Engineered apoptotic nucleases for chromatin research

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INTRODUCTION

Eukaryotic genomic DNA is organized into repeating arrays of nucleosomes that are the structural units of chromatin (1). The nucleosome is also known to play a central role in regulating gene transcription from promoters (2). For more than 30 years, micrococcal nuclease (MNase), DNase I and restriction endonucleases have been essential tools for chromatin research (1). MNase in particular has been the gold standard for characterizing nucleosomal structures in chromatin, because of its relative high specificity toward the internucleosomal linker region. However, this enzyme also digests DNA within nucleosome core particles, attacks single-stranded nucleic acids, possesses exonuclease activity and has significant DNA sequence cleavage preferences (3–5).

One of the hallmarks of theterminal stages of programmed cell death or apoptosis is internucleosomal DNA breakdown (6). The endonuclease primarily responsible for mediating apoptotic nucleosomal DNA laddering is DNA fragmentation factor-40 (DFF40), also called caspase-activated deoxyribonuclease (CAD) (7–10). DFF is a heterodimer in its inactive form, composed of a 45-kDa inhibitor and chaperone subunit (DFF45), also called inhibitor of CAD (ICAD), and a 40-kDa latent endonuclease subunit (DFF40/CAD). Upon caspase-3 cleavage of DFF45, DFF40 forms active endonuclease homo-oligomers. Although Saccharomyces cerevisiae lacks DFF, expression of caspase-3 is lethal in this organism, but expression of the highly sequence-specific tobacco etch virus protease (TEVP) is harmless. Therefore, we inserted TEVP cleavage sites immediately downstream of the two caspase-3 cleavage sites within DFF45, generating a novel form of DFF (DFF-T) whose nuclease activity proved to be exclusively under the control of TEVP. We demonstrate that co-expression of TEVP and DFF-T under galactose control results in nucleosomal DNA laddering and cell death in S. cerevisiae. We also created synthetic DFF genes with optimized codons for high-level expression in Escherichia coli or S. cerevisiae. We further demonstrate the excellence of the synthetic gene products for in vitro mapping of the nucleosome positions and hypersensitive sites in specific genes such as the yeast PHO5.

ABSTRACT

We have created new genomics tools for chromatin research by genetically engineering the human and mouse major apoptotic nucleases that are responsible for internucleosomal DNA cleavage, DNA fragmentation factor (DFF). Normally, in its inactive form, DFF is a heterodimer composed of a 45-kDa chaperone inhibitor subunit (DFF45 or ICAD), and a 40-kDa latent endonuclease subunit (DFF40 or CAD). Upon caspase-3 cleavage of DFF45, DFF40 forms active endonuclease homo-oligomers. Although Saccharomyces cerevisiae lacks DFF, expression of caspase-3 is lethal in this organism, but expression of the highly sequence-specific tobacco etch virus protease (TEVP) is harmless. Therefore, we inserted TEVP cleavage sites immediately downstream of the two caspase-3 cleavage sites within DFF45, generating a novel form of DFF (DFF-T) whose nuclease activity proved to be exclusively under the control of TEVP. We demonstrate that co-expression of TEVP and DFF-T under galactose control results in nucleosomal DNA laddering and cell death in S. cerevisiae. We also created synthetic DFF genes with optimized codons for high-level expression in Escherichia coli or S. cerevisiae. We further demonstrate the excellence of the synthetic gene products for in vitro mapping of the nucleosome positions and hypersensitive sites in specific genes such as the yeast PHO5.

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yeast is lethal, but expression of the highly sequence-specific TEVP is harmless (26–28). Thus, TEVP expression per se should not cause any artificial alterations in chromatin structure and gene expression.

We demonstrate here the successful expression of recombinant forms of these proteins in either Escherichia coli or Saccharomyces cerevisiae and that after TEVP cleavage highly active DFF endonuclease is generated. We further demonstrate that DFF-T is an excellent reagent for mapping nucleosome positions and hypersensitive sites in specific genes as revealed by chromatin footprinting of the well-studied PHO5 gene, whose promoter and upstream region displaces four nucleosomes upon transcriptional induction via chromatin remodeling and binding of Pho4p (29–33).

