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Disruption of ATP binding destabilizes NPM/B23 and inhibits anti-apoptotic function

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Nucleophosmin/B23, a major nucleolar phosphoprotein, is overexpressed in actively proliferating cells. In this study, we demonstrate that B23 exclusively localizes in the nucleolus, whereas ATP depletion results in the redistribution of B23 throughout the whole nucleus and destabilizes B23 via caspase-3 mediated cleavage. Interestingly, ATP binding precedes PI(3,4,5)P_3 binding at lysine 263 and ATP binding mutants fail to restore the anti-apoptotic functions of B23 in PC12 cells. Thus, the ATP-B23 interaction is required for the stability of the B23 protein and regulates cell survival, confining B23 within the nucleolus in PC12 cells. [BMB reports 2008; 41(12): 840-845]

INTRODUCTION

B23/NPM is an abundant phosphoprotein which localizes within the granular region of the nucleolus (1), where it is recognized as the site of active rRNA transcription and ribosomal biogenesis (2). Other studies have suggested that B23 transport may be crucial for its functions in the cell. Indeed, not only does B23 shuttle between the nucleus and cytoplasm (3), but also translocates from the nucleolus to the nucleoplasm during the S phase of cell growth (4). The depletion of the GTP pool in culture conditions caused B23 translocation from the nucleolus to the nucleoplasm (5). B23 translocation can also be induced by certain anti-cancer drugs which cause growth inhibition (6), but such drug-induced translocations are blocked when ATP content is reduced by antimycin A in a glucose-free medium (7). Moreover, ATP promotes the translocation of B23 from the nucleolus to the nucleoplasm in isolated permeabilized cells rather than whole culture cells (8), and positively charged lysine residues in the C-terminus of B23 have been implicated in the binding of negatively charged ATP (9). Recently, we have reported that B23 inhibits apoptosis (10) via interaction with CAD and prevents its DNA fragmentation activity via specific binding to PI(3,4,5)P_3, and we have also identified lysine 263 in B23 as a specific binding site of ATP (11).

Mitochondria in the cytoplasm regulate cell death (12), and the nucleolus in the nucleus appears to be of similar importance in deciding cell fate. Many nucleolar components are involved in the mediation of apoptosis, and the nucleolus has been demonstrated to be a degradation substrate for caspase-3 (13). B23 has been demonstrated to be a target for caspase-3 dependent proteolysis (6), thereby implying that it may affect its anti-apoptotic effects.

In the current study, we demonstrate that the disruption of ATP binding prohibits the efficient nucleolar localization of B23, thereby resulting in an increase in the sensitivity of B23 against capase-3 dependent cleavage and the inhibition of cell survival. This shows that the ATP binding ability of B23 is crucial for nucleolar retention and protein stability, and is involved in biological functions within the nucleolus, including cell survival.

RESULTS

Disruption of B23-ATP interaction impedes B23 stability

B23 localizes principally to the nucleolus. However, the mechanism underlying the nucleolar localization of B23 remains unknown. We considered that B23 degradation has not been detected in the nucleolus (14). With the finding that the ATP binding mutant, B23 K263N, was delocalized from the nucleolus, we wonder whether the B23-ATP interaction regulates B23 stability. In order to assess the effect of ATP on the nucleolar localization of B23, we conducted an experiment involving the deficiency of cellular ATP contents employing sodium azide (AZ) or antimycin A, a metabolic ATP synthesis inhibitor in an effort to reduce ATP level in culture conditions. Myc-B23 WT cells were treated for 1 hour with AZ or antimycin A at several dosages (0, 1, 2.5, 5 μg/ml). Consistent with our previous observation (11), endogenous B23 was located principally in the nucleolus, where Fibrillarin is specifically expressed. AZ was shown to elicit the increased nucleoplasmic staining of B23 in a dose-dependent manner (Fig. 1), as well as antimycin A treatment (data not shown). Notably, as the result of treatment with 5 μg/ml of AZ or antimycin A to

