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Fifty C-terminal amino acid residues are necessary for the chaperone activity of DFF45 but not for the inhibition of DFF40

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Apoptotic DNA fragmentation, the hallmark of apoptosis, is mediated primarily by caspase-activated DFF40 (CAD) nuclease. DFF40 exists as a heterodimer with DFF45 (ICAD), which is a specific chaperone and inhibitor of DFF40 under normal conditions. To understand the mechanism through which the DFF40/DFF45 system is regulated, we analyzed the structural and biochemical properties of apoptotic DNA fragmentation mediated by DFF40/DFF45. Using limited proteolysis, we show that residues 1-281 of DFF45 form a rigid, crystallized domain, whereas the loop formed by residues 277-281 is accessible by trypsin. These results show that the C-terminal helix formed by residues 281-300 is dynamic and necessary for the chaperone activity of DFF45, but not for inhibition of DFF40. [BMB reports 2009; 42(11): 713-718]

INTRODUCTION

Apoptosis is a process by which cells are removed in response to various programmed stimuli that occur during development, immune response and tissue homeostasis (1). The apoptosis-signaling pathway is activated and inhibited by several chemicals (2, 3) as well as by small domains known as the Death-Domain superfamily (4, 5). DNA fragmentation, a key biochemical feature of apoptotic cell death (6), is primarily controlled by the heterodimeric DNA fragmentation factor (DFF) complex, DFF40/DFF45 (7). DFF40 is a caspase-activated endonuclease that cleaves naked and chromosomal DNA, whereas DFF45 is an inhibitor that suppresses the nuclease activity of DFF40 via a tight interaction (7-9). In addition, DFF45 specifically exhibits molecular chaperone activity on the folding of DFF40 (7, 10). Both DFF40 and DFF45 contain a conserved N-terminal CIDE-N domain (cell-death-inducing DFF45-like effector domain) that is involved not only in their interaction (11, 12), but also in the inhibition of DFF45 on DFF40 (13).

When apoptotic stimuli activate effector caspases such as caspase-3, the DFF45 becomes cleaved at residues 117 and 224, resulting in the dissociation of DFF45 from its binding partner DFF40 (14, 15). Dissociated DFF40 is then activated and moves to the nucleus where it cleaves chromosomal DNA (14).

Human DFF45 consists of 331 amino acid residues with a CIDE domain (about 80 residues) at the N-terminus. DFF35, a short isoform of DFF45 comprised of 268 amino acids, can inhibit the nuclease activity of DFF40 both *in vitro* and *in vivo*, but lacks chaperone activity (16). Furthermore, analysis of the solution structure of DFF45 from residues 225-307 has revealed that this C-terminal region consists of 4 α-helixes (17).

Currently, all structural approaches designed to elucidate the mechanism by which the DFF40/DFF45 system affects apoptotic DNA fragmentation have been unsuccessful. This lack of success is due to difficulty in producing crystals comprised of the stable DFF40/DFF45 heterodimeric complex, although several groups have been able to purify it (18, 19). Proper characterization of the DFF40 protein requires it to be co-expressed with its chaperone, DFF45. Recent structural studies on DFF40 have been conducted by removing DFF45 from the DFF40/DFF45 complex after co-expression, followed by co-purification (19). These studies showed that while DFF45 is critical for the correct folding of DFF40, it also interferes with crystallization of the DFF40/DFF45 complex for reasons still unknown.

Here, we showed through limited proteolysis that residues 1-281 of DFF45 form a rigid, crystallizable domain. Furthermore, it was shown that the loop formed by residues 277-281 is accessible to trypsin, and that the last C-terminal helix formed by residues 281-300 is dynamic and critical for chaperone activity, but not for inhibition of DFF40.

RESULTS AND DISCUSSION

50 amino acid residues at the C-terminus of DFF45 are flexible and hinder crystallization

Crystallization of the DFF40/DFF45 complex for structural study has proven difficult despite several groups' success in purifying the stable DFF40/DFF45 heterodimeric complex (18).

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The results of previous studies indicate that the flexibility of DFF45 may prevent the complex and DFF45 itself from forming a crystal (unpublished).

