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Production and characterization of recombinant protein preparations of Endonuclease G-homologs from yeast, *C. elegans* and humans

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ABSTRACT

Nuc1p, CPS-6, EndoG and EXOG are evolutionary conserved mitochondrial nucleases from yeast, *Caenorhabditis elegans* and humans, respectively. These enzymes play an important role in programmed cell death as well as mitochondrial DNA-repair and recombination. Whereas a significant interest has been given to the cell biology of these proteins, in particular their recruitment during caspase-independent apoptosis, determination of their biochemical properties has lagged behind. In part, biochemical as well as structural analysis of mitochondrial nucleases has been hampered by the fact that upon cloning and overexpression in *Escherichia coli* these enzymes can exert considerable toxicity and tend to aggregate and form inclusion bodies. We have, therefore, established a uniform *E. coli* expression system allowing us to obtain these four evolutionary related nucleases in active form from the soluble as well as insoluble fractions of *E. coli* cell lysates. Using preparations of recombinant Nuc1p, CPS-6, EndoG and EXOG we have compared biochemical properties and the substrate specificities of these related nucleases on selected substrates in parallel. Whereas Nuc1p and EXOG in addition to their endonuclease activity exert 5′–3′-exonuclease activity, CPS-6 and EndoG predominantly are endonucleases. These findings allow speculating that the mechanisms of action of these related nucleases in cell death as well as DNA-repair and recombination differ according to their enzyme activities and substrate specificities.

Introductory statement

Programmed cell death is a mechanism to eliminate damaged, surplus or infected cells from tissues of metazoan organisms as well as certain cells from populations of unicellular organisms such as yeast [1,2]. In this process mitochondrial proteins can play a decisive role [3]. Among mitochondrial factors involved in programmed cell death, the pro-apoptotic nuclease activity of Endonuclease G-proteins apparently is conserved from unicellular eukaryotes to mammals (Fig. 1A) [4–8]. In yeast and *Caenorhabditis elegans* the Endonuclease G-homologs Nuc1p and CPS-6, respectively, efficiently trigger apoptotic cell death in a caspase-independent manner when released from mitochondria [7,9,10]. Similar results have been obtained for mammalian Endonuclease G with conflicting reports demonstrating caspase-dependent as well as caspase-independent mechanisms of mitochondrial release of this enzyme [11–13]. More recent results suggest that Nuc1p and mammalian Endonuclease G may also play a role in normal cell proliferation and DNA recombination in addition to their well established functions in programmed cell death [7,14]. Gene knock-out studies in yeast had suggested earlier that Nuc1p is involved in mitochondrial DNA recombination and it had been speculated that the enzyme produces recombinogenic DNA ends by introducing single stranded gaps into double stranded DNA using its 5′–3′-exonuclease activity [15,16]. Nuc1p is a so-called mitochondrial endo/exonuclease exhibiting both endo- and exonuclease activities. In the case of Nuc1p the polarity of the exonuclease activity is 5′–3′ [17]. It is not known whether all homologs of mitochondrial Nuc1p in Eukarya are also endo/exonucleases and to which extent and under which conditions one or the other activity dominates the cleavage of a given substrate. Since mammalian Endonuclease G seems to lack a detectable 5′–3′-exonuclease activity the mechanism of processing DNA in order to produce recombinogenic DNA ends by this enzyme is likely to be different from that of its yeast homolog [17–19]. Moreover, we have recently characterized a paralog of Endonuclease G in humans,

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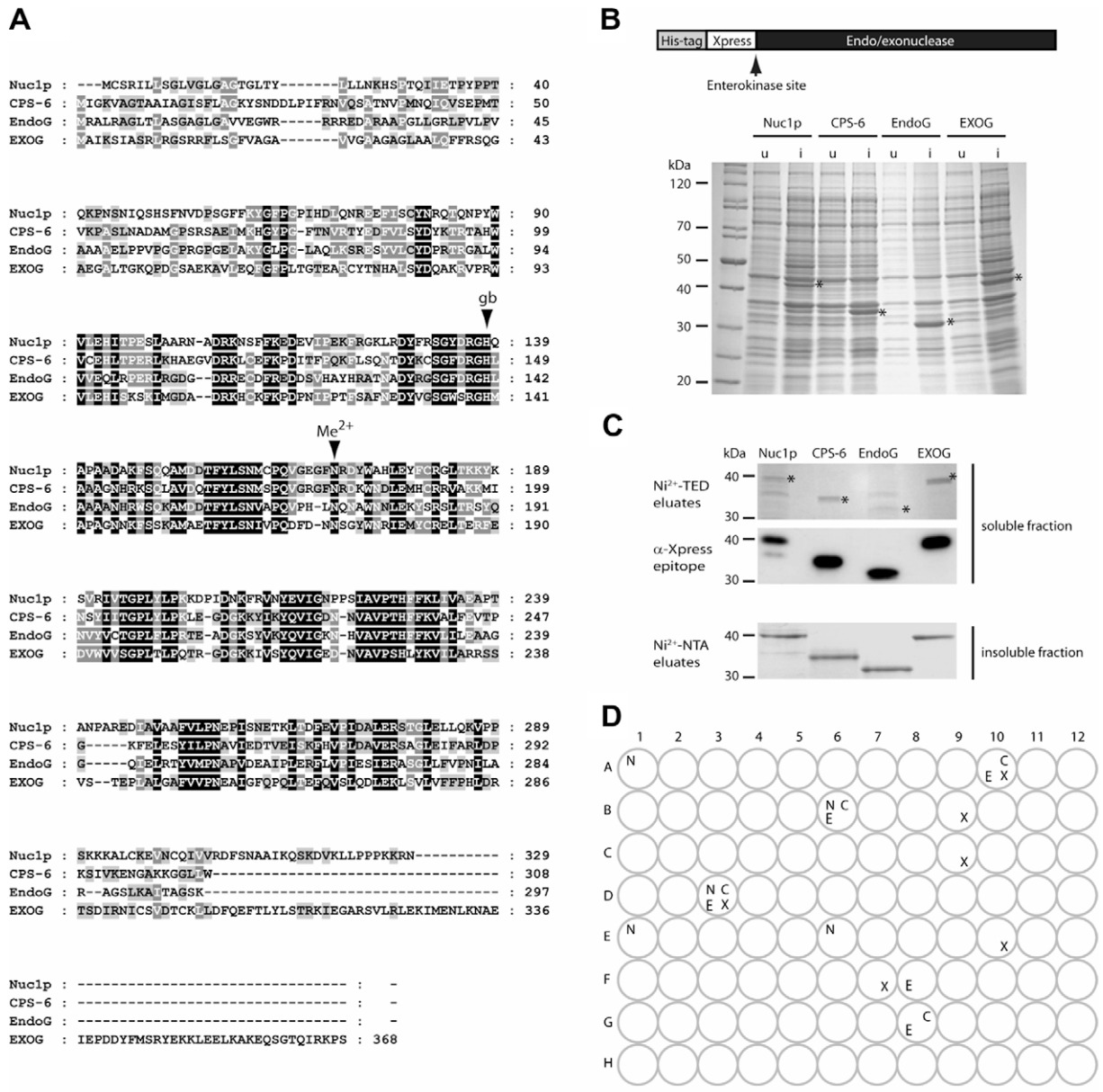