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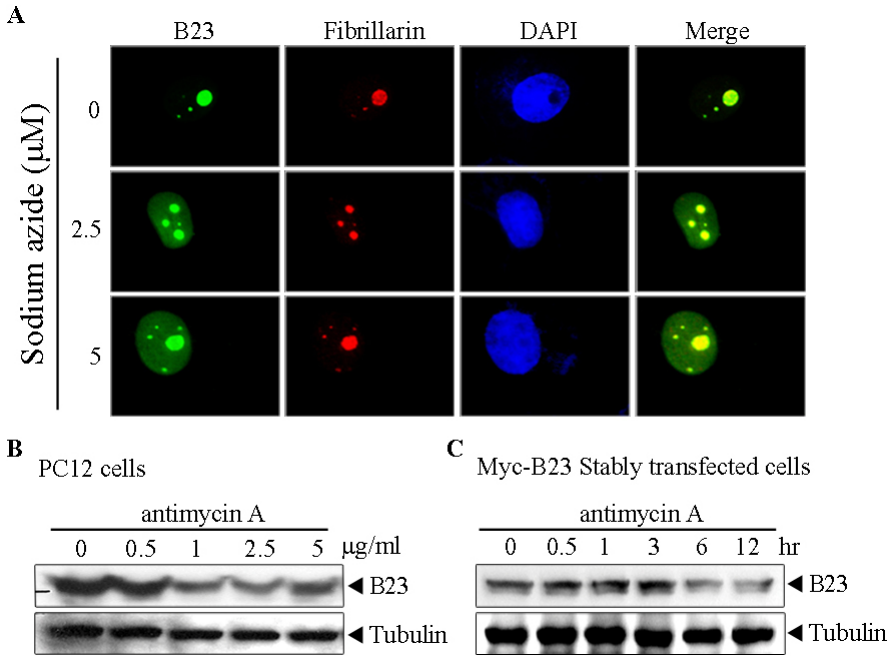

Fig. 1. ATP depletion leads to B23 delocalization and instability. (A) Myc-B23 WT cells were treated with 0, 2.5, 5 μM of sodium azide (AZ) for 1 hour. Immunofluorescence staining revealed that endogenous B23 delocalized from nucleolus in response to sodium azide (AZ) treatment. Nucleolus was stained with anti-Fibrillarin antibody and nucleus was stained with DAPI. (B) PC12 cells were stimulated with antimycin A in a dose-dependent manner at the indicated concentrations for 12 hours. (C) Myc-B23 WT cells were treated with 5 μg/ml of antimycin A in a time-dependent manner. Cell lysates were immunoblotted with anti-NPM antibody (upper band) and anti-Tubulin antibody (lower band). B23 was degraded in a dose-dependent and time-dependent manner with antimycin A.

B23 WT cells, B23 delocalized from the nucleolus is comparable to GFP-B23 K263N (Fig. 1A bottom panels), thereby indicating that the nucleolar localization of B23 requires ATP binding. In an effort to further determine whether the delocalization of B23 from the nucleolus as the result of ATP depletion or failure to bind to ATP affects B23 stability, we assessed endogenous B23 stability following the administration of antimycin A treatment to PC12 cells. In the absence of antimycin A or very low concentrations of antimycin A (0.5 μg/ml), B23 remained intact, whereas the level of B23 protein was reduced in a dose-dependent manner (Fig. 1B). In order to confirm B23 instability under ATP depletion conditions, we employed Myc-B23 WT stably transfected cells and administered 5 μg/ml of antimycin A in a time-dependent manner. As is shown in Fig. 1C, the level of exogenous B23 decreased gradually after an hour. Thus, one of the probable reasons for the increased instability of B23 might involve its inability to bind to ATP.

ATP-depletion enhances B23 susceptibility for caspase-3

B23 overexpression protects cells against apoptosis and inhibits DNA fragmentation upon binding to $PI(3,4,5)P_3$ (15). The results of a recent study have demonstrated that the disruption of B23-ATP interaction induces B23 proteasomal degradation and ubiquitination, thereby enhancing the instability of the B23 protein. Nevertheless, B23 has also been reported as a substrate of caspase-3 (16). In an effort to determine whether or not ATP depletion performs any role in mediating its proteolytic degradation, we transfected GFP-B23 WT and K263N mutants into PC12 cells and treated them with staurosoporine. The quantification of apoptotic cells with MR-