Therefore, in order to find a rigid fragment capable of being crystallized and used in structural studies, we conducted a limited proteolysis experiment using a purified form of the DFF40/DFF45 complex. Briefly, pure DFF40/DFF45 heterodimeric complex proteins were prepared by co-purification as described by Dezevenu *et al*. (2004) (18). Subtilisin and Trypsin were used to perform a limited digestion, after which the digested samples were separated by SDS-PAGE. The results of this analysis clearly demonstrated that DFF40 is quite resistant to protease digestion, whereas DFF45 is not and is cleaved even by low concentrations of protease (Fig. 1A). A 1/10 (v/v) reaction mixture of trypsin/DFF complex, which produced the most amount of distinct DFF45 fragment, was applied to the gel-filtration column (Superdex-200) to further separate the fragment (Fig. 1B). The fractionated samples were subsequently subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad) for N- terminal sequencing and mass spectrometry for identification of the stable fragment. The DFF45 fragment produced by the limited trypsin digestion experiments was identified as amino acid residues 1-281 (1-281DFF45). Therefore, we subcloned 1-281DFF45 into the pOKD5 vector (18) and then purified the resulting protein by His-tag affinity chromatography and gel-filtration chromatography in 20 mM Hepes-NaOH (pH 7.0), 2 mM $MgCl_2$, 100 mM KCl and 5 mM DTT. The final product was concentrated to 10 mg/ml using a concentration kit provided by Millipore (Fig. 2A). The limited proteolysis revealed that 50 amino acid residues at the C-terminus of DFF45 are flexible and may hinder crystallization of the DFF40/DFF45 complex. The regions on DFF45 that may hinder crystallization most likely constitute a smaller part. Conversely, DFF40 is a rigid protein that is resistant to trypsin and subtilisin digestion.

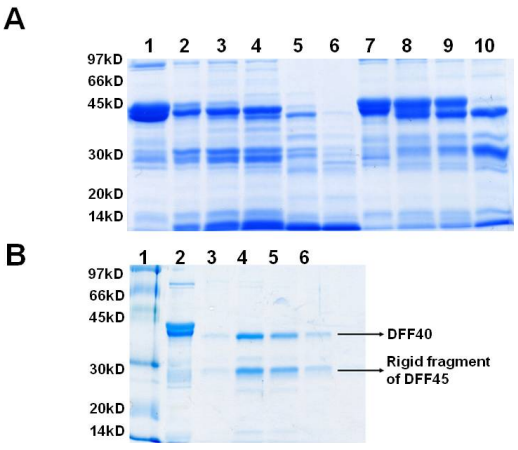

Fig. 1. Limited proteolysis of the DFF40/45 heterodimeric complex. (A) DFF45 digestion dependent on the protease concentration was monitored by SDS-PAGE. A stock solution of 0.5 mg/ml was prepared for both proteases in 20 mM Tris-HCl (pH 7.4). Lane 1: control (undigested sample). Lanes 2-6: Trypsin digestion. Stock solution was diluted 1/500, 1/100, 1/50, 1/10 and 1/1, respectively. Lanes 7-10: Subtilisin digestion. Stock solution was diluted 1/500, 1/100, 1/50 and 1/10, respectively. (B) Isolation of the proteolytic products using a Superdex-200 gel filtration column. Lane 1: Marker, Lane 2: Before digestion, Lanes 3-6: Fractions produced by gel filtration.

Crystallization of the stable fragments of DFF45

We searched for conditions that would produce protein crystals of the stable fragments of DFF45 (1-281DFF45). Crystallization conditions were initially screened at 20°C using sitting-drop vapor-diffusion. Crystals were grown by equilibrating a mixture containing 1 µl of protein solution (9-10 mg/ml protein in 20 mM Hepes-NaOH pH 7.0, 2 mM $MgCl_2$, 100 mM KCl, 5 mM DTT) and 1 µl of reservoir solution (30-35% PEG 400, 100 mM Hepes-NaOH pH 7.0-7.6) against 0.5 ml of reservoir solution. Crystals appeared within 7 days and grew to a maximum dimension of 0.05 × 0.05 × 0.1 mm (Fig. 2B). For diffraction, the crystals were cryo-cooled at 110 K using a liquid nitrogen stream (Cryocool, Cryo Industries, New Hampshire, USA). The high concentration of PEG in the droplet was sufficient to act as a cryo-protectant. Diffraction tests were performed at the X4A beamline of the National Synchrotron Light Source (NSLS) at Brookhaven national laboratory in New York, USA. Since crystals were diffracted to 8-9 Å, the diffraction data could not be indexed by HKL2000 due to the poor diffraction limit and quality. However, crystallization of 1-281DFF45 revealed that the rigid domain produced by limited proteolysis is well-folded and able to be crystallized, despite the low quality. This indicates that correct folding of DFF45 is not dependent