Fig. 1. Expression and purification of recombinant mitochondrial endo/exonucleases. (A) Multiple sequence alignment of the conserved mitochondrial endo/exonucleases Nuc1p from yeast, CPS-6 from *C. elegans* and human EndoG and EXOG. The conserved histidine residue acting as general base (gb) from the DRGH-motif and the asparagine residue binding the divalent metal ion cofactor (Me^{2+}) are marked by arrow heads. (B) SDS–PAGE of cell lysates from *E. coli* BL21Star(DE3) cells harboring pET-200 expression plasmids encoding the cDNAs for Nuc1p, CPS-6, EndoG and EXOG before (u, uninduced) and after (i, induced) induction with 1 mM IPTG (final concentration). (C) SDS–PAGE (upper panel) and Western blot (middle panel) of recombinant mitochondrial endo/exonucleases purified from the soluble fraction of overproducing *E. coli* cells. Lower panel, SDS–PAGE of mitochondrial endo/exonucleases purified from inclusion bodies and refolded using buffer D3. (D) Schematic representation of successful refolding of recombinant mitochondrial endo/exonucleases using the i-FOLD 2 matrix. A1 to H12 denote different buffers including controls. N, Nuc1p; C, CPS-6; E, EndoG; X, EXOG.

termed EXOG (Endonuclease G-like 1)[1], that in addition to endonuclease activity exerts strong 5′–3′-exonuclease activity on single and double stranded DNA producing mono- as well as di-nucleotides [20]. This finding suggests that in mammals EndoG and EXOG are mitochondrial twins that might share the cellular functions that are executed by the single Nuc1p enzyme in yeast. Here, we report the successful overexpression, purification and comparative biochemical characterization of four homologous so-called mitochondrial endo/exonucleases, Nuc1p, CPS-6, EndoG and EXOG, reported to be involved in cell death and DNA-repair and recombination in yeast, *C. elegans* and mammals. Our results demonstrate that these enzymes differ in their endo/exonuclease activities pointing to different roles and/or mechanisms of action during the execution of apoptotic DNA-fragmentation as well as DNA-repair and recombination in their host organisms. The availability of recombinant mitochondrial endo/exonucleases should facilitate future comparative structural and functional analyses of this class of enzymes.

[1] *Abbreviations used:* 6-FAM, 6-Carboxyfluoresceine; BHQ-1, Black Hole Quencher-1; cpm, counts per minute; ds, double stranded; DTT, DL-dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylenedinitrilotetraacetic acid; EGL-1, EndoG-like 1 (EXOG); HRP, horseradish peroxidase; IPTG, isopropyl thiogalactosid; NTA, nitrilotriacetic acid; oc, open circled; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PEG, polyethylene glycol; Pfu, *Pyrococcus furiosus*; PNK, polynucleotide kinase; rpm, revolution per minute; RT, room temperature, sc, supercoiled; SDS, sodium dodecyl sulfate; ss, single stranded; STE, NaCl/Tris/EDTA; TBE, Tris–borat–EDTA; TCEP, Tris(2-carboxyethyl) phosphine hydrochloride; TED, Tris-carboxymethyl ethylene diamine; TSS, transformation storage solution; TTE, Tris–taurine–EDTA.

Materials and methods

Multiple sequence alignments

Sequences of the aligned mitochondrial endo/exonucleases were retrieved using the basic local alignment search tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the primary sequence of human Endonuclease G as the query. Alignment of the primary structure of selected proteins was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The alignments were then visualized using the GeneDoc (http://www.nrbsc.org/gfx/genedoc/) software package.

