of a mononucleotide, the exonuclease acts by removing the terminal mononucleotide. As insect cells are known to contain endogenous exonucleases, control purifications with mock-transfected cells were done in parallel to ensure that the IMAC elution fractions were free of endogenous exonuclease activity (Figure 1D, lane 12). A gel

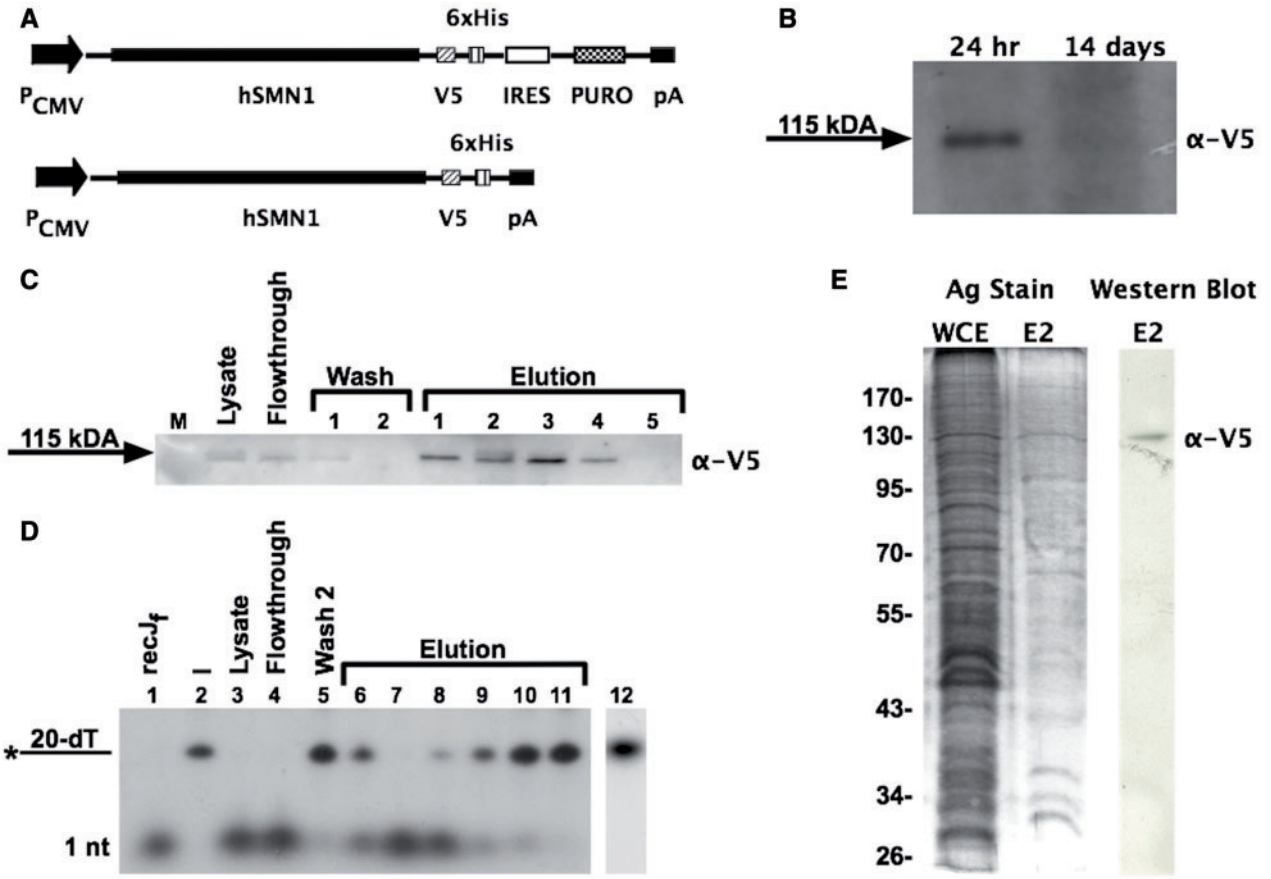

Figure 1. Affinity purification of His-tagged hSNM1Protein. (**A**) pAc5.1-hSNM1 and pIRESpuro-hSNM1 constructs. (**B**) Immunoblot of HEK293 cells transfected with pIRESpuro-hSNM1; 25 µg total protein loaded per lane. (**C**) Immunoblot of hSNM1-V5His fractions from IMAC. Lane 5 is an elution fraction 2 from mock-transfected cells, carried out in parallel. (**D**) Assay of hSNM1-V5His fractions for 5′ exonuclease activity. Lanes: 1, $RecJ_f$; 2, Substrate alone; 3, Cleared lysate; 4, Flowthrough; 5, Wash 2; 6–11, Elution fractions 1–6; 12, IMAC fraction 2 from mock-transfected Kc 167 cells. (**E**) Silver stained SDS-PAGE of pAc5.1-SNM1-transfected KC167 cleared lysate (WCE), and IMAC elution fraction 2, alongside an anti-V5 western blot of IMAC elution fraction 2.

electrophoresis analysis of a typical extract and Fraction 2 from IMAC is shown (Figure 1E) along with an immunoblot to detect the V5 epitope. No intrinsic cross-reacting material was detected, verifying the position of the hSNM1 protein.

The hSNM1 exonuclease functions best at slightly alkaline pH and low salt (Figure 2A), and is dependent on Mg^{2+}, showing no activity in excess EDTA, Fe^{3+} or Zn^{2+}. The activity is inhibited by ATP or NEM, indicating requirement for cysteine residues and suggesting the catalytic site binds phosphate. The 5′-exonuclease acts equally well on longer length substrates, including 40-mers (data not shown). Partial reactions with 5′-labeled substrate yielded single-nucleotide product and no other intermediates (Figures 1D and 2A); therefore, the enzyme has no apparent endonucleolytic activity.