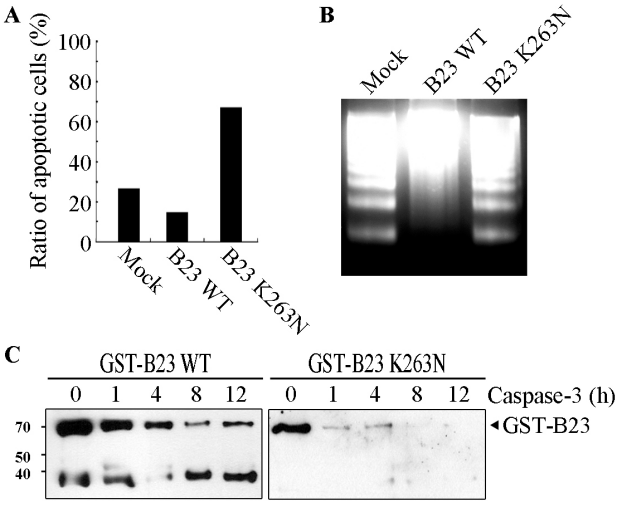

Fig. 2. ATP-depletion enhances B23 susceptibility for caspase-3. (A) Quantitative analysis of apoptosis in the GFP-B23 WT and K263N transfected cells. (B) *In vitro* DNA fragmentation assay. PC12 cells were transfected with GFP-B23 WT or GFP-B23 K263N for 24 hours, followed by 18 hours of treatment with 250 nM STS. 10 micrograms of genomic DNA was loaded onto 2% agarose gel. (C) Purified GST-B23 WT and K263N proteins were incubated with 25 ng/ml of active caspase-3 protein administered at the indicated times. The reaction mixture was then analyzed with anti-B23 antibody.

(DEVD)2, a cell-permeable fluorescent dye activated by active caspase-3 or -7, demonstrated that 70% of K263N cells were apoptotic, whereas 25% and 15% of the control and WT cells

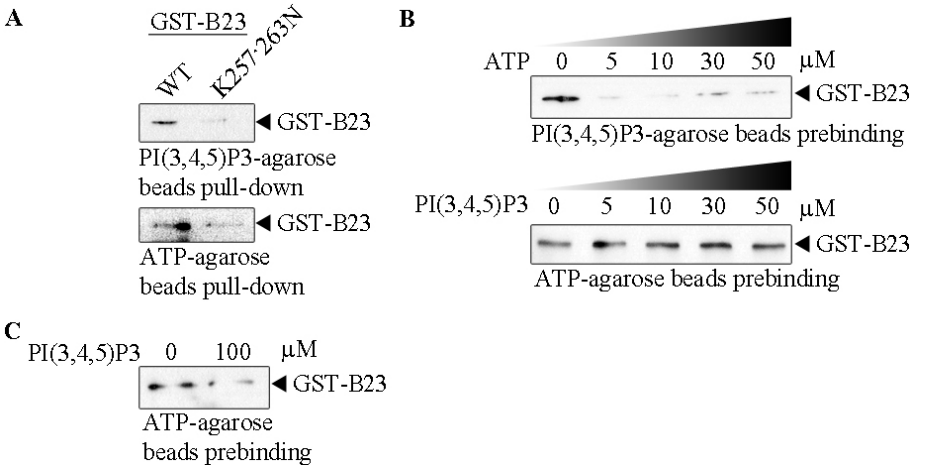

Fig. 3. ATP competes with PI(3,4,5)P_3 on lysine 263 residue for binding of B23. (A) Verification of B23 binding activity with ATP or PI(3,4,5)P_3, respectively. (B) *In vitro* competition assay. Purified recombinant GST-B23 was pre-incubated with PI(3,4,5)P_3- beads and chased by gradually increasing amounts of PI(3,4,5)P_3 or ATP (0-50 μM), respectively. (C) *In vitro* competition assay with higher concentrations (100 μM) of PI(3,4,5)P_3.

were apoptotic (Fig 2A). As compared with WT B23, the results of a DNA fragmentation assay showed that B23 overexpression diminished DNA fragmentation. By way of contrast, evident DNA fragmentation was detected in K263N transfected cells (Fig. 2B). *In vitro* apoptotic cleavage with active caspase-3 and purified GST-B23 recombinant proteins showed that WT-B23 was processed with the active caspase-3 for 4 to 12 hours. By way of contrast, the K263N mutant was cleaved completely within an hour, thereby implying that the disruption of ATP binding from B23 protein enhances its sensitivity to caspase-3, destabilizing it.