Expression cloning of endo/exonuclease genes

The cDNAs coding for mitochondrial Nuc1p from *Saccharomyces cerevisiae* and CPS-6 from *C. elegans* were kindly provided by Frank Madeo (University of Graz, Austria) and Ding Xue (University of Colorado at Boulder, USA), respectively [7,9]. The cDNA encoding human Endonuclease G (IMAGE: 4080883) was obtained from imaGenes GmbH (Berlin, Germany). Cloning of human EXOG (Endonuclease G-like 1) cDNA was described earlier [20]. The cDNAs encoding Nuc1p, CPS-6, human EndoG and EXOG without the coding regions for the respective N-terminal mitochondrial targeting sequences were amplified by PCR using *Pfu* DNA-polymerase and primers adding a CACC-end 5′ to the first requisite codon of each open reading frame in order to perform a directed cloning reaction employing vaccinia virus topoisomerase-activated pET-200/D-TOPO-expression vector using the Champion™ pET200 Directional TOPO® Expression Kit (Invitrogen). Expression vectors containing the desired inserts were sequenced and subsequently used to express recombinant forms of the mitochondrial endo/exonucleases in *Escherichia coli* BL21Star(DE3) cells.

Overexpression of endo/exonuclease genes

In order to produce recombinant mitochondrial endo/exonucleases in *E. coli*, BL21Star(DE3)-cells were transformed with the respective pET-200/D-TOPO-expression vectors and plated on agar plates containing 25 μg/ml kanamycin. A single colony was then picked and used to grow a pre-culture at 37 °C over night in 25 ml LB-broth containing 25 μg/ml of kanamycin. About 10 ml of the pre-culture were used to inoculate 500 ml LB-broth containing 25 μg/ml kanamycin. When the cell density reached an OD^{600} of 0.3–0.6, overexpression was induced by addition of 1 mM (final concentration) of IPTG. After 3 h of induction cells were harvested by centrifugation at 3600*g* at 4 °C. The cells were then washed using STE-buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA) and the wet weight of the cell pellet was measured after recentrifugation and removal of residual STE-buffer.

Purification of recombinant endo/exonucleases from the soluble fraction of E. coli cell lysates

In order to purify recombinant endo/exonucleases from the soluble fraction of *E. coli* cell lysates, cells were lyzed by sonication in buffer A (20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10 mM DTT, and 10% sucrose) supplemented with 20 mM imidazole. The lysate was then transferred into a high speed centrifuge tube and spun 1 h at 4 °C at 31,400*g*. After centrifugation the supernatant containing the soluble fraction of the recombinant enzymes was loaded onto a Protino®Ni-TED column (1000) (Macherey & Nagel) equilibrated with 2 ml buffer A containing 20 mM imidazole. Subsequently the column was washed twice with 2 ml of the same buffer and finally the nucleases were eluted with a total of 1.5 ml buffer A containing 200 mM imidazole.

Extraction and purification of recombinant endo/exonucleases from inclusion bodies

After sonication and centrifugation of the cells most of the protein was found in the pellet due to accumulation in inclusion bodies. For denaturation the inclusion bodies were dissolved over night in buffer B (10 mM Tris–HCl, pH 8.2, 6 M guanidinium chloride, 20 mM 2-mercaptoethanol, 200 mM NaCl, 10 mM imidazole). Insoluble particles were removed by centrifugation and the denatured proteins were batch purified over Ni^{2+}–NTA agarose beads as described elsewhere. In brief, denatured proteins were bound to Ni^{2+}–NTA agarose beads, washed with buffer B containing only 3.5 M guanidinium chloride and eluted with buffer B containing 3.5 M guanidinium chloride, 1 mM EDTA and 200 mM imidazole. All four nucleases were refolded using the iFOLD protein refolding system 2 (Novagen). About 10 μl of the denatured protein were added to 490 μl iFOLD buffer and incubated at room temperature over night. Then activity assays were made using plasmid DNA pBluescript SK(+) as a substrate. Among all buffers from the i-FOLD2 system buffer D3 (50 mM HEPES, pH 7.5, 1.5 M sorbitol, 1 mM TCEP, 24 mM NaCl, 1 mM KCl) gave the best results.

Protein concentration determination

The concentrations of soluble and denatured nucleases were measured using the Bradford assay. Proteins were incubated with Bradford buffer, containing 10 mM Tris–HCl, pH 8.0 and 50 mM NaCl, and Bradford-reagent for 10 min. For calibration a BSA standard was recorded. Measurements were done using a NanoDrop ND1000 spectrophotometer. Percent protein production were calculated using an average *E. coli* cell wet weight of 1×10^{-12} g, an average total cell volume of 1×10^{-15} l as well as assuming an average protein content of 200 g/l of cell wet weight according to statistical information on *E. coli* (http://gchelpdesk.ualberta.ca/CCDB/cgi-bin/STAT_NEW.cgi).

Activity assay with recombinant endo/exonucleases using a molecular beacon as fluorescent substrate

A molecular beacon (5′-6-FAM-CAAGATATCCAGCTGCCCCCCCC CCAGCTGGATATCTTG-BHQ-1-3′) labeled at its 5′ end with 6-FAM (6-Carboxyfluoresceine) and at its 3′ end with BHQ-1 (Black Hole Quencher-1) was used to determine the activities of purified enzymes under various conditions. About 50 nM beacon were incubated with 50 nM of each enzyme in a buffer consisting of 50 mM Tris–HCl, pH 7.0, supplemented with 2.5 mM each of $MgCl_2$ and $MnCl_2$ and 0.001% Tween-20. The change in fluorescence over time was recorded at ambient temperature at a wavelength of 517 nm using a FluoroMax-4 fluorometer (Horiba Jobin Yvon, NJ, USA). The initial slopes of the progress curves were used to calculate reaction velocities.