To evaluate whether the activity is able to hydrolyze DNA from the 3′-terminus, a substrate was constructed with the ^{32}P label at the 3′-end. The hSNM1 enzyme does not hydrolyze substrate detectably from the 3′-terminus. This test also showed that the exonuclease activity strictly requires a 5′-phosphate group (Figure 2B), whereas the $RecJ_f$ enzyme is active without a 5′-phosphate group, although with reduced activity. The final product for hSNM1 is in the range of 6–8 nt, indicating that the exonuclease hydrolyzes from the 5′-terminus, with much decreased affinity for the substrate once the oligonucleotide is shorter than an octamer (Figure 2B, lane 6). Better resolution of the reaction was obtained by running the reaction products on a 16% acrylamide–42% urea gel (Figure 2C) using an equimolar mix of 5′-labeled oligonucleotides differing by 5 nt in length. The reaction went nearly to completion with the 20-dT substrate, the 15-dT was ~75% digested, the 10-dT substrate was digested <20% and there was no detectable digestion of the 5-dT substrate, supporting loss of binding for short substrates (Figure 2B, lane 6). At this resolution, the single-nucleotide product clearly co-migrated with the product of the $RecJ_f$ reaction verifying that the product is a mononucleotide. On the 16% gel, the products of digestion of a 3′-labeled substrate migrated as a ladder with single-nucleotide spacing ranging from 6 to 20 nt (Figure 2C, lane 7). A 5′-phosphate is required (Figure 2C, lane 9).