ATP competes with PI(3,4,5)P_3 on lysine 263 for binding of B23

Our previous study showed that the C-terminus of B23 is required for interaction with PI(3,4,5)P_3 binding, thereby preventing DNA fragmentation. We have also shown that Lysine 263 is a critical docking site for ATP binding. Our current data confirmed that the GST-B23 K263N mutant was unable to bind either to PI(3,4,5)P_3 or ATP using an in vitro binding assay with PI(3,4,5)P_3-agarose conjugated beads and ATP-agarose conjugated beads, while GST-B23 WT evidenced obvious binding with PI(3,4,5)P_3 or ATP (Fig. 3A). Based on this observation, we hypothesized that one of the PI(3,4,5)P_3 binding sites in B23 may either share or compete with ATP. To test this hypothesis, we conducted an *in vitro* competition assay using the purified recombinant GST-B23, preoccupied with PI(3,4,5)P_3-beads. The aforementioned procedure was followed by a gradual increase in ATP (0-50 μM) or a gradual increase in PI(3,4,5)P_3 (0-50 μM) to GST-B23 that had been pre-coupled with ATP-beads. We noted that the binding of GST-B23 to PI(3,4,5)P_3 was reduced as ATP was increased. Moreover, the binding of GST-B23 to ATP remained unaffected by a gradual increase in PI(3,4,5)P_3 (Fig. 3B). Even at higher concentrations of PI(3,4,5)P_3, only a slight effect on B23-ATP interaction was noted (Fig. 3C), suggesting that the prior occupation of Lys 263 residue by ATP binding in B23 prevents PI(3,4,5)P_3 binding.

ATP binding ability of B23 is required for cell survival

As the ATP binding site of B23 is shared with PI(3,4,5)P_3 and a previous study has demonstrated that B23 mediates cell survival via PI(3,4,5)P_3 binding, we have attempted to determine whether the interference of ATP binding in B23 induces cell death. The immunodepletion of endogenous B23 from PC12 cells with anti-B23 antibody triggered severe DNA fragmentation and the adding back of 3 μg of purified GST-B23, but not GST-B23 K263N mutant or GST alone, restored the inhibitory activity (Fig. 4A). This result is consistent with our previous observation that 1 μg of purified GST-B23 was sufficient to reconstitute the inhibitory effect of DNA fragmentation from B23-deprived nuclear extract (15). Immunoblotting analysis demonstrated that B23 was removed to a substantial degree from the nuclear extract by B23 antibody, but not control mouse IgG (Fig. 4A bottom). In order to further elucidate the effects of the inability of ATP binding in mediating the suppression of apoptosis by B23, we elicited the depletion of B23 from PC12 cells using shRNA/B23 followed by rescue with the transient expression of GFP-B23 WT or GFP-B23 K263N mutant in staurosporine, general protein kinase inhibitor, induced an apoptotic condition. Staurosporine treatment results in robust cell death in control PC12 cells (Fig. 4B left) and the knockdown of B23 by shRNA enhanced nuclear condensation under staurosporine-induced apoptotic conditions (Fig. 4B right), indicating that the depletion of B23 significantly abolishes its anti-apoptotic effects. Notably, GFP-B23 WT expression in the endogenous B23-deficient cells reconstituted the protective effects of cell death, clearly evidencing a reduced level of nuclear condensation and apoptotic ratio, whereas GFP-B23 K263N or GFP alone had no effect, displaying condensed chromatin morphology with a higher ratio of apoptotic cell death (Fig. 4C), indicating that the K263N mutant is defective in the prevention of apoptosis. GFP-B23 and mutant ex-