Activity assays with recombinant endo/exonucleases on radioactively labeled oligonucleotide substrates

The cleavage of radioactively labeled oligonucleotides was analyzed using 25% polyacrylamide gels containing 7 M urea in 0.5× TTE-(Tris–taurine–EDTA) buffer. To analyze whether the recombinant mitochondrial endo/exonucleases exhibit exonuclease activity, endonuclease activity or both, a hairpin oligonucleotide identical in sequence to the molecular beacon, a 14-mer single stranded oligonucleotide (EXOUP-3′: 5′-CGCCAGATCTTCCC-3′) and a gapped substrate consisting of three annealed oligonucleo-

tides (EXOUP-5′: CCAAGATATCAG; EXOUP3′sh2a: CGCCAGATCTTCCC; EXOLO: GGGAAGATCTGGCGCCTGATATCTTGG) were, as indicated by an asterisk in the respective figure, radioactively labeled at the 5′ end with [γ^{32}P]-ATP using polynucleotide kinase or at their 3′ end using [α^{32}P]-dATP and terminal nucleotidyl transferase and subjected to cleavage by the enzymes. Enzymatic assays were done in 50 mM Tris pH 7.0, supplemented with 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$ and 0.001% Tween. The restriction endonucleases EcoRV and PvuII as well as the exonucleases $RecJ_f$ and ExoI were used where appropriate to digest the labeled oligonucleotides in single or double stranded form according to the recommendation of the supplier (NEB), leading, depending on the label, to the production of mono- and tri-nucleotides ($RecJ_f$), mono- and di-nucleotides (ExoI), hexa- and hepta-nucleotides (EcoRV), and dodecanucleotides and 13-mers (PvuII) used as size markers. After the separation of cleavage products by denaturing polyacrylamide electrophoresis data were collected by electronic autoradiography using an Instant Imager (Packard).

Mg^{2+} optima and salt requirements

In order to determine Mg^{2+} and Ca^{2+} optima for the mitochondrial endo/exonucleases the molecular beacon was used as substrate as described above with different $MgCl_2$ or $CaCl_2$ concentrations (0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 25.0 and 50.0 mM) in the reaction buffer. In order to determine salt requirements kinetics were done under optimum Mg^{2+} concentrations for each enzyme with NaCl concentrations from 0, 10, 25, 50, 75, 100, 150, 200–300 mM.

Activity assays with recombinant endo/exonucleases on phage DNAs

Enzymatic assays with supercoiled, open circular, and circular single stranded DNA from phage ΦX174 were done in 50 mM Tris pH 7.0, supplemented with 2.5 mM each of $MgCl_2$ and $MnCl_2$. Cleavage reactions were performed with the indicated amount of recombinant enzymes and 70 ng/μl DNA. Cleavage products were separated by agarose gel electrophoresis and the program ImageJ (http://rsb.info.nih.gov/ij/) was used to quantify individual bands from ethidium bromide stained gels.

pH optima

In order to determine pH optima Na-acetate, Na-phosphate and glycine buffers containing 5 mM Mg^{2+} were used to cover a range from pH 5 to 9 (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0) with single stranded virion DNA as substrate analyzed on agarose gels as described above.

Results and discussion

Expression and purification of recombinant mitochondrial endo/exonucleases

For expression of recombinant mitochondrial endo/exonucleases we cloned the respective cDNAs into the T7-promoter containing vector pET-200/D-TOPO by directed topoisomerase-mediated recombination. Previous attempts to use less stringently controlled *E. coli* expression systems led to genetic instability of transformands pointing towards cytotoxic effects by mitochondrial endo/exonucleases due to leaky expression of a cloned cDNA (data not shown). Using the T7 expression system we could overexpress all four nucleases, Nuc1p, CPS-6, EndoG and EXOG in amounts from 9% up to 18% of total *E. coli* protein (Fig. 1B and Table 1). Though most of the overexpressed protein accumulated in inclusion bodies we were able to recover all four nucleases in active form from the soluble fractions of *E. coli* cultures expressing the cDNAs, albeit in small amounts (Fig. 1C and Table 1). Nevertheless, these soluble enzymes were active and stable for several weeks when kept at 4 °C and stable for several months when stored at −20 °C. The identity of the recombinant enzymes was checked by Western blotting using an antibody directed against the N-terminal α-Xpress epitope fused to the enzymes. Critical to a successful recovery of the nucleases from the soluble fraction was the use of a buffer containing high amounts of the zwitterionic detergent CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), DTT (1,4-dithiothreitol) and sucrose (buffer A, see Materials and methods section). In addition to trying different host-vector systems and protein fusions including the glutathione S-transferase (GST) and the maltose binding protein (MBP) we also tried to express mitochondrial endo/exonucleases at low temperature or with varying IPTG-concentrations employing the Tuner™ strain, a lacZY deletion mutant of BL21. None of these attempts resulted in a higher fraction of soluble enzyme than overexpression of the four target proteins via the established system presented here. We can only speculate that the particular host-vector system used (pET-200/D-TOPO and BL21Star(DE3)), which is based on selection by resistance to kanamycin, provides a superior control over the cloned genes and that the combination of the His-tag together with the Xpress-epitope tag rich in aspartic acid residues exerts a kind of "supercharging effect" that helps the nucleases to gain solubility. This theory is supported, though not proven, by the fact that the same enzymes do not behave as well when expressed from the pET160/GW/D-TOPO vector that is based on ampicillin selection and provides a His-tag with a different amino acid composition.

Since the predominant fraction of the overexpressed enzymes accumulated in inclusion bodies we also pursued a strategy to

Table 1
Yield and relative activity of recombinant mitochondrial endo/exonucleases.