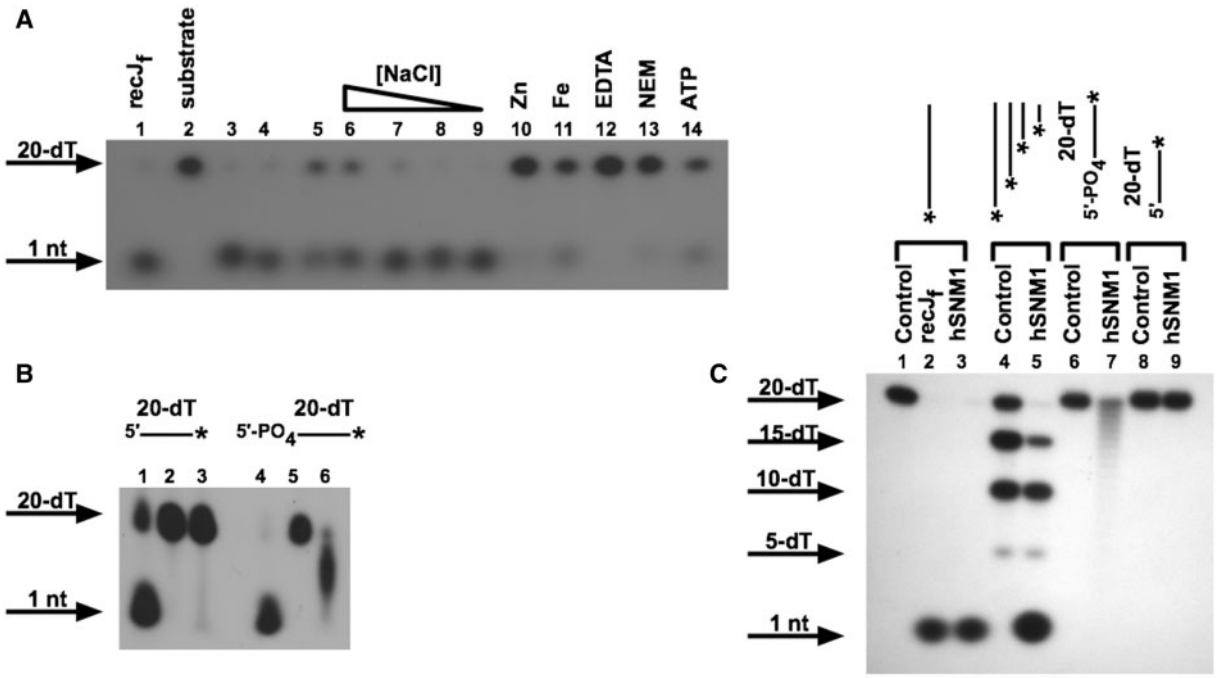

Figure 2. Characterization of hSNM1. (**A**) hSNM1 activity requires Mg^{2+} as a cofactor. 5′-end-labeled substrate was tested with IMAC fraction 2 and various buffers. Lanes: 1, $RecJ_f$ control; 2, Substrate only control; 3, Buffer F (see Materials and Methods section); 4, Buffer F, as for lane 3, but 2-fold dilution; 5, Buffer P, (10 mM $NaPO_4$, 10 mM $MgCl_2$, 1 mM DTT), pH 6.4 (100 mM NaCl); 6, Buffer P, pH 7.2 (100 mM NaCl); 7, Buffer P, pH 7.2 (50 mM NaCl); 8, Buffer P, pH 7.2 (15 mM NaCl); 9, Buffer P, pH 7.2 (No NaCl); 10, Buffer P, pH 7.2 (100 mM NaCl, 2 mM $ZnCl_2$); 11, Buffer P, pH 7.2 (100 mM NaCl, 0.1 mM $FeCl_3$); 12, Buffer P, pH 7.2 (100 mM NaCl, 20 mM EDTA); 13, Buffer P, pH 7.2 (100 mM NaCl, 2 mM *N*-ethyl maleimide); 14, Buffer P, pH 7.2 (100 mM NaCl, 1 mM ATP) (**B**) Assay of 3′-end-labeled substrate. Lanes 1–3, 3′-end-labeled, 5′-non-phosphorylated substrate; lanes 4–6, 3′-end-labeled, 5′-phosphorylated substrate. Lanes: 1 and 4, $RecJ_f$; 2 and 5, NEB2 Buffer only; 3 and 6, ~140 ng IMAC-purified hSNM1. (**C**) Exonuclease assays comparing 5′-phosphorylated and 5′-unphosphorylated substrates. Assays used ~140 ng IMAC-purified hSNM1, and were incubated 60 min at 37°C. Controls consisted of substrate and buffer only. The substrate in lanes 4 and 5 was an equimolar mix of 5′-labeled 5-dT, 10-dT, 15-dT and 20-dT (the 5-dT oligo did not label well with polynucleotide kinase). Products were resolved on a 16% acylamide–42% urea gel.

Table 1. Comparative analysis of hSNM1 on ssDNA and dsDNA substrates

	Protein concentration (μg/μl)	Total protein	Specific activity (u/μg)	
			ssDNA	dsDNA
Cleared lysate	20.7	10.4 mg	<5*	ND
IMAC fraction 2	0.070	17.5 μg	1.5×10^4	1.8×10^2
FPLC	<0.005	<2.5 μg	$>8 \times 10^4$	$>6 \times 10^2$

ND = not determined.
*Specific activity of mock-transfected cleared lysate was not significantly different from pAc5.1-hSNM1-transfected cleared lysate, due to endogenous nuclease activity.

