Characterization of DNA end-binding activities in higher plants

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Abstract

DNA double-strand-breaks (DSB) are the most severe lesion in cells exposing to ionizing radiation and many other stress environments. Repair of DNA DSB is therefore critical to cellular survival. In this work, we observed the double-stranded DNA end-binding (DEB) like activities in rice (Oryza sativa L. cv. TN5) suspension cells and hypocotyls from etiolated mung bean (Vigna radiata L. TN5) seedlings. Higher plant DEB-like protein binds primarily to linearized double-stranded DNA ends. Competition of unlabeled probe was examined in double-stranded DEB assay of cell extracts from rice and mung bean. DEB-like activities of higher plants did not depend on sequence and types of double-stranded DNA ends. Distinct electrophoretic mobility shift patterns and binding features further indicate that DEB-like factors from various sources might not share identical structure and function, and probably belong to different types of DEB proteins from higher plants. Our evidence suggests that DEB proteins are certainly ubiquitous in all organisms probably for repairing and processing double-stranded DNA breaks from formidable lethal lesion.

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1. Introduction

DNA double-strand-breaks (DSB) are the most severe lesion in cells exposed to ionizing radiation as well as other stress environments. Repair of DSB is therefore critical to cellular survival. At least two independent pathways are proposed for repairing DSB, i.e., homologous recombination and non-homologous DNA end joining [5,15]. DNA DSB in plants are primarily repaired via non-homologous end joining [16]. In mammalian cells, various nuclear DNA-binding proteins are believed to participate in the non-homologous DNA end joining which is the dominant mechanism of DSB repair [5,25]. A body of evidence demonstrated that the DNA-dependent protein kinase (DNA-PK) is involved in DSB repair and V(D)J recombination (variable region, diversity, and joining segment recombination) [13,27,31]. DNA-PK, which phosphorylates several chromatin-bound proteins in vitro, is composed of a 460-kDa catalytic complex (DNA-PKcs) and a Ku protein.

Ku protein consists of two subunits of approximately 70 and 86 kDa [22,24]. Further characterization indicated that Ku protein binds primarily to double-stranded DNA ends (with either cohesive or blunt ends) and other structural discontinuities in DNA, such as nicks, gaps, and hairpins [1,7,23,26]. Some reports later showed that under several conditions Ku protein displays a possible sequence-specific binding to DNA [14,20,30].

Current literature shows that Ku is an abundant protein in most human cells. Homologues of Ku protein, moreover, have also been found in other animals, such as monkey [26], rodents [10], Xenopus [17], and Drosophila [18], and in yeast [9]. These Ku protein homologues are similar in overall size, subunit structure, and DNA binding properties [6,8]. The cDNA sequence of Arabidopsis Ku proteins has been cloned just recently [2,11,12,28,29,32,35]; albeit, detailed studies on plant DEB activity are still lacking. In this communication, we observed the presence of DEB-like activities recog-
nizing double-stranded DNA ends in higher plant cells. Distinct electrophoretic mobility shift patterns and binding features further indicate that DEB-like factors from various sources might not share identical structure and function, and probably belong to different types of DEB-like proteins in monocotyls and dicotyls, respectively. Our evidence suggests that proteins showing DEB activities are certainly ubiquitous in all organisms probably for repairing and processing double-stranded DNA ends from formidable lethal lesions.

2. Results

2.1. DEB-like activities of plant cell extracts

To verify the existence of the DEB activity in cell extracts of higher plants, a purified Ku protein from human placenta was routinely used as gel shift standard marker [4, 19, 27]. Fig. 1 depicts that human placenta Ku protein produced a mobility shift of the DNA probe, indicating the feasibility of this system for following studies. Crude extracts from rice cell line and hypocotyl of etiolated mung bean seedlings were incubated with the linear 32P-labeled double-stranded DNA probe and then subjected to electrophoretic mobility shift assay (EMSA). Mobility shifts of the probe by cell extracts from rice and mung bean were also detected at the position similar to human placenta Ku protein (Fig. 1). Plant cell extracts, both from monocotyl rice and dicotyl mung bean, apparently contain DNA binding activities with retardation in the mobility of a linear, double-stranded DNA probe. These factors that bind to linear double-stranded DNA were further demonstrated as the DNA end-binding (DEB) factors in the following studies (see below). In addition to the predominant band that resembles purified Ku protein from human placenta, plant cell extracts also produced several minor bands with different mobility shifts. The existing pattern of minor bands as DEB proteins was likewise observed in wild-type Chinese hamster ovary (CHO) cells and high mobility group 1 protein [27, 34]. Nevertheless, the intensity of retarded bands for the DEB-like proteins increased concomitantly with the increase in amounts of plant cell extracts or radioactively labeled double-stranded DNA probes added, respectively (data not shown). The interaction of plant DEB-like protein with radioactively labeled double-stranded DNA probe could be carried out appropriately at temperature between 4 and 25 °C (data not shown). High ionic strength, such as 200 mM KCl, was required for double-stranded DNA binding to plant DEB-like factors. However, EMSA patterns were not significantly changed in the presence of N,N,N′,N′-ethylenediamine tetraacetic acid (EDTA) or adenosine 5’-triphosphate (ATP), suggesting that divalent cations are not indispensable and the reaction is energy-independent (see below). On the contrary, when the unlabeled double-stranded DNA probe was added in the reaction mixture, there was a loss in intensities of DNA binding activity of plant DEB-like proteins (data not shown). The degree of competition depends on the concentration of unlabeled double-stranded DNA in the binding reaction medium. At approximately 6.0 fmol µl–1 of unlabeled double-stranded DNA, the radioactively labeled probe at the position similar to Ku was completely competed under our conditions. Thus, we unambiguously observed the presence of DEB-like activities in cell extracts of monocotyl and dicotyl plants.