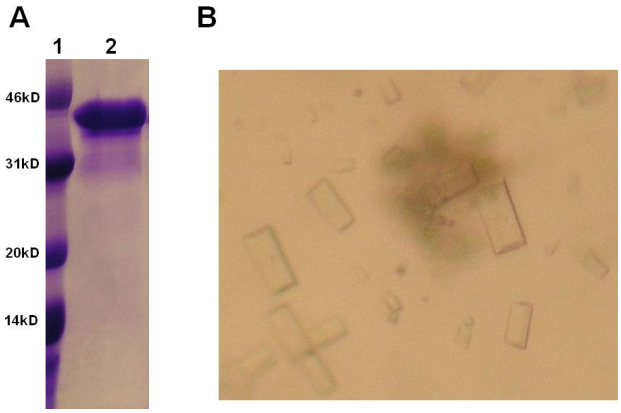

Fig. 2. Expression, purification and crystallization of 1-281DFF45. (A) 281DFF45 protein was purified by Ni-NTA affinity chromatography, followed by gel filtration chromatography and analysis by SDS-PAGE and coomassie blue staining. (B) Rectangular shaped crystals of 1-281DFF45. Crystals were grown in 15-18% PEG 8,000 and 100 mM Hepes-NaOH at a pH of 7.0-7.6. The crystals appeared within 7 days and grew to a maximum dimension of 0.05 × 0.05 × 0.1 mm.

on DFF40, contrary to the solution structure of the DFF40/DFF45 N-terminal domain complex that seem to confirm co-operative chaperone activity (12).

50 amino acid residues at the C-terminus of DFF45 are dynamic and critical for chaperone activity on DFF40

DFF35, an isoform of DFF45 comprised of amino acid residues 1-268, exhibits no chaperone activity on DFF40. Therefore, we evaluated 1-281DFF45 to determine if any chaperone activity on DFF40 was present. This was accomplished by cloning five different DFF45 constructs with C-terminal deletions of various lengths into a pET26b vector containing a His-tag at the N-terminus (Fig. 3A). Constructs of DFF40 were also cloned into a pOKD5 vector lacking any tags. The four DFF45 constructs with C-terminal deletions were designed based on the secondary structure predicted by the GOR IV program (20) as well as on the solution structure of the C-terminal domain of DFF45 (DFF-C), which contains residues 225-307 (17). The 1-281DFF45 construct was made based on the limited proteolysis experiment. The five different DFF45 constructs were then evaluated for chaperone activity upon co-transformation and co-purification with DFF40. If any of the 5 DFF45 constructs lost their ability to chaperone DFF40, no co-elution would be detected. Therefore, each his-tagged DFF45 protein was eluted from Ni-NTA beads using elution buffer containing 300 mM imidazole, followed by SDS-PAGE of the sample to verify DFF40 pull-down. Except for 1-281DFF45, all of the DFF45 constructs co-eluted with DFF40 (Fig. 3A). These results strongly suggest that the C-terminal residues 310-331 of DFF45, which are predicted to form a coiled-coil structure by the GOR IV secondary structure prediction program, are not necessary for chaperone activity. However, residues 280-309 are critical for the chaperone activity of DFF45. Previous structural studies have shown that residues 281-300 of DFF45 form a long α-helix that connects to another α-helix through a loop formed by residues 277-281 (17) (Fig. 3B). This implies that the chaperone activity of DFF45 requires this last, C-terminal α-helix formed by residues 281-300.