MATERIALS AND METHODS

Generation of mouse or human DFF nuclease activities dependent on TEVP cleavage by mutagenesis and expression in E. coli

The TEVP cut site (25), encoding the amino acid sequence ENLYFQS, was inserted between amino acid positions 117–118 and 224–225, immediately downstream of each of the two caspase-3 cleavage sites in the coding sequence of mouse DFF45. We created all three possible combinations of these insertion mutations, termed I1WT, WT12 and I1I2. Similarly, we created the corresponding I12 human DFF45 species, and also substituted the caspase-3 cleavage sites with TEVP cut sites in all combinations in mouse DFF45. Using the two-plasmid E. coli expression system for DFF (34) kindly provided by Gregor Meiss, the individual modified forms of mouse DFF45 along with the corresponding wild-type control were co-expressed with mouse GST-DFF40. The resulting GST-fusion protein-containing complexes were purified on GSH-Sepharose. Human DFF species were cloned into pRSF2duet™ for co-expression in E. coli (Novagen) and purification by nickel chromatography.

Assay of DFF45 cleavage and DFF40 endonuclease activity on plasmid DNA substrate

Recombinant caspase-3 was prepared as previously described (9), and TEVP was purchased from Invitrogen. One microgram of either wild-type, swap or insertion mutated DFF40/DFF45 heterodimer were incubated with caspase-3 or 0.5 unit of TEVP at 37°C in reaction buffer consisting of 10 mM KCl, 100 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol and 20 mM Tris–Cl, pH 7.5. After 20 min of incubation, samples were separated on 12% polyacrylamide–SDS gels, and then proteins were electrophoretically transferred onto nitrocellulose membranes. Membrane-immobilized proteins were probed with the following commercial antibodies: rabbit anti-human DFF40 polyclonal antibodies and/or rabbit anti-human DFF45 N-terminus polyclonal antibodies (Pharmingen). The antigen–antibody complexes were visualized using enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham Biosciences). For the endonuclease activity assay, 1 μg of naked plasmid DNA was incubated for 30 min at 37°C with DFF nuclease species pre-incubated with appropriate protease, as indicated in the legends of Figure 1. Aliquots of the endonuclease reaction were stopped by gel loading buffer containing 0.6% SDS, 50 mM EDTA, 30% glycerol and samples were then separated on 1.5% agarose gels using TAE as the running buffer. After electrophoresis, DNA was stained with ethidium bromide and gels were scanned with a FluorImager (Molecular Dynamics Inc., Sunnyvale, CA, USA).

Expression of mouse DFF-T and TEVP in S. cerevisiae

The TEVP gene’s coding region with SV40 nuclear localization signals added to its N- and C-termini, under the control of the GAL1 promoter, and cloned into a TRP1 integration vector (28), which was kindly provided by Kim Nasmyth and used to stably transform the yeast P2 strain (35). DFF40 and wild-type DFF45 or the other three insertion mutants, I1WT, WT12 and I1I2, were cloned into pESC-His dual yeast expression vector (Novagen) containing two divergently orientated galactose inducible GAL1 and GAL10 promoters. The pESC-DFF vectors were introduced into yeast P2 strains containing or lacking integrated TEVP via standard lithium acetate transformation and selection on synthetic complete medium lacking histidine and tryptophan, supplemented with 2% dextrose. To assay the effects of TEVP and DFF expression on cell viability, exponentially growing cultures of individual transformants were serially diluted 10-fold in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 5 μl aliquots were spotted onto selective media supplemented with 2% raffinose and 2% galactose. The number of viable cells forming colonies at 30°C was determined. For in vivo endonuclease activity assay, yeast genomic DNA was isolated and analyzed. Briefly, 10 ml cultures of yeast cells transformed as indicated in Figure 2A, were harvested by centrifugation following galactose induction for 6 h. The cells were washed with 1 ml of distilled H2O, re-suspended in 200 μl of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–Cl, pH 8.0, 1 mM EDTA, pH 8.0, vortexed at highest speed for 3 min with 200 μl glass beads and 200 μl phenol/chloroform/isoamyl alcohol, followed by the addition of 200 μl TE buffer and centrifugation for 5 min. The genomic DNA in the aqueous layer was ethanol precipitated and re-suspended and treated with 100 μg/ml RNase for 20 min at 37°C. The DNA was phenol extracted, ethanol precipitated and re-suspended in 50 μl TE buffer. The integrity of 5 μg DNA samples was assessed by agarose gel electrophoresis.