Purified hSNM1 appears to function as a monomer

The overproduction and IMAC purification of hSNM1 produces over 1000-fold purification as measured by the exonuclease assay (Table 1). This would represent a lower estimate due to mixed nuclease and phosphatase activities in the extract. IMAC purified hSNM1 fraction 3 was fractionated on a gel filtration column for further purification and to estimate the apparent molecular weight of the active species. Elution of the detectable epitope tag occurred at a position consistent with the molecular weight of 115 kDa for hSNM1 [range 130–200 kDa] (Figure 3A). 5′-Exonuclease activity was detected in a peak centered around the same fraction (Figure 3B). Therefore it appears most likely that the activity of hSNM1 occurs in monomer form. Specific activity was determined by serial dilution of an active fraction, as shown in Figure 4. The purification, by specific activity, after Superose chromatography was greater than 5-fold over the IMAC eluate (Table 1).

hSNM1 does not hydrolyze double-strand DNA

The hSNM1 5′-exonucleolytic activity was tested on double-strand DNA. In order to avoid over-estimating the activity due to 'breathing' of the duplex termini, an internal label was used. λ exonuclease, active on double-strand DNA was used as a control (Figure 4). ySNM1, as reported (9), shows activity on the double-strand substrate, but hSNM1 activity is reduced to ~1% of that on single-strand DNA (Table 1). Results for IMAC-purified hSNM1 (Figure 4A and B) were comparable to the further-purified Superose 6 active fraction (Figure 4C and D).

hSNM1 is inactivated with loss of a conserved aspartate residue

Mutagenesis of the aspartate at residue 252 in a conserved domain in the yeast SNM1 protein inactivates the

5′-exonuclease (9). The comparable site, residue 736, in hSNM1, was changed to an alanine (Figure 5A). The mutant protein was expressed normally and at appropriate molecular weight as verified by anti-V5 immunoblot (Figure 5B). However, the expressed protein did not have detectable activity in the standard assay, even with overloading (Figure 5C). Thus the β-lactamase domain is required for the 5′-exonuclease function. This observation is in agreement with findings for ySNM1 and demonstrates a functional basis in the conservation. The absence of mononucleotide product in fractions 1–3 of the D736A protein (Figure 5C, lanes 5–7) underscores the lack of a contaminating insect cell exonuclease.

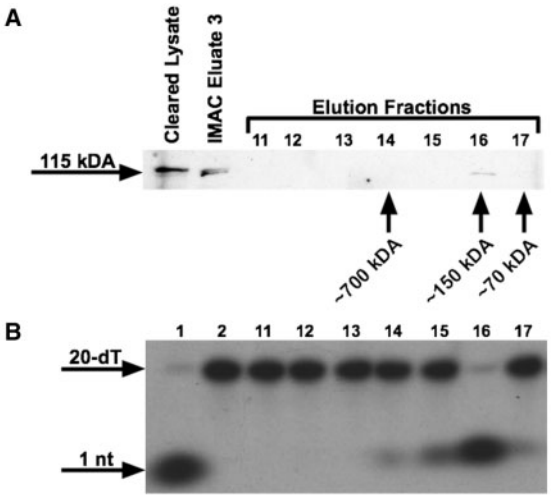

Figure 3. Gel filtration of hSNM1. (**A**) Immunoblot of Superose 6 fractions. IMAC fraction 3 was further purified on a Superose 6 column as described in Materials and Methods section. (**B**) Assay of Superose 6 fractions 11–17. Lanes: 1, $RecJ_f$ control; lane 2, substrate only.

The exonuclease also hydrolyzes single-strand RNA to approximately the same extent as DNA (Figure 6D).

hSNM1 has no intrinsic endonuclease activity on flap or hairpin substrates

Due to the homology between hSNM1 and Artemis, and the ability of Artemis to incise single-stranded DNA at junctions between single strands and double strands, in association with DNA-PK_{cs} (12), hSNM1 was tested with various substrates to see if it could make similar incisions. A pseudoflap structure consisting of two annealed oligonucleotides sharing 23 bp of complementarity and 18 nt of non-complementary bases was incubated with hSNM1 over a 30-min time course (Figure 6A). Whereas hSNM1 removed the 5′ label as a single nucleotide, there was no evidence for the generation of a 23-mer intermediate. Similarly, a 21-bp hairpin substrate and a 21-bp hairpin with a 10 nt loop were not incised by hSNM1 (Figure 6B), in contrast to Exo 1 from yeast (21). Taken together, this suggests that hSNM1 has no single-strand endonucleolytic activity. This does not preclude the possibility of activation by post-translational modification or interaction with a partner protein, but at face value hSNM1 appears to be a purely single-strand exonuclease.