2.2. Plant DEB-like proteins bind primarily double-stranded DNA ends

Several binding properties of DEB-like factors in plant cell extracts were further investigated. Firstly, we examined the binding affinity of plant DEB-like factor to single-stranded DNA with different sequences under our conditions (Fig. 2). The presence of single-stranded DNA in the reaction mixture did not compete the DNA binding activity of mung bean DEB-like protein. However, several single-stranded DNA could compete against labeled double-stranded DNA probe for the rice DEB-like protein. From EMSA patterns and distinct binding affinities, we believe that DEB-like proteins from monocotyls and dicotyls might be different.

Fig. 1. Identification of plant DEB-like activities. DEB activities of human placenta Ku protein and cell extracts from rice cell line and mung bean seedlings were determined as described in Section 4. Lane B, without cell extract. F, free probe.

Fig. 2. DEB-like activities of plant cell extracts in the presence of single-stranded DNA. DEB-like activities of cell extracts from mung bean seedlings and rice cell line were determined, as described in Section 4, in the presence of various single-stranded DNAs (10 fmol µl–1). Lanes B, without cell extract; C, no addition.
Circular DNA could not compete efficiently the binding of labeled double-stranded DNA probe to mammalian Ku protein. Similarly, circular form of plasmid DNA, such as pGEM1, could not compete effectively for DEB activities of plant cell extracts, demonstrating the true substrate of DEB-like factor containing free ends of double-stranded DNA (Fig. 3). However, when the pGEM1 was cut once with a restriction enzyme HindIII and then the digest added in reaction medium, there was a significant competition in plant DEB-like activities (data not shown). For instance, at higher concentration of HindIII-digested plasmid, most labeled double-stranded probe could be completely removed. Obviously, linear and doubled-stranded DNA ends are presumably prerequisite for DEB-like activities of plant cell extracts, also a diagnostic feature of mammalian Ku protein. We thus surely observed the existence of DEB-like activities in cell extracts of mung bean and rice cell line under our conditions.

2.3. Plant DEB-like proteins show no sequence specificity

To examine the preference of plant DEB-like factors to the types and sequences of free double-stranded DNA ends, we investigated the competition in DEB-like activity with different forms of DNA. Restriction enzymes PvuII (1), BglI (3), and AvaII (8) can generate blunt ends, 3′ overhang, and 5′ overhang of DNA fragments from pBR322 with cut number shown in parenthesis, respectively. Each enzyme-digested DNA fragment obtained above showed appropriately high competition to plant DEB-like factor (Fig. 4), revealing that DEB activity is independent of the sequence and types of free DNA ends. However, intensities of retarded bands of each treatment are different. It is likely that the degree of competition increased proportionally with the increase in the number of free ends of DNA fragments generated by restriction enzymes. This possibility was further confirmed by using different plasmids cut by the same restriction enzyme, which produces identical sequence of DNA ends but various number of free ends (Fig. 5). Our current results suggest that DEB activities of plant DEB-like factors might not depend on sequences and types of double-stranded DNA ends. Nevertheless, the verification of this speculation requires further investigations.

2.4. Ion effects on plant DEB-like proteins

Ion effects on plant DEB-like activities were investigated. First of all, we found that DEB-like factor from rice cell line did not absolutely require monovalent cations for its DEB activity (Fig. 6). However, the presence of several monovalent cations in the medium could still enhance their DEB activity to certain extent. On the other hand, monovalent cations are certainly indispensable for DEB activity of DEB-like protein from mung bean seedlings. As for divalent cations scrutinized, 5 mM Mg2+, Ca2+, and Mn2+ could augment
Moreover, chelating agents, such as EDTA and ethyleneglyco-bis(β-aminoethyl ether)N,N',N",N"'-tetraacetic acid (EGTA), did not exert any significant effects on the binding affinity of plant DEB-like proteins to double-stranded DNA ends, implying that divalent cations are not absolutely necessary for this reaction. Nevertheless, the exact mechanism of monovalent cations in stimulation and that of divalent cations in inhibition of DEB-like activities of higher plants requires further determination.