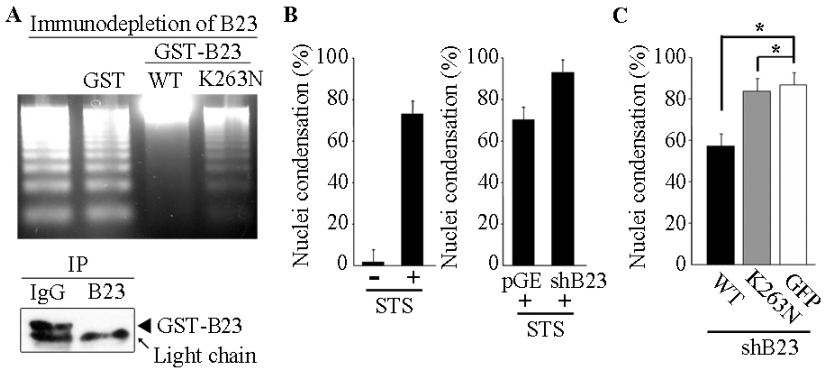

Fig. 4. K263N mutation impairs anti-apoptotic activity of B23. (A) *In vitro* DNA fragmentation assay. PC12 cell nuclear extract was subjected to immunodepletion with specific B23 antibody. 3 μg of purified GST-B23 WT, GST-B23 K263N and GST were incubated with supernatant followed by DNA fragmentation assays with apoptotic solution consisting of purified DFF45/40 and purified caspase-3. The reactants were analyzed on 2% agarose gel. The immunodepletion of B23 was verified via immunoblotting (bottom). (B) Quantification of apoptotic ratio. PC12 cells were incubated with or without 250 nM of staurosporine for 12 hours or depleted endogenous B23 via the transfection of pGE or shB23 for 36 hours followed by 12 hours of treatment of staurosporine. Apoptotic cells were measured by counting nuclei condensed cells after DAPI staining. (C) In order to rescue cells from apoptosis, PC12 cells were transfected for 36 hours with shB23, followed by transfection with GFP-B23 WT, GFP-B23 K263N and GFP. After 24 hours, the cells were incubated with staurosporine and counterstained with DAPI. Three independent experiments evidenced similar results. *$P < 0.01$ (Student's *t*-test).

pression were verified under a fluorescence microscope. The prominent effect in K263N-transfected cells raises the possibility that the mutation may induce a structural alteration, and may somehow prove to be toxic to the cells, resulting in an unstable form, and thereby enhancing apoptosis. Thus, our results indicate that ATP binding ability is crucial for the role of B23, which prevents apoptotic cell death.

DISCUSSION

B23 is an abundant nuclear protein which is localized in both the nucleolus and the nucleoplasm (17), and functions in a variety of roles in multiple cellular processes, including ribosome processing, cell proliferation, apoptosis, and cytoplasmic/nuclear shuttling. It has been also demonstrated that B23 is translocated from the nucleolus to nucleoplasm during the stationary growth phase (4) or during exposure to certain anti-tumor drugs (4, 18). Under lower ATP contents under culture conditions, the delocalization of B23 from the nucleolus is blocked, and newly synthesized rRNA is retained within the nuclei (7), thereby suggesting that B23 translocation occurs in a regulated fashion. In this study, we demonstrate that the interruption of ATP binding extrudes B23 from the nucleolus and enhances protein instability, subsequently causing apoptotic cell death.

Although B23 shuttles continuously between the nucleolus and nucleoplasm as a major nucleolar protein, over 80% of B23 is generally localized within the nucleolus. Notably, our data showed that disruptions in the interaction between B23 and ATP delocalize B23 from the nucleolus and reserve B23 within the nucleoplasm. However, the molecular mechanisms underlying B23 shuttling and ATP binding remain poorly understood. As the nucleolus is well known to function as a ribosome assembly point (14) and B23 has been implicated in pre-rRNA processing (17), we initially speculated as to the effects of B23-ATP interaction on ribosome biogenesis. However, we failed to detect impeded rRNA processing following the interruption of ATP binding to B23. Using PC12 cells that were transfected with Myc-B23 WT and Myc-B23 K263N, the cells were pulse-labeled with [^{3}H]uridine and chased for 0, 2, and 4 hours. The pulse-labeled 47S rRNA precursor could be readily detected in all samples at the beginning of chasing (0 min), thereby indicating that the transcription of the rRNA precursor was not blocked by the lack of ATP. After 60 min of chasing, the quantity of mature 28S rRNA was similar to that of the 32S intermediate in the cell-transfected control vector or B23 WT or B23 K263N (data not shown). This suggests that active processing of the 32S precursor and the disruption of ATP binding to B23 did not influence the processing of the 32S intermediate for the subsequent maturation of 28S rRNA.