DISCUSSION

The hSNM1 protein has been overproduced in an insect cell vector system and partially purified, with definition of a single-stranded DNA 5′-exonclease activity, dependent on the conserved β-lactamase domain. Thus the protein shares the basic activity of its orthologs, ySNM1 and Artemis, which show 5′-exonuclease activity. Other members of the β-CASP family, CPSF73 and CPSF100, process the 3′ termini of mRNA (22). As described above,

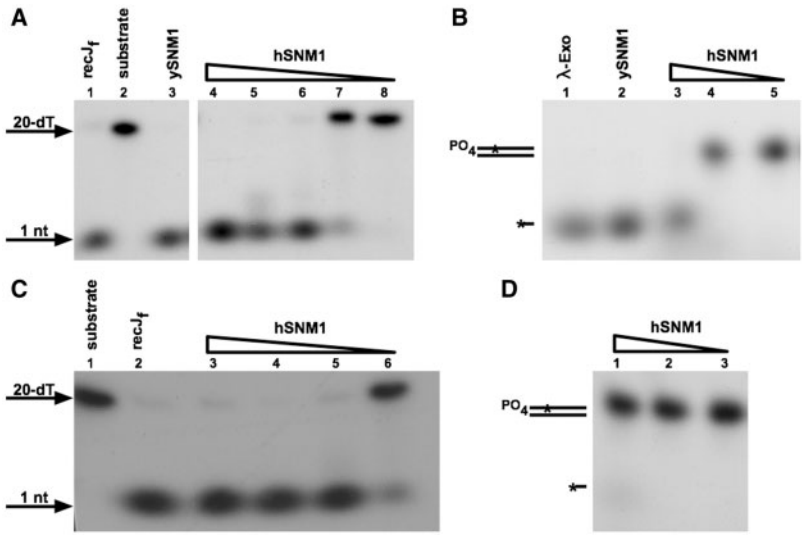

Figure 4. hSNM1 is a single-strand-specific exonuclease. (**A**) Assay of hSNM1 IMAC fraction 3 on 20-dT substrate. Lanes: 1, $RecJ_f$; 2, substrate only; 3, ySNM1; Lanes 4, 116 ng protein 5, 29 ng protein; 6, 7.25 ng protein; 7, 1.8 ng protein; 8, 0.45 ng protein. (**B**) Assay of IMAC fraction 2 on internally labeled double-stranded substrate. Lanes: 1, λ exonuclease control; 2, ySNM1; 3, 140 ng protein; 4, 35 ng protein; 5, 4.4 ng protein. (**C**) Assay of FPLC fraction 16 on 20-dT substrate. Lanes: 1, substrate only; 2, $RecJ_f$ control; 3, fraction 16 (~10 ng); 4, ~2.5 ng; 5, ~0.65 ng; 6, ~0.17 ng. (**D**) Assay of FPLC fraction 16 on internally labeled ds substrate. Lanes: 1, fraction 16 (~10 ng); 2, ~2.5 ng; 3, ~0.65ng.