Generation of synthetic genes for high-level expression of DFF in E. coli

We took advantage of the ‘Center for Invention’ resource at UT Southwestern to generate a computer-assisted prediction for the design of synthetic genes and their robotic synthesis from pairs of overlapping 40-mer oligonucleotides (36). We have created, through this core, synthetic genes for both mouse and human forms.
of DFF40 and DFF45. After ligation of oligonucleotides, we amplified the products with high-fidelity PCR in preparation for cloning and sequencing. Any mutations that occurred were cured by additional high-fidelity PCR reactions with appropriate primers and repeated subcloning and sequencing. We have designed these synthetic genes to possess hexa-his tags at the C-terminal ends of DFF40 species, to either lack or possess the TEVP-cut sites as described below (sequences will be made available upon request), as well as the appropriate restriction sites for cloning into pRSFDuet™ E. coli expression vectors (Novagen). Escherichia coli BL21(DE3) cells transformed with this plasmid were induced with 0.5 mM IPTG for 3 h when the optical density at a wavelength of 600 nm (OD600) was 0.5. Cells were lysed by thawing in wash buffer solution (WB; 50 mM NaH2PO4, 300 mM NaCl containing lysozyme (1 mg/ml), RNase A (10 μg/ml) and DNaseI (5 μg/ml). Lysates were clarified by centrifugation and allowed to bind to nickel affinity resin (Ni-NTA superflo, Qiagen) for 1 h at 4°C with gentle mixing. After washing with WB, the protein was eluted with elution buffer solution (10 mM NaH2PO4, 100 mM NaCl, pH 8.0, 250 mM imidazole).

Mapping nucleosome positions on the inactive and induced PHO5 gene’s upstream and coding regions