Additional biochemical and structural studies show that the chaperone activity of DFF45 may also depend on C289, as chaperone activity is present at highly reduced conditions, and as DFF35, a splicing variant containing residues 1-262, does not possess chaperone activity (14, 17). However, C289 is completely buried within the hydrophobic core formed by the C-terminal α-helix bundle of DFF45, according to the solution structure (17). To explain the contribution of C289 to chaperone activity, many speculate that a dramatic conformational change exposes C289 to DFF40. Interestingly, C289 is located in the last α-helix of the C-terminus formed by residues 281-300. Our results strongly suggest that C289 is critical for chaperone activity and is exposed to DFF40 by dynamic movement of the helix.

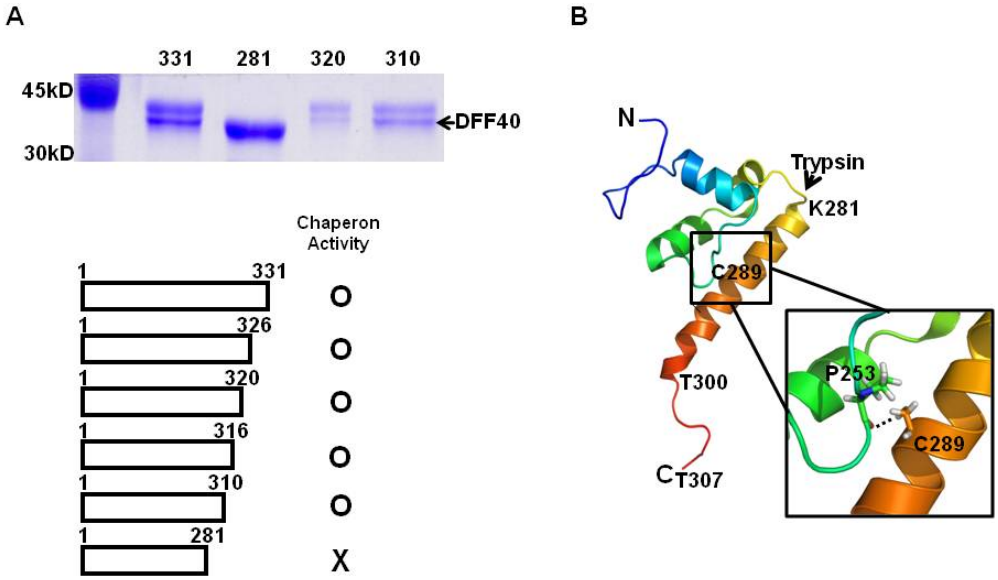

Fig. 3. Different DFF45 constructs and their chaperone activity. (A) Bars indicate the different lengths of DFF45 constructs used for testing of chaperone activity. Lengths of the constructs were designed based on limited proteolysis and secondary structure prediction. Chaperone activities were investigated by co-expression experiments with DFF40. Unlike the other DFF45 constructs, which clearly pulled down DFF40 after co-expression in *E. coli*, 1-281DFF45 did not pull down DFF40. (B) Previously identified solution structure of the C-terminal domain of DFF45 containing residues 225-307(PDB ID:1IYR) (Fukushima *et al*., 2002). The arrow head indicates the trypsin cleavage site determined during the limited proteolysis experiment. The black box magnifies the region including the cysteine residue in the last helix as well as a possible residue that is involved in the intramolecular interaction with cysteine 289.

Previous studies have shown that four C-terminal helices located between residues 225-307 are important for the chaperone activity of DFF45, based on the lack of chaperone activity exhibited by DFF35, which does not contain the four helices. In addition, our biochemical analysis clearly showed that the last α-helix formed by residues 281-300, along with 30 more C-terminal amino acids, is critical for the chaperone activity of DFF45. Therefore, the critical portion of DFF45 may either be the last helix formed by residues 281-300 or the 50 amino acid C-terminal end (residues 281-331). Further characterization studies are necessary in order to clarify this point.