Protein	Average pellet wet weight per 500 ml *E. coli* culture[a] (g)	% protein[b]	Soluble protein[c] (μg g^{-1} wet weight)	Relative activity soluble protein[d]	Insoluble protein[e] (mg g^{-1} wet weight)	Relative activity solubilized protein[d,f]
Nuc1p	0.99	18.4	52	0.97	36.7	1.00
CPS-6	1.06	9.7	129	0.47	19.1	0.16
EndoG	1.24	10.2	47	0.02	20.3	0.04
EXOG	1.06	14.6	96	0.08	29.0	0.69

[a] From 3× 500 ml *E. coli* cell culture.
[b] % overexpressed protein of total protein calculated using an average *E. coli* cell wet weight of 1×10^{-12} g, an average total cell volume of 1×10^{-15} l as well as assuming an average protein content of 200 g/l of cell wet weight according to statistical information on *E. coli* available at: http://gchelpdesk.ualberta.ca/CCDB/cgi-bin/STAT_NEW.cgi.
[c] After IMAC.
[d] As determined from the initial velocity in the fluorescence assays.
[e] After solubilization from inclusion bodies.
[f] After IMAC and refolding in buffer D3.

prepare active nucleases by extraction from inclusion bodies and subsequent refolding of the solubilized proteins (Fig. 1C). To this end we batch purified the solubilized inclusion body proteins via IMAC over Ni^{2+}–NTA agarose under denaturing conditions using guanidinium chloride containing buffers. We then used commercially available medium throughput refolding assays (i-FOLD systems) for renaturation. Using the i-FOLD system 2 comprising 96 different buffers including controls we found several conditions under which the individual recombinant nucleases could be renatured either alone or in different combinations (Fig. 1D). Two hits were found for buffers allowing refolding of three nucleases under the same conditions and one hit was found allowing the refolding of all four nucleases under a single buffer condition (buffer D3, Fig. 1D). A successful refolding was monitored by the digestion of plasmid DNA with the refolded enzymes and analysis of the cleavage products by electrophoresis on 0.8% agarose gels and subsequent ethidium bromide staining (data not shown). To be more sensitive in the detection of nuclease activity in this cleavage assay, and in following assays if not stated otherwise, we used a mixture of Mg^{2+} and Mn^{2+} as cofactors for the mitochondrial endo/exonucleases since it is known from previous studies of related nucleases that *in vitro* these show highest activity with transition metals such as Mn^{2+} or Co^{2+} instead of Mg^{2+} without changing their substrate or sequence preferences [20,21].

Activity of recombinant mitochondrial endo/exonucleases

To determine the relative activity of the purified enzymes we used a molecular beacon as a substrate in a fluorescence activity assay (Fig. 2A). Upon cleavage of the substrate that forms a hairpin structure by the endo/exonucleases, the quencher (BHQ-1) and the fluorescent dye (6-FAM) become separated and a fluorescence signal can be detected. This assay revealed that nucleases purified from the soluble fraction of *E. coli* cell lysates as well as those extracted from inclusion bodies and refolded in buffer D3 were all able to cleave the molecular beacon substrate, albeit with different activities (Fig. 2B and Table 1). It should be noted that we chose the molecular beacon as a fluorescent substrate in order to establish a convenient assay to quickly monitor the activity of preparations of recombinant endo/exonuclease in real time, though the beacon is an artificial substrate bearing highly modified ends. The relative activities towards the molecular beacon thus do not reflect relative activities towards natural substrates preferred by a particular mitochondrial endo/exonuclease. As can be seen below (Fig. 3) the enzymes indeed cleave the molecular beacon at different positions displaying different sequence and/or structural preferences. Under the conditions applied, Nuc1p showed the highest cleavage activity towards the beacon substrate with nearly identical values for the refolded and the soluble recombinant enzyme, whereas EndoG showed the lowest activity. Notably, EXOG from the soluble fraction was nearly 8-fold less active than renatured EXOG, strongly suggesting that the enzyme purified from the soluble fraction contained a substantial amount of soluble molecules, which did not acquire a catalytically competent state and hence are inactive. With the exception of Nuc1p none of the refolded enzymes displayed a higher catalytic activity when refolded in any other buffer than buffer D3. However, when Nuc1p is refolded in buffer B6 an approximately 9-fold higher activity towards the molecular beacon substrate can be detected, again suggesting that the preparation of soluble protein contains high amounts of catalytically incompetent molecules. Whether this is due to a molten globule state and/or an impaired quaternary structure of the enzyme is not clear. In contrast, CPS-6 was nearly 3-fold more active when purified from the soluble fraction as compared to the refolded enzyme (for details see Table 1).

