

## Characterization of a Novel Ionizing Radiation-Induced Nuclease

Ji-Hyun Lee, Eunhee Kim, Su-Jae Lee, Yun-Sil Lee and Sangwoo Bae\*

Laboratory of Radiation Effect, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Korea

\*Correspondence: Sangwoo Bae, e-mail: swbae@kcch.re.kr

### 1. Introduction

Radiation can produce cell death by either necrosis or apoptosis. Necrosis is a passive process where cells are characterized by a loss of membrane integrity, cell swelling and random degradation of DNA [1]. In contrast, apoptosis is an active process characterized by programmed cell death in which a series of events is triggered in response to stress conditions including ionizing radiation [2]. Apoptosis is characterized by nuclear DNA fragmentation, condensed chromatin, and a fragmented nucleus.

Activation of various apoptotic nucleases is implicated in the process following IR. For instance, Caspase-activated DNase (CAD) and DNaseI are involved in IR-induced apoptosis [3] by generating blunt-end double strand breaks or DNA strand breaks containing 3'-OH end groups respectively. Moreover cooperation among nucleases has been observed during apoptosis. CAD is involved in apoptosis in synergy with DNase  $\gamma$ -like endonucleases whereas EndoG is along with ExoIII, and DNaseI.

Considering complexities of the mechanisms that are involved in irradiation-induced apoptosis, there might be still unknown genetic factors. Therefore, we set out to identify novel factors involved in the process.

### 2. Materials and Methods

Subtractive hybridization, sequence analysis:

Suppressive subtractive hybridization analysis was carried out using kits from Clontech (USA) following instruction manual (PCR-select cDNA subtraction (K1804-1) Manual PT1117-1). Isolated clones from subtractive hybridization analysis were sequenced and analyzed using Blast search program from NCBI.

Preparation of cell extracts and immunoprecipitation:

Cells were collected and lysed in lysis buffer (40 mM Tris-Cl, pH 8.0, 0.1% Nonidet P-40, 100 mM NaCl, 2.5 mM Na<sub>3</sub>VO<sub>4</sub>, and 10  $\mu$ M leupeptin, aprotinin, AEBS). After centrifugation (for 15 min. at 14000rpm), supernatants were collected and used as whole cell extracts for immunoprecipitation. For immunoprecipitation, cell extracts were incubated with 2  $\mu$ g of Flag antibody (Sigma) overnight at 4°C. Protein A was added and incubated further for 3hr at 4°C and centrifuged. Precipitated beads were washed three times in PBS and used in nuclease assay.

DNA constructs: The following primers were used to make plasmid constructs of clone A. Sequence is from 5' to 3'. A Eco 5': CGGAATTC ATG GTACCCCG GGAGGCCCT. A Eco 3': CGGAATTCTCAATTC CTTCTGT CCTGTGCCT. Clone A cDNA was cloned by RT-PCR using A Eco 5' and A Eco 3' primer set. Amplified cDNA was inserted into EcoRI site of pCDNA3 vector to make pCDNA3-A.

Nuclease assay: A nuclease activity of clone A was assayed by following the protocol [4].

Immunofluorescence microscopy: Clone A protein in the cell was detected by immunofluorescence microscopy. Briefly, NCI-H460 cells grown on glass coverslips in 35 mm dishes were either unirradiated or gamma-irradiated with 10 Gy. After 1 or 2 h, cells were fixed with ethanol. Flag M2 (Sigma) primary antibody and Alexa fluor 488 labeled secondary antibody (Molecular probes) were used to detect flag tagged clone A whereas nuclear DNA was stained with propidium iodide (PI).

### 3. Results and Discussion

We have conducted subtractive hybridization assay to identify genes that are induced upon exposure of cells to gamma-ionizing radiation.

#### 3.1 A novel ionizing radiation-induced nuclease clone A

The isolated complementary DNA of the clone A encodes a polypeptide of 325 amino acids with unknown function and is identical to the gene in Genbank (Accession no. [NM\\_022767](#)). We hypothesized that highly expressed genes in irradiated cells might affect cellular radiation response. In order to investigate how clone A affects cellular radiation response, we first analyzed its nucleotide sequence. BLAST analysis and alignment of clone A nucleotide sequence with homologues revealed significant homology to various exonucleases. In particular, we noticed the highest homology to exonuclease domain of EXO III, Exonuclease X-T and DnaQ.

#### 3.2 DNase activity of clone A

In order to confirm whether clone A protein has a nuclease activity, we immunoprecipitated clone A protein for enzyme assay.