1-281DFF45 inhibits DFF40

Next, we evaluated the inhibition of DFF40 by 1-281DFF45 by generating a DFF40/1-281DFF45 complex. Since 1-281DFF45 lost its chaperone activity, the complex could not be prepared by co-expression. Instead, the DFF40/1-281DFF45 complex was obtained by separately mixing purified proteins. The nuclease activities of DFF40, DFF40/1-281DFF45 and DFF40/1-281DFF45 + Caspase-3 were then evaluated by measuring their degradation of linearized plasmid DNA. The results revealed that plasmid DNA incubated with DFF40 alone or DFF40/1-281DFF45 + Caspase-3 was digested (Fig. 4). For the DFF40/1-281DFF45 + Caspase-3 sample, 1-281DFF45 was cleaved by caspase-3, followed by release of DFF40 for DNA digestion. Conversely, DFF40/1-281DFF45 did not display any activity toward the plasmid DNA (Fig. 4). These findings indicate that the nuclease activity is derived from DFF40 and that 1-281DFF45 blocks the activity of DFF40. Therefore, we conclude that 50 C-terminal residues of DFF45 are dispensable for the inhibition of DFF40.

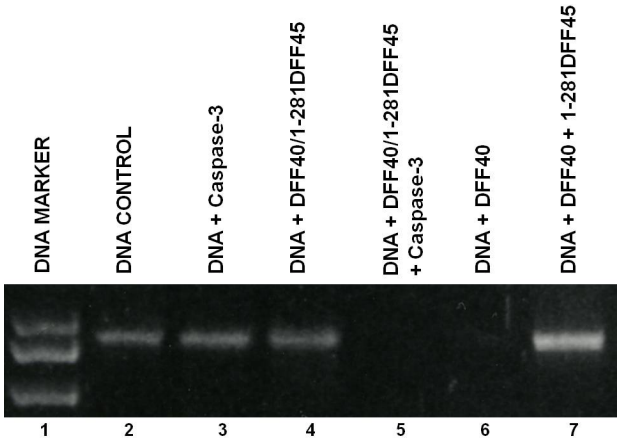

Fig. 4. Nuclease activity assay as measured by the degradation of linearized plasmid DNA. Lane 2 is DNA control. Lane 3 contains caspase-3 and DNA. Lane 4 contains DNA and the DFF40/1-281DFF45 complex. Lane 5 contains the DFF40/1-281DFF45 complex, caspase-3 and DNA. Lane 6 contains DFF40 and DNA. Lane 7 contains 1-281DFF45, DNA and DFF40. All prepared samples were incubated at 37ºC for 30 min prior to being loaded onto the 2% agarose gel.

MATERIALS AND METHODS

Limited proteolysis of DFF40/DFF45

A stable DFF40/DFF45 complex was prepared by co-purification as described by Dezevenu *et al*., 2004 (18). Subtilisin and trypsin were used for the limited digestion (stock concentration of 0.5 mg/ml in 20 mM Tris-HCl at pH 7.4 for both proteases). The DFF40/DFF45 complex was prepared at a concentration of approximately 2 mg/ml in 20 mM Tris-Hcl at pH 8.0, 150 mM NaCl and 2 mM DTT. Samples with different ratios (v/v) of subtilisin/DFF complex and trypsin/DFF complex were then prepared and incubated at room temperature for 30 min. Next, all reactions were terminated by boiling with SDS- loading buffer for 5 min, after which the reaction mixture was subjected to 15% SDS-PAGE.

Following protease digestion, the DFF45 fragment was separated and further purified by gel-filtration chromatography (Superdex-200, GE healthcare). The fractionated samples were subsequently subjected to 15% SDS-PAGE, after which the desired bands were transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad). N-terminal sequencing and mass spectrometry were then performed at the UTMB Cancer Center at Galveston, Texas, USA.