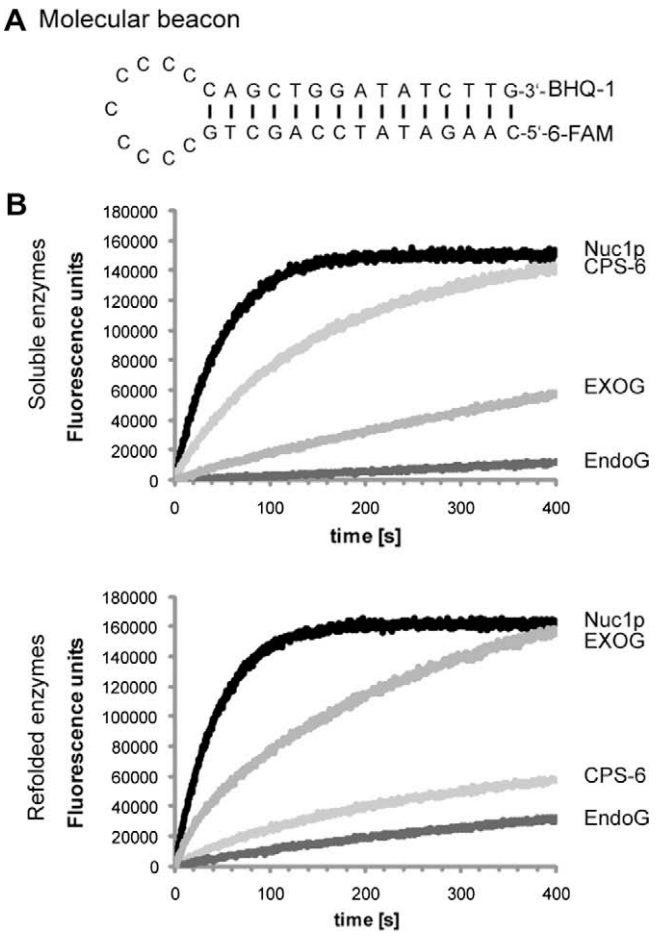

Fig. 2. Fluorescence activity assay. (A) Molecular beacon used as a substrate for recombinant mitochondrial endo/exonucleases. (B) Progress curves (fluorescence units *vs.* time) for the cleavage of the molecular beacon substrate (50 nM final concentration) by the recombinant endo/exonucleases (50 nM final concentration each; upper panel, soluble enzymes; lower panel, refolded enzymes).

Biochemical properties of recombinant mitochondrial endo/exonucleases

In order to compare the biochemical properties such as cofactor concentration optima, salt requirements and pH-dependencies of Nuc1p, CPS-6, EndoG and EXOG we used the fluorescence assay described above as well as the cleavage of single stranded virion DNA under varying conditions. The data revealed that with the exception of CPS-6 all other nucleases exhibit a very broad Mg^{2+}-concentration optimum with highest activity at 2.5 mM Mg^{2+} for Nuc1p, 0.2 mM for CPS-6, 1.0 mM for EndoG and 5.0 mM for EXOG. Thus at physiological concentrations of free Mg^{2+} (0.8–1.0 mM) all enzymes are able to catalyze phosphodiester bond cleavage with almost maximum activity (Table 2). EXOG was the only enzyme to show considerable activity in the presence of Ca^{2+} with an optimum concentration of 1.0 mM (Table 2). Interestingly, although these related nucleases share a similar ββα-Me-finger active site structure their affinity for individual metal ions can vary strongly, as also nicely demonstrated for the restriction endonuclease KpnI, which also contains the ββα-Me-finger motif [22–24]. As reported earlier for EndoG and EXOG we found that all four mitochondrial endo/exonucleases require no or relatively low concentrations of monovalent cations for maximum activity [25,26]. At physiological salt concentrations (100–150 mM NaCl) all enzymes exhibit cleavage activities below 50% of their individual maximum activities. Apparently, monovalent cations electrostatically interfere strongly