Addition of the immunoprecipitate from the FL clone A-transfected cells cleaved a 5'-labeled blunt end double stranded DNA substrates (Fig. 1) whereas that from the  $\Delta$  EX clone A or from vector alone transfected-control did not. These results indicate that clone A encodes a DNase and that the conserved exonuclease domain is critical for the enzyme activity. Clone A might be involved in cellular radiation response by digesting DNA substrates generated by cell's exposure to ionizing radiation. For instance ionizing radiation (IR) generates ~35 double strand breaks (DSBs) and ~1000 single-strand breaks (SSBs) per diploid G1 cell per Gy as well as numerous base damages. Or DNA fragmentation by IR-induced apoptosis might provide DNA substrates for clone A.

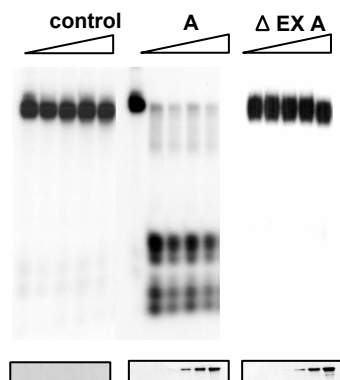


Figure 1. Nuclease assay for clone A and  $\Delta$  EX A which has exonuclease domain deletion. Only wild type clone A has a nuclease activity. Lower panels are immunoblots to show equal protein amounts that were used in the assay.

### 3.3 Co-localization of clone A protein with AIF and CAD following IR

DNA degradation is a critical step in apoptosis and requires involvement of several endo- and exonucleases. For example, Caspase-activated DNase (CAD) is activated by gamma irradiation and is involved in the nuclear DNA fragmentation. CAD cleaves double stranded DNA substrates and generates blunt-end double strand breaks whereas EndonucleasesG cleaves single strand and double strand DNA or RNA to produce single strand nicks. Moreover DNaseI has been implicated in radiation induced lymphoid cell death by producing DNA strand breaks containing 3'-OH end groups-the same end groups found in DNA fragmentation products during apoptosis. Since the cleaved DNA products by apoptosis have structural features on which clone A can act, clone A protein might facilitate further cleavage of DNA with SSB or DSB generated by the apoptotic nucleases. These apoptotic nucleases move into the nucleus or are activated in the nucleus from inactive state and attack chromatin to yield 50-300kb cleavage products as well as internucleosomal DNA fragmentation.

In order to test the possibility whether clone A is involved in apoptotic DNA fragmentation with the aforementioned nucleases, we examined whether clone A colocalizes with apoptotic proteins such as AIF and CAD.

We found that AIF localized in cytoplasm in unirradiated cells (Fig. 2) as expected. Upon  $\gamma$ -irradiation, AIF moved into the nucleus and colocalized with most of clone A. CAD overlapped with clone A in the nucleus in unstressed cells consistent with previous result that CAD is present in the nucleus as an inactive form and activated following stress. Following gamma irradiation, we found more extensive overlapping between clone A and CAD. This result suggests that FL clone A might be involved in apoptotic process upon ionizing irradiation in concert with other apoptotic nucleases such as CAD and AIF/EndoG.

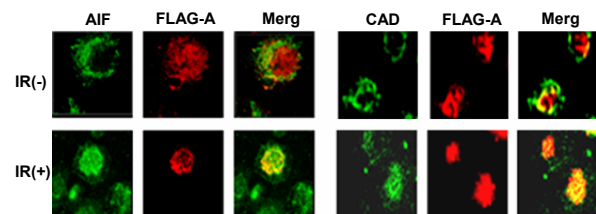


Figure 2. Colocalization (yellow) of clone A protein with AIF and CAD suggesting its role in apoptosis. AIF or CAD (green) and flag tagged clone A (red) were detected with corresponding antibodies in immunofluorescence.

Our results do not provide explanation for regulatory mechanism how clone A expression is tightly regulated by the presence or absence of IR. Moreover the nature of clone A cooperation with other apoptotic nucleases is still open for further investigation. Our findings, however, reveal a role of clone A as a DNase in IR-induced apoptosis and suggest requirement of tight regulation in cellular radiation response.

### Acknowledgement

This work was supported by the Nuclear R&D program from the Ministry of Science and Technology of Korea.

### References

- [1] E.C. Jonathan, E.J. et al., How does radiation kill cells?, *Curr Opin Chem Biol.* 3 (1999) 77-83.
- [2] S. Nagata, Apoptosis by death factor, *Cell* 88 (1997) 355-365.
- [3] D. McIlroy, H. et al., Involvement of caspase 3-activated DNase in internucleosomal DNA cleavage induced by diverse apoptotic stimuli, *Oncogene.* 1999 18 (1999) 4401-4408.
- [4] A. Yoshida, and T. Ueda, Human AP endonuclease possesses a significant activity as major 3'-5' exonuclease in human leukemia cells, *Biochem. Biophys. Res. Commun.*, 310 (2003) 522-528.