Saccharomyces cerevisiae strain W303 was grown over night at 30°C in yeast nitrogen base phosphate-free medium. Cells were centrifuged, suspended in fresh medium, either phosphate-free or supplemented with 14 mM KH2PO4 and grown for an additional 9 h. Cells were centrifuged and washed with water. One g of wet cell pellet was suspended in 2 ml of yeast lysis buffer (1 M sorbitol, 5 mM β-mercaptoethanol supplemented with 2 mg/ml of Zymolase 100T, ICN) and incubated at room temperature for 3–4 min with occasional agitation in 2-ml round-bottom microcentrifuge tubes. Cells were centrifuged for 15 s, washed in 1 M sorbitol, then suspended in 4 ml of digestion buffer (1 M sorbitol, 50 mM NaCl, 10 mM Tris–Cl pH 7.6, 5 mM MgCl2, 1 mM CaCl2, 1 mM β-mecaptoethanol, 0.5 mM spermidine, 0.075% NP-40) supplemented with 2 μg/ml of RNaseA and incubated for 15 min at room temperature (DFF is inhibited by RNA) (37). Aliquots measuring 400 μl of the resulting suspension were mixed with 100 μl of MNase ( Worthington, 1 or 2 U) in digestion buffer. Alternatively, 400 μl aliquots of the resulting suspension were diluted with 400 μl of digestion buffer (1 M sorbitol, 50 mM NaCl, 10 mM Tris–Cl pH 7.6, 5 mM MgCl2, 1 mM CaCl2, 1 mM β-mecaptoethanol, 0.5 mM spermidine, 0.075% NP-40) supplemented with 2μg/ml of RNaseA and incubated for 15 min at room temperature (DFF is inhibited by RNA) (37). Aliquots measuring 400 μl of the resulting suspension were mixed with 100 μl of MNase ( Worthington, 1 or 2 U) in digestion buffer. Alternatively, 400 μl aliquots of the resulting suspension were diluted with 400 μl of digestion buffer and mixed with 500 μl solution of TEVP-activated human DFF-T (100 or 200 pmol) in digestion buffer (100pmol of DFF-T was pre-incubated with 50 U of AcTEV protease for 15 min at room temperature). Digestion mixtures were incubated for 10 min at 33°C, and then reactions were stopped by adding 1/5 vol of stop solution (2% SDS, 100 mM EDTA and 2 mg/ml protease K). Mixtures were incubated for 6 h at 50°C and for additional 4 h at 65°C, then phenol/chloroform extracted and isopropanol precipitated. DNA was...
purified by routine RNaseA treatment, phenol/chloroform extractions and isopropanol precipitation. DNA concentration was assessed by fluorometry; usually 25 mg of DNA was recovered from 0.1 g of wet yeast cell pellet. Ten micrograms of purified DNA was digested with ApaI restriction enzyme (50 U for 15 h at 28°C), electrophoretically separated on a 1.6% agarose gel and transferred onto Zeta-probe (Bio-Rad) nylon membrane using alkali (0.4 M NaOH, 1.5 M NaCl). The membrane was prehybridized for 4 h at 64°C with 10% SDS, 1 mM EDTA and then hybridized over night in the same buffer supplemented with 0.1 mg/ml of denatured calf thymus DNA and 10 ng/ml of 32P-labeled PCR-amplified PHO5 probe, which was a 200-bp fragment adjacent to the ApaI site generated with the following primers 5'-GTC TTC AGC GTC AAC TTT AG-3' and 5'-TAA CGA TGA TGG TCC CTT AA-3' (32).

**RESULTS AND DISCUSSION**

Generation of TEVP-cleavage-dependent DFF nuclease activity

In an attempt to convert mouse DFF's nuclease activity to be dependent on TEVP cleavage, we first swapped one or both of the caspase-3 cleavage sites within DFF45 with a TEVP recognition sequence (Figure 1A). After co-expression of the different recombinant forms of DFF in E. coli and purification of the heterodimers, we separately assayed the cleavage pattern of DFF45 mutant forms by either caspase-3 or TEVP (or both) via western bloting, and the activity of DFF40 nuclease by digestion of a plasmid DNA substrate. As shown in Figure 1B, the various forms of DFF45 were cleaved as expected by the appropriate proteases. However, in spite of the fact that the double swap mutant was effectively cleaved by TEVP (Figure 1B, lane 12), DFF40 nuclease activity was only weakly generated compared with the wild-type control after cleavage by caspase-3 ± TEVP (Figure 1C, compare lanes 8 and 12 with 9). Interestingly, the SWT but not the WTS2 mutant was also fully activated after cleavage by both proteases (Figure 1C, compare lanes 10 and 11). It therefore appears as if maintenance of the second caspase-3 cleavage site is most important for maintaining DFF45 chaperone function. We conclude that the swapped amino acid sequence at caspase-3 cleavage sites within DFF45 apparently plays a chaperone role in folding DFF40 into a potential nuclease capable of activation.