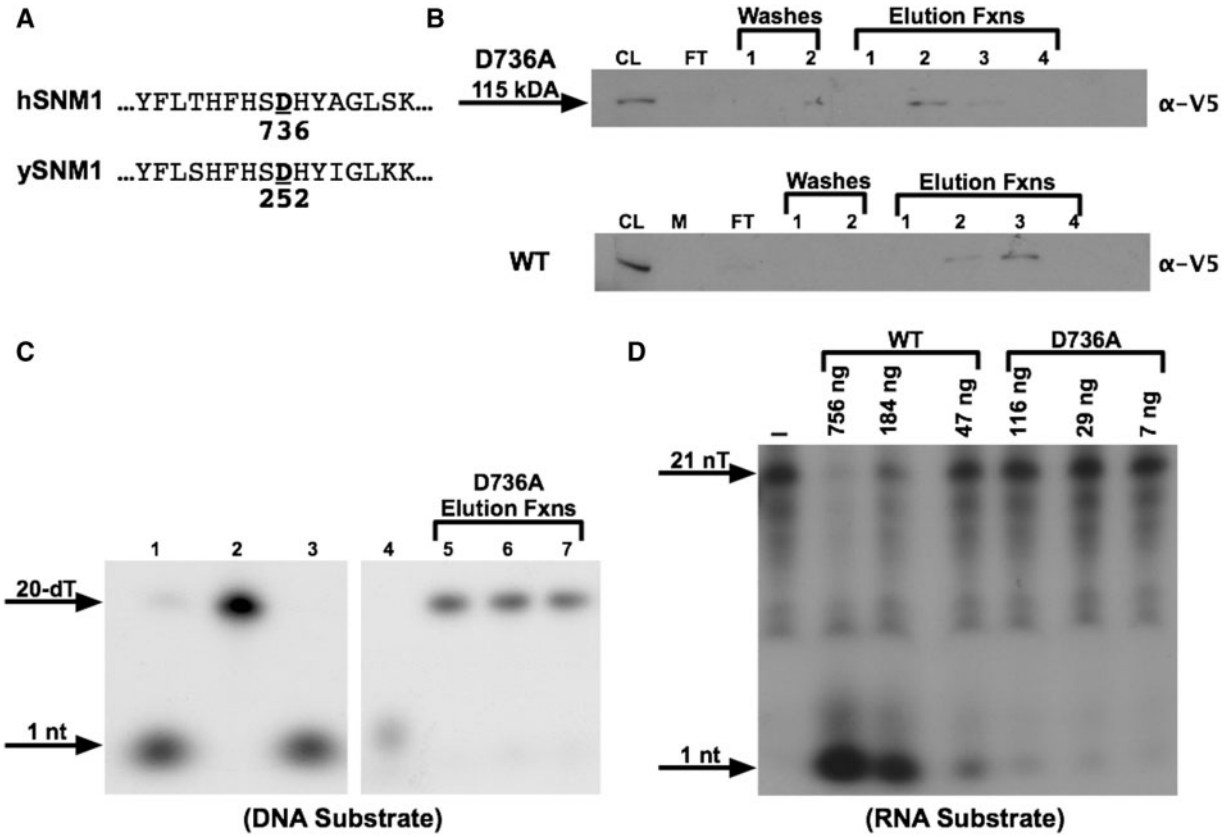

Figure 5. hSNM1 requires a conserved domain and also digests RNA. (**A**) Alignment of human and yeast SNM1 proteins in the vicinity of a conserved aspartate which is required for exonuclease function. (**B**) Immunoblot of hSNM1 D736A mutant protein and wild-type IMAC fractions, performed in parallel. Lanes: CL, cleared lysate; M, Markers; FT, Flowthrough. (**C**) Assay of D736A IMAC fractions. Lanes: 1, $RecJ_f$ control; 2, substrate alone; 3, ySNM1; 4, Cleared lysate; 5–7, eluates 1–3. (**D**) Assay of IMAC-purified wild-type and D736A mutant hSNM1 (fraction 2) with a single-strand RNA substrate.