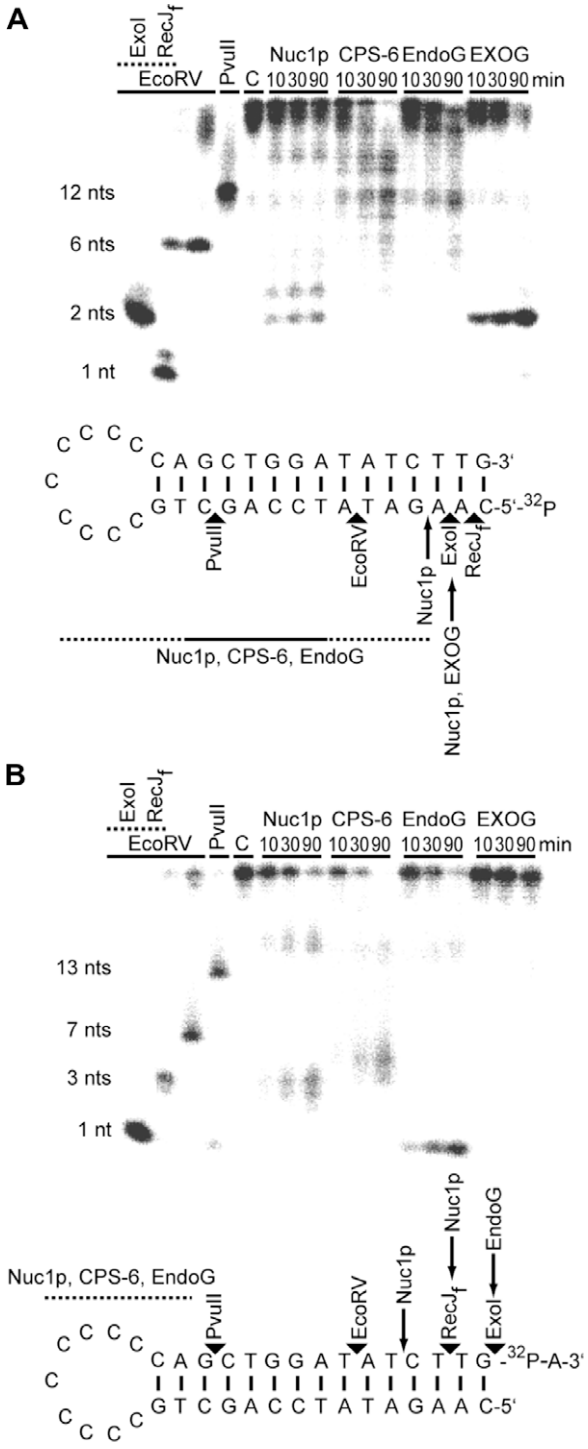

Fig. 3. Cleavage of a hairpin substrate similar in sequence to the molecular beacon. (A) A hairpin substrate similar in sequence to the molecular beacon radioactively labeled at its 5′ end with [γ^{32}P]-ATP was subjected to cleavage with the four nucleases (70 nM Nuc1p, 140 nM CPS-6, 120 nM EXOG, and 600 nM EndoG for 10, 30 and 90 min). Whereas Nuc1p and EXOG preferentially produce fragments arising from cleavage close to the 5′ end of the labeled substrate (di- and tri-nucleotides), CPS-6 and EndoG predominantly show endonucleolytic cleavage. As a control the substrate was also cleaved with the restriction enzymes EcoRV or PvuII to produce 6 nt and 12 nt markers and with ExoI and $RecJ_f$ after EcoRV cleavage to produce 2 nt and 1 nt markers. (B) Cleavage of the hairpin substrate radioactively labeled at its 3′ end with [α^{32}P]-dATP. As a control the substrate was also cleaved with the restriction enzymes EcoRV or PvuII to produce 7 nt and 13 nt markers and with ExoI and $RecJ_f$ after EcoRV cleavage to produce 1 nt and 3 nt markers. In this assay concentrations of the endo/exonucleases were normalized to their relative activities towards the molecular beacon as measured in the fluorescence assay (see Table 1).

Table 2
Biochemical properties of recombinant mitochondrial endo/exonucleases.

Protein	Mg^{2+} optima (mM)	Ca^{2+} optima (mM)	NaCl requirement (mM)	pH optima (ssDNA)
Nuc1p	2.5 (0.2–7.5)[a]	n.a.	0[b]	7.5
CPS-6	0.2 (0.2–1.0)[a]	n.a.	0[b]	6.0
EndoG	1.0 (0.5–5.0)[a]	n.a.	0[b]	5.5
EXOG	5.0 (1.0–12.5)[a]	1 (0.2–2.5)[a]	25[b]	5.0

[a] Range with more than 75% activity.
[b] Above 100 mM NaCl all nucleases range below 50% of their individual maximum activity.

with the mode of substrate binding of this class of non-specific nucleases which contact nucleic acid substrates mainly through electrostatic interactions with the phosphodiester backbone. As known from previous studies all recombinant mitochondrial endo/exonucleases cleaved single stranded, not, however, double stranded DNA with maximum activity at slightly acidic pH, further confirming previously reported data for these enzymes isolated from other sources [17,21,27].

Recombinant mitochondrial endo/exonucleases exhibit different degrees of endo- and exo-nuclease activity

In order to determine the substrate specificity of evolutionary conserved mitochondrial endo/exonucleases we used ΦX174-DNA as a substrate and subjected it to cleavage by all four recombinant nucleases. All of them preferred the single stranded virion DNA over the double stranded replicative forms I (supercoiled) and II (open circular) confirming results obtained previously for Nuc1p, EndoG and EXOG. In this assay we used different concentrations of the mitochondrial endo/exonucleases in order to obtain similar rates for the cleavage of the preferred single stranded substrate (data not shown). In addition, we also used a DNA substrate similar in sequence to the molecular beacon yet without fluorescent dye and quencher and radioactively labeled it at its 5′ or 3′ end (Fig. 3). In these assays we also used different concentrations of each enzyme normalized to their relative activities towards the molecular beacon as determined in the fluorescence assays, in order to be able to detect cleavage products also from those nucleases that displayed very little activity towards this substrate. The analyses of the cleavage of the radioactively labeled hairpin substrate by denaturing polyacrylamide gel electrophoresis showed that the four related nucleases exhibit marked differences in their endo- and exo-nuclease activities. As can be seen from the autoradiography of a denaturing (7 M urea) 25% polyacrylamide gel Nuc1p and EXOG predominantly cleave the 5′-labeled substrate close to the 5′ end mainly producing di- (Nuc1p and EXOG) and tri-nucleotides (Nuc1p) in addition to endonucleolytic cleavage in the double stranded (Nuc1p and EXOG) and single stranded (Nuc1p) region of the substrate. In contrast, CPS-6 and EndoG display endonuclease activity within the single and double stranded region of the substrate with no detectable (EndoG) or very little (CPS-6) production of trinucleotides (Fig. 3A). When the 3′-labeled substrate was analyzed it turned out that Nuc1p produces tri- and tetra-nucleotides and also cleaves the substrate in the single stranded region (Fig. 3B). CPS-6 cleavage of the 3′-labeled substrate results in products of a length of 4–6 nucleotides and similar to Nuc1p the enzyme also cleaves in the single stranded region. Whereas EXOG only produces fragments running close to the substrate band, as indicated by a smear below which very likely results from cleavage close to the 5′ end, EndoG very efficiently cleaves off the 3′-overhanging label in addition to cleavage in the

single stranded region of the substrate. This 3′ overhang is a result of the 3′ labeling with [α^{32}P]-dATP and terminal transferase and thus not present in the fluorescent dye and quencher labeled molecular beacon.

Since the enzymes preferred single stranded virion DNA and cleaved the single stranded region of the hairpin substrate we also investigated a small single stranded 5′-labeled oligonucleotide substrate in order to determine the ability of the four nucleases to cleave a single stranded substrate endo- or exo-nucleolytically (Fig. 4). As observed for the 5′-labeled hairpin substrate Nuc1p and EXOG showed a pronounced 5′–3′-exonuclease activity whereas CPS-6 displayed endonuclease activity only (Fig. 4A, upper panel). Notably, Nuc1p produces dinucleotides due to preferred cleavage close to the 5′ end in addition to endonucleolytic products, whereas EXOG only produces mono and dinucleotides. Intriguingly, though being active towards the molecular beacon at high enzyme concentration (see above) and also highly active towards the single stranded virion DNA (Fig. 4B), EndoG did not cleave the small single stranded DNA substrate even at high enzyme concentration, suggesting that in contrast to all other endo/exonucleases investigated in this study EndoG from our preparation is not able to bind and process short single stranded oligonucleotides. Whether this reflects an aspect of the division of labor between the paralogs EndoG and EXOG in mammals currently remains unclear and needs further investigation.