Because the caspase-3 sites within DFF45 are important for its chaperone activity, we created another series of DFF45 mutants in which the TEVP cleavage sequence was inserted immediately downstream of the caspase-3 cleavage sites (Figure 1D). Surprisingly, even though the caspase-3 recognition sequences DEPD and DAVD in mouse DFF45 were not altered by the adjacent insertions of the TEVP sites, the corresponding caspase-3 sites are no longer cleavable by that enzyme, as demonstrated by the maintenance of intact DFF45 I1I2 mutant protein after caspase-3 treatment, as revealed by western blotting (Figure 1E, lane 8), and by the inability of caspase-3 alone to activate the nuclease (Figure 1F, lanes 2–4). We conclude that the ability of caspase-3 to cleave at these sites is dependent on the amino acid sequence context and that the additional seven amino acids may affect the spatial properties of the caspase-3 cleavage sites. As shown in Figure 1F (lane 8), only the heterodimer containing the double-TEVP-site-insertion mutant I1I2 could be activated by TEVP treatment alone to a specific activity nearly equal to that of the caspase-3 ± TEVP treated wild-type enzyme (Figure 1F, lanes 1 and 9). We conclude that a novel modified form of mouse DFF nuclease (DFF-T) has been successfully generated, expressed and purified, whose activation is specifically under the control of TEVP.

Co-expression of TEVP and DFF-T in S. cerevisiae

We intended to develop a highly reproducible system in yeast for the regulated expression and activation of
DFF-T for the purposes of in vivo footprinting of the positions of nucleosomes and transcription factors on specific genes. Previously, Simpson and Wang (38) were successful in developing a DNase I expression system in yeast for in vivo footprinting experiments by putting the DNase I gene into a high copy shuttle expression vector under the control of the GAL1 promoter. Here we have taken advantage of a system developed by Nasmyth and co-workers (28), who engineered the TEVP gene’s coding region by adding SV40 nuclear localization signals to its N- and C-termini, which was placed under the control of the GAL1 promoter, and integrated into the trp1 locus. We therefore created a yeast strain with a galactose-inducible TEVP gene. We next inserted various engineered forms of mouse DFF45 and mouse DFF40 genes into the bicistronic, high copy number shuttle expression vector pESC-HIS (Stratagene), which divergently expresses introduced sequences from GAL1-10 promoters, and transformed yeast strains that either lacked or possessed the TEVP gene under GAL control with these expression vectors, and optimized expression in this system by titration with different concentrations 3-amino-1,2,4-triazole. As shown by the plating assay in Figure 2A, expression of TEVP together with various engineered forms of mouse DFF results in cell death only when the DFF45 I1I2 mutant and DFF40 are co-expressed together with TEVP. It is significant that this in vivo result is in agreement with the in vitro activation data for the corresponding recombinant proteins expressed in E. coli (Figure 1F above). Furthermore, yeast DNA undergoes nucleosomal laddering in vivo in a galactose-dependent fashion, only in the strain harboring the genes encoding the DFF45 I1I2 mutant, DFF40 and TEVP (Figure 2B, lane 8). We conclude that this form of DFF expression could be useful as a genomics tool to study yeast chromatin structure in vivo. It should be noted, however, just as in the case of the DNase I yeast expression system developed by Simpson and Wang (38), there is ca. 6-h time lag after GAL induction before significant DNA breakdown occurs, and it would be most desirable to be able to much more rapidly activate the nuclease to obtain a snapshot of the in vivo chromatin structure. This might be achieved by the high-level constitutive expression of codon-optimized DFF-T prior to GAL induction of TEVP. In addition, it may be possible to engineer TEVP for rapid regulated transport from the cytoplasm to the nucleus. A further limitation of DFF, particularly in the yeast system, is that these cells have a very high RNA/DNA ratio and RNA is an inhibitor of the enzyme (37).