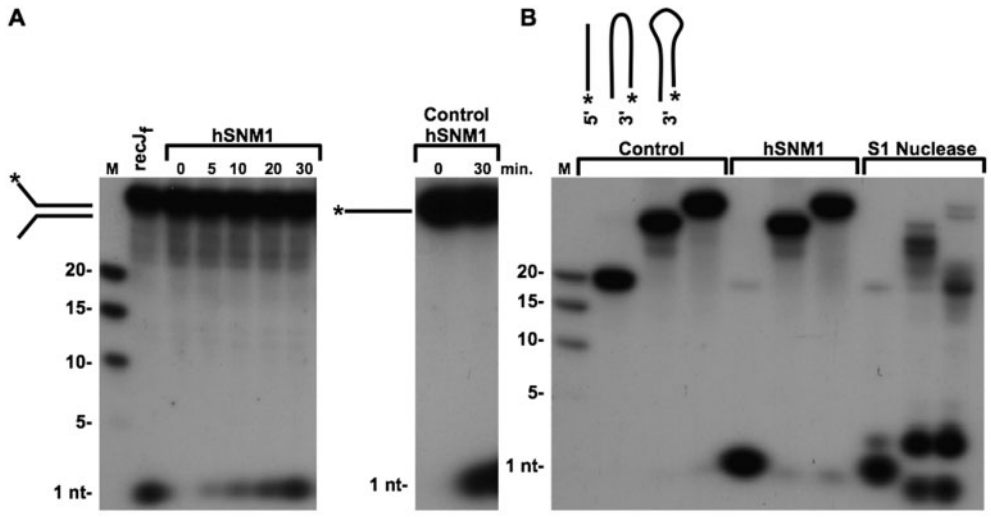

Figure 6. hSNM1 does not cleave pseudoflap structures or hairpins. (**A**) Timecourse of digestion of pseudoflap. There is no endonucleolytic activity at the ss–ds junction. (**B**) Assay of hSNM1 with hairpin substrates. Control reactions with the standard 5′-labeled 20-dT substrate went virtually to completion, whereas no intermediate-sized products were released from the hairpin substrates; for comparison, S1 nuclease released a 20-mer endonucleolytic product from the hairpin-loop substrate. Samples were boiled prior to loading and analyzed on a 16% denaturing acrylamide gel.

mutation of a conserved site in the β-lactamase domain (D736A) inactivates the 5′-exonuclease activity. Mutagenesis of the same site (D37N) in Artemis, changing an aspartate to asparagine, caused loss of the DNA-PKcs endonuclease activity of the protein, but did not cause loss of the 5′-exonuclease activity (23). The discrepancy for loss of the exonuclease may reflect the different amino acid substitutions, or it may reflect a fundamental difference in structure and activity of the two proteins.

The ySNM1 protein is known to act in ICL repair after incision (unhooking) at the site of the ICL, and to be required for reformation of the intact strand of DNA. It is possible that the yeast enzyme is involved in terminus modification of intermediates in the ICL repair pathway, facilitating strand rejoining (9). The EXO1 enzyme of yeast, involved in several DNA processing pathways in yeast is also a 5′ exonuclease (21). Therefore the activity of hSNM1 is a candidate to be involved in modifying termini at the site of DNA repair. Since hSNM1 is specific for single-stranded DNA, such a substrate might be generated during the repair of DNA interstrand cross-links, possibly in concert with a helicase, analogous to what has been suggested for Exo1 and WRN or RecQ1 (24,25).

There is no defined group of patients defective in hSNM1, nor is there an easily identifiable defect in DNA repair in animal models lacking SNM1. This may be due to the existence of multiple closely related members of the β-CASP family, SNM1A, SNM1B and Artemis. Disruption of SNM1B in chicken DT40 cells renders them sensitive to cisplatin, and a double disruption of SNM1A and SNM1B is additive, suggesting that SNM1B may be a redundant function in ICL repair (26). A knockdown of hSNM1B/Apollo also sensitized human cells to cross-linking agents as well as ionizing radiation (27). hSNM1B/Apollo was recently characterized as a 5′-exonuclease interacting with TRF2 in telomere maintenance, suggesting that hSNM1 and hSNM1B/Apollo may have partially overlapping functions (11,28), but that their association with different proteins or complexes may determine the pathway in which they operate. In view of these results collectively, identification of a phenotype in animal models lacking SNM1 may require multiple deficiencies. While hSNM1 has not been shown to be significant for cell survival with multiple DNA cross-linking agents, we have found that its deficiency leads to chromosome breaks and radial formation, an index of genome instability with ICL formation (unpublished data).

We found no evidence for phosphorylation of the protein (data not shown). This does not eliminate the possibility that modification of SNM1 occurs in human cells with DNA damage. The enzyme reported here was recombinant and produced in insect cells and so may not reflect the state of hSNM1 in human cells, with or without damage. Artemis is notably modified, acquiring an endonucleolytic activity cleaving putative recombination intermediates when phosphorylated and bound by DNA-PK. Thus it is possible that we observed only one activity associated with hSNM1.

The hSNM1 protein cannot be stably overproduced in a human cell. Since disruption of SNM1 has been reported to alter a mitotic checkpoint (18), it may be that overproduction is lethal to the cell due to disruption of the mitotic checkpoint. Alternatively, since we demonstrate here that SNM1 is a 5′-exonuclease, it is possible that overexpression is destroying an intermediate in normal DNA processing, leading to genome instability. If SNM1 is modified in human cells, then it might be that the modified form possesses an additional activity, which is deleterious when overexpressed.

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