Construction, expression and purification of 1-281DFF45

The 1-281DFF45 expression construct was produced by the following method. The cDNA of full length DFF45 was used as a template for PCR while the plasmid vector pET28a (Novagen) enabled affinity purification by adding a hexa-histidine tag to the N-terminus of 1-281DFF45. The sub-cloned plasmid was then transformed into BL21 (DE3) *E. coli* competent cells, after which expression was induced by culturing of bacteria in the presence of 0.5 mM IPTG overnight at 20°C. The bacteria were then collected, resuspended and lysed by sonication in 50 ml of lysis buffer (20 mM Tris buffer at pH 7.9, 500 mM NaCl and 5 mM imidazole). The bacterial lysate was then centrifuged at 16,000 rpm for 1 hour at 4°C. Next, the supernatant fraction was applied to a gravity-flow column (Bio-Rad) packed with Ni-NTA affinity resin (Qiagen). The unbound bacterial proteins were then removed from the column using washing buffer (20 mM Tris buffer at pH 7.9, 500 mM NaCl, 60 mM imidazole and 10% glycerol). Next, the N-terminal hexa-histidine-tagged 1-281DFF45 was eluted from the column by elution buffer (20 mM Tris buffer at pH 8.0, 500 mM NaCl and 250 mM imidazole). Purity of the protein was further improved by passing the samples through a Superdex 200 gel filtration column 10/30 (Pharmacia) that had been pre-equilibrated with a solution of 20 mM Hepes-NaOH at pH 7.0, 2 mM $MgCl_2$, 100 mM KCl and 5 mM DTT. Purified 1-281DFF45 was then concentrated to 10-12 mg/ml using a Millipore concentration kit (Millipore) and then used for subsequent crystallization trials.

Crystallization of 1-281DFF45

1-281DFF45 was crystallized at room temperature using sitting-drop vapor-diffusion and screening kits from Hampton Research and deCODE Biostructures Group. Briefly, crystals were grown on a sitting-drop plate by equilibrating a mixture containing 1 μl of protein solution (10-12 mg/ml protein in 20 mM Hepes-NaOH at pH 7.0, 2 mM $MgCl_2$, 100 mM KCl and 5 mM DTT) and 1 μl of a reservoir solution (30-35% PEG400, 100 mM Hepes-NaOH at pH 7.0-7.6) against 0.5 ml of reservoir solution. Crystals apparing within 7 days were grown to a maximum dimension of 0.05 × 0.05 × 0.1 mm. For diffraction experiments, the crystals were cryo-cooled at 110 K using a liquid nitrogen stream (Cryocool, Cryo Institutes, New Hampshire, USA). Diffraction tests were then conducted at the X4a beamline of the National Synchrotron Light Source (NSLS).

Co-expression and His-Tag pulldown assay

Five different DFF45 constructs (amino acid residues 1 to 331, 1 to 326, 1 to 320, 1 to 316 and 1 to 281) were cloned into a pET26b vector and then individually co-transformed into BL21 (DE3) *E. coli* competent cells with DFF40, which was cloned into the pOKD5 vector. A stop codon was placed immediately after the DFF40 sequence in the pOKD5 vector so that no tags could be added. Conversely, all DFF45 constructs that were ligated into the pET26b vector contained a C-terminal hexa-histidine tag. Protein expression was then induced by incubating the cells overnight at 20°C in the presence of 0.5 mM IPTG. The bacteria were then collected, resuspended in 10 ml of lysis buffer and lysed by sonication (20 mM Tris buffer at pH 7.9, 500 mM NaCl and 5 mM imidazole). Next, the bacterial lysate was centrifuged at 16,000 rpm for 1 hour at 4°C, followed by incubation of the supernatant with Ni-NTA beads (Qiagen). The unbound bacterial proteins were then washed out using washing buffer (20 mM Tris buffer at pH 7.9, 500 mM NaCl, 60 mM imidazole and 10% glycerol). The various C-terminal hexa-histidine-tagged DFF45 proteins were then eluted from the beads by elution buffer (20 mM Tris buffer at pH 8.0, 500 mM NaCl, and 300 mM imidazole). Finally, the presence of DFF40 pulled down by His-tagged DFF45 was confirmed by SDS-PAGE.

Nuclease activity assay

Caspase-3 was prepared according to the method described by Stennicke and Salvesen 1997 (21, 22). DFF40 was prepared by the purification method described by Woo *et al*. (19). DFF40/1-281DFF45 samples were prepared by separately mixing purified DFF40 and 1-281DFF45, followed by concentration with a Millipore concentration kit (Millipore). The prepared DFF40/1-281DFF45 complex (10 μg) was then incubated with 50 ng of caspase-3 at room temperature for 30 min in 20 μl of buffer comprised of 20 mM Hepes at pH 7.2, 5 mM $MgCl_2$, 50 mM NaCl, 1 mg/ml BSA and 5 mM EGTA. The same buffer was used for all enzyme reactions. Three μg of linearized plasmid DNA were added to each lane and the reaction mixtures were analyzed by electrophoresis on 2% agarose gels.

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