Creation of synthetic DFF genes and their high-level expression in E. coli

Many codons in DFF cDNAs are rare with respect to their cognate tRNA abundances in E. coli, and only a few micrograms of recombinant proteins are routinely obtained upon expression of recombinant forms of DFF in this organism per liter of culture. To provide a more robust source of DFF proteins to the scientific community we have created codon-optimized synthetic genes for wild-type and I1I2 mutants of the mouse and human DFF proteins through their robotic synthesis from pairs of overlapping 40-mer oligonucleotides (36). After expression of the proteins encoded by these synthetic genes in the pETDuet™ E. coli vector (Novagen), more than 20% of the total E. coli protein corresponds to DFF bands in Coomassie blue-stained SDS–PAGE gels (Figure 3A). This level of expression is highly significant because our previous expression systems using the corresponding wild-type cDNA coding sequences gave no visible new bands after induction in such gels of total E. coli cell protein (data not shown). Furthermore, DFF nuclease activities produced from these synthetic genes were fully dependent on cleavage by the appropriate protease, and both wild-type and insertion mutant proteins have similar nuclease activities (Figure 3B).
Mapping hypersensitive sites and nucleosome positions on the inactive and active yeast PHO5 gene with DFF-T produced from synthetic genes

We have demonstrated here that DFF-T could be fully and specifically activated by TEVP both in vitro and in vivo. To further investigate the utility of DFF-T we mapped nucleosome positions and hypersensitive sites within the promoter region of PHO5 gene, one of the most thoroughly studied yeast genes with respect to chromatin structure (29–32). Upon induction of PHO5 gene transcription by phosphate starvation, four nucleosomes are displaced from the upstream promoter region through the binding of Pho4p to UASp1 and UASp2 and the recruitment of chromatin remodeling complexes (Figure 4, right diagram). To evaluate the effectiveness of DFF-T to detect hypersensitive sites, and both nucleosome positioning and displacement, we made a direct comparison on the same chromatin samples with MNase cutting patterns by indirect end-labeling analysis. Here we utilized human DFF-T produced in high-yield from codon-optimized synthetic genes in an E. coli expression system (Figure 3). This comparison reveals a striking degree of similarity with respect to: (i) the positions of hypersensitive sites HS2 and HS3 on the uninduced PHO5 gene, which correspond to the accessible UASp1 between nucleosomes −2 and −3, and the 3′ end of the PHO5 gene, respectively (29,30); (ii) the positions of nucleosome footprints (Figure 4, compare lanes 1 and 4) and (iii) in the displacement of nucleosomes −1 to −4 after gene induction (Figure 4, compare lanes 2 and 5). The partial occupancy by nucleosomes at the −1 position of the activated PHO5 promoter may be attributed to a steady state of disassembly and reassembly of nucleosomes as previously described (39). There is some subtle difference, however, in the hypersensitive sites in the HS1 doublet (Figure 4, compare lanes 1 and 2 with lanes 4 and 5), which corresponds to the upstream region of the YBR094W gene. In summary, this comparison allows us to conclude that DFF-T is an excellent reagent to map the chromatin structures associated with specific inactive or active genes. We speculate that this enzyme may detect chromatin structures missed by other conventional enzymes.

Other potential uses for DFF in functional genomics

In this study, we have shown that DFF-T is an excellent reagent for chromatin structure investigations. The enzyme should also prove to be valuable in other types of footprinting experiments. From our previous and current studies, we also know DFF is extremely suitable for fragmenting chromatin to prepare nucleosomes without them possessing internal DNA nicks. We also propose that DFF nuclease is ideally suited to serve as a substitute for sonication to shear chromatin to nucleosome-sized fragments for the chromatin immunoprecipitation (ChIP) technique, because it only cuts between and not within nucleosomes and thus it cannot over-digest the chromatin DNA as other nuclease can. This nuclease should also be ideal for the purpose of generating genomic libraries for gene cloning/subcloning and shotgun sequencing, as it creates nearly exclusively double-stranded blunt DNA ends, and leaves 5′-phosphate and 3′-hydroxyl groups that are ideal for blunt-end cloning by ligation with T4 DNA ligase. Controlled digestion of DNA to yield fragments of various sizes should be easy to achieve with this enzyme. Thus, DFF-T should be a valuable tool for several lines of investigation.

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REFERENCES