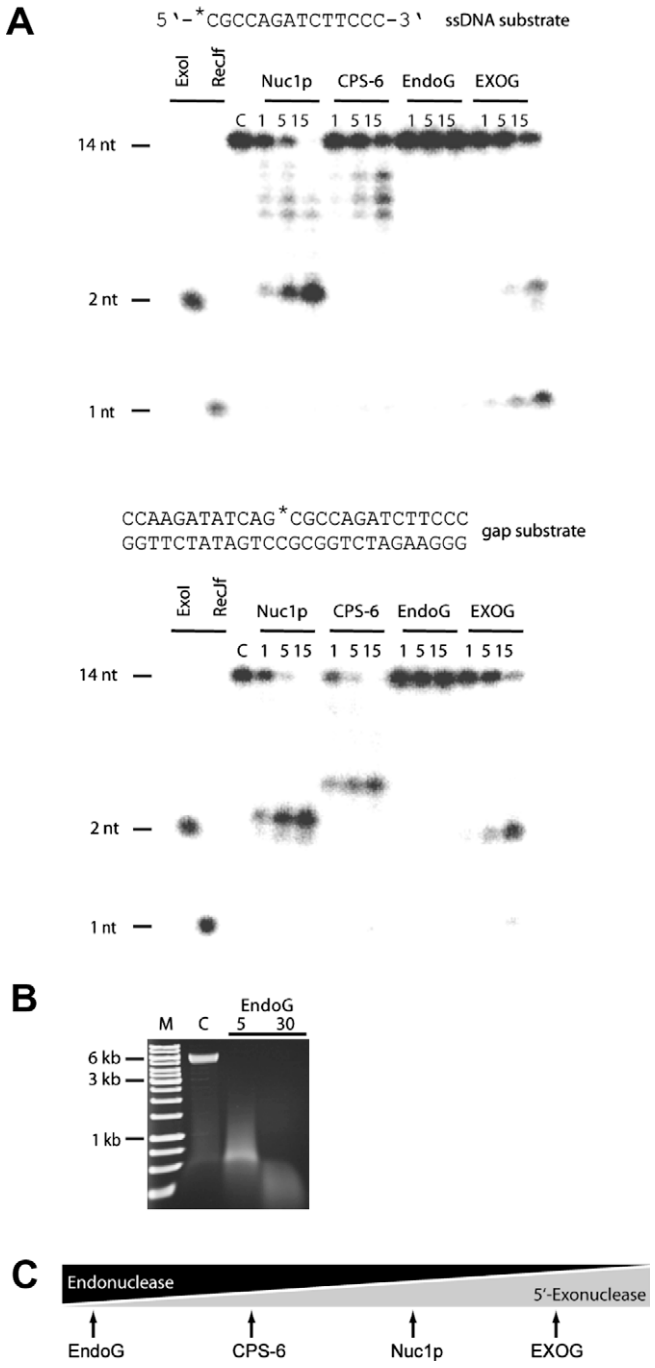

Fig. 4. Cleavage of a 5′-labeled single stranded oligonucleotide and a gapped substrate. (A) Nuc1p and EXOG show 5′–3′-exonuclease activity producing mono- and di-nucleotides upon cleavage of single stranded DNA whereas CPS-6 only displays endonuclease activity (upper panel). Nuc1p, CPS-6 and EXOG also show activity on the gapped substrate whereas EndoG does not. Under the conditions applied EndoG is neither able to process the short single stranded oligonucleotide nor the gapped double stranded substrate, though the enzyme from the same preparation is highly active towards single stranded virion DNA as shown in (B). The asterisk denotes the position of the 5′-label. (C) Schematic representation of the distribution of endo- and 5′–3′-exo-nuclease activity in the mitochondrial endo/exonucleases Nuc1p, CPS-6, EndoG and EXOG.

Nuc1p, CPS-6 and EXOG, not, however, EndoG, exhibit activity on gapped substrates

It was previously suggested that Nuc1p uses its 5′–3′-exonuclease activity to widen gaps in double stranded DNA, an activity that could support mitochondrial DNA-repair and/or recombination. We have therefore generated a gapped substrate using oligonucleotides and investigated the potential of Nuc1p, CPS-6, EndoG and EXOG to extend the gap. As can be seen from Fig. 4A (lower panel), Nuc1p, CPS-6 and EXOG exhibit activity on gapped substrates, producing di- and tri-nucleotides. In contrast, EndoG does not process this substrate.

Taken together we achieved the successful overexpression, purification and comparative biochemical characterization of Nuc1p, CPS-6, EndoG and EXOG, four related members of the class of so-called mitochondrial endo/exonucleases. The established expression and purification protocol should facilitate future structural and functional studies of these enzymes, which play an important role in apoptotic DNA-fragmentation as well as DNA-repair and recombination. Our comparative biochemical analyses demonstrates that the evolutionary conserved mitochondrial nucleases differ in their ability to process DNA substrates endo- and exo-nucleolytically (Fig. 4C) and suggest that the mechanisms of action of these related nucleases in cell death as well as DNA-repair and recombination might differ according to their enzyme activity and substrate specificities.

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