B23 has a relatively long half-life, more than 24 hours (11, 17). The nucleolar localization of B23 is believed to play a role in its stability (17, 19). Our observation that the B23 mutant, which does not bind to ATP, or the depletion of ATP in the culture medium, results in a substantial extrusion of its nucleolar localization (Fig. 1) and a reduction in B23 protein stability caspase-dependent degradation coupled with an increase in apoptosis (Fig. 2). These phenomena demonstrate that ATP-B23 interaction is essential for B23 stability. Presumably, the failure of ATP-B23 interaction triggers B23 delocalization and subsequently increases the instability of B23 as the consequence of its increased sensitivity for caspase-3.

The interaction between B23 and PI(3,4,5)P_3 is reduced rapidly by ATP competition (Fig. 3B). On the other hand,

$PI(3,4,5)P_3$ is incapable of disrupting B23-ATP interaction, even at high concentrations of $PI(3,4,5)P_3$, thereby suggesting that the primary role of B23 may reside in its ability to bind to ATP in the nucleolus. The manner in which cells manage the balance between the $PI(3,4,5)P_3$ binding activity to protect against NGF treatment-mediated DNA fragmentation, and ATP binding for the maintenance of nucleolar localization and stability, remains to be determined.

MATERIALS AND METHODS

Cell cultures and reagents

PC12 cells were maintained in medium A (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 5% horse serum, and 100 units of penicillin-streptomycin) at 37°C, under a 5% CO_2 atmosphere. The Myc-tagged B23 (Myc-B23) stably-transfected PC12 cells were cultured in medium B (medium A with 100 μg/ml of G418, 100 μg/ml of hygromycin B, and 2 μg/ml of tetracycline). The transfected cell lines were then induced via 24 hours of culturing in medium B without 2 μg/ml of tetracycline. Anti-NPM, anti-HA, anti-PARP, and anti-Myc antibodies were obtained from Cell Signaling (Danvers, MA). Anti-α-Tubulin and anti-Fibillarin were acquired from Santa Cruz (Santa Cruz, CA). Adenosine triphosphate-agarose conjugated beads (ATP-beads) were acquired from Fluka (Milwakuee, WI). Phosphatidylinositol (3,4,5)-triphosphate ($PI(3,4,5)P_3$) and $PI(3,4,5)P_3$-agarose conjugated beads were obtained from Echelon Bioscience, Inc (Salt Lake City, UT). All chemicals not listed above were obtained from Sigma (St. Louis, MO).

ATP or PIP_3 competition assay

ATP or $PI(3,4,5)P_3$-beads were incubated with 1.5 μg of purified GST-B23 proteins in 500 μl of binding buffer. The beads were washed three times and the associated protein was analyzed via Western Blot with anti-GST antibody. For the lipid competition assay, the binding of associated protein onto ATP or $PI(3,4,5)P_3$-beads was competed with gradually increased soluble ATP, or $PI(3,4,5)P_3$ was used.

Immunostaining and Confocal analysis

PC12 cells grown on coverslips in 6-well plates were treated for 1 hour with antimycin A (5 μg/ml). The cells were fixed for 15 minutes with 4% paraformaldehyde and permeabilized for 15 minutes in 0.5% Triton X-100 in PBS. Immunolocalization was conducted using anti-B23 and anti-fibrillarin antibodies with the fluorescent dye-conjugated antibody, Alexa Flour 488 goat anti-mouse and Alexa Flour 594 goat anti-rabbit. The cells were visualized using a Zeiss (Jena, German) LSM confocal fluorescence microscope.

B23 proteolytic cleavage assay

25 ng of active caspase-3 was preincubated with assay buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, and 10% glycerol at 37°C. After 10 min, caspase-3 was incubated with 2 μg of purified GST B23 WT and K263N, as indicated.

DNA fragmentation assay

DNA fragmentation assays were conducted as previously described (20). The total quantity of extracted DNA from each sample was normalized and an equal quantity of DNA was positioned in each lane. A representative of the DNA fragmentation assay from multiple repetitions is presented. Similar results were obtained each time.

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