3. Discussion

Repair of DNA DSB is essential for cellular survival upon exposure to damaging agents, such as free radicals and ionizing irradiation. Ku protein has been identified from mammals and yeasts as a key component presumably involved in the non-homologous DNA end joining, a dominant mechanism of double strand break repair [5]. Present study, using EMSA, provides the evidence showing that plant cell extracts, both from monocotyl rice cell line and dicotyl mung bean seedlings, apparently contain also a DEB-like activity. EMSA analysis shows that plant cell extracts produced a predominant band that likewise resembles purified Ku protein from human placenta. Besides, several minor bands were also observable, as shown in wild-type CHO cells [27]. Nevertheless, mobility shift of minor bands from various sources is different. Distinct EMSA patterns presumably suggest the species-specificity of double-stranded DEB proteins from various sources.

Ku proteins from mammals and yeast preeminently bind to linear and double-stranded DNA ends, though a report recently showed its possibility to react with sequence-specific single-stranded DNA under certain conditions [33]. For mung bean cell extract, all single-stranded DNA scrutinized could not show significant competition against its DEB activity. However, several single-stranded DNA could compete against the DEB-like activity of rice cell extract. From EMSA patterns and distinct binding affinities toward single-stranded DNA, we believe that both plant DEB-like proteins might not share identical structure and function, probably indicating distinct types of DEB proteins in monocotyls and dicotyls. Furthermore, circular form of plasmid DNA, such as pGEM1 and pBR322, could not compete efficiently for DEB-like activities of cell extracts from both rice cell line and mung bean seedlings. Tentatively, we believe the true substrate of plant DEB-like protein should contain free ends of double-stranded DNA. In mammals, neither could circular DNA compete efficiently the binding of labeled double-stranded DNA probe to Ku protein. However, a report recently showed a few circular plasmid DNA with appropriate supercoiled structure could still bind to double-stranded DNA for Ku protein, albeit, with lower affinity [27]. At present, we did not observe the competition of circular form of plasmid DNA against the binding of labeled probe in plant cell extracts. Our observations in further verify the species-specificity of double-stranded DEB-like proteins from various sources. Furthermore, plant DEB-like protein primarily binds to double-stranded DNA ends, independently of the detailed structure of the ends. The DNA structures recognized by plant DEB-like proteins include blunt ends, 5′ overhangs, and 3′ overhangs generated by restriction enzyme cleavage. Moreover, plant DEB-like protein binds to double-stranded DNA ends without any obvious preference. This is not unexpected, since higher plants are immobile and subjected to diverse stress factors in the open field in which various types of double strand breaks might be generated. The lack of preference may offer advantages for plant DEB proteins to efficiently access any damaged ends of double-stranded DNA, probably for their repair. The possibility of the involvement of DEB-like proteins in DNA repair systems requires further verification.

Our studies indicate that monovalent cations are required for optimal DEB-like activities of plant DEB proteins. How-
ever, diverse effects of divalent cations were observed. For instance, Mg$^{2+}$ and Ca$^{2+}$ showed no significant influence on DEB-like activity of plant DEB protein, whereas other divalent cations, such as Cu$^{2+}$, Cd$^{2+}$, Sn$^{2+}$, and Zn$^{2+}$, exhibit inhibitory effects. It is possible that Mg$^{2+}$ and Ca$^{2+}$ are more common in plant cell, but over-dose of other divalent cations may induce configurational change of DEB-like protein, resulting in a decrease in its DEB activity. Nevertheless, it is likely that divalent cations are not absolutely required since the presence of chelating agents, such as EDTA and EGTA, did not alter the binding affinity of plant DEB-like proteins to double-stranded DNA ends.

Taken together, the results presented in this report demonstrated that plant cell extracts, both from monocotyl rice cell line and dicotyl mung bean seedlings, apparently contain DEB-like activities but with several different characteristics of their own. It is possible that DEB factors from various sources belong to different types of DEB proteins with diverse structure and function, respectively. The ubiquitous existence of DEB proteins in higher plants implicates its essential roles in repairing and processing double-stranded DNA breaks from formidable lethal lesions. Notwithstanding, further purification of DEB protein from various higher plants is recommended for more detailed characterization and investigation of these interesting double-stranded DEB proteins.

4. Methods

4.1. Materials and cell line

Seeds of mung bean (V. radiata L. TN5) were soaked overnight in tapwater and then germinated at room temperature in the dark for 7 d. Hypocotyls of etiolated seedlings were excised, chilled on ice, and then used for further preparation of cell extracts. Rice cell line (O. sativa L. cv. TN5) was grown in the culture medium (Murashige and Skoog basal salt mixture (Gibco/BRL, Grand Island, NY, USA) (pH 5.7), 3% (w/v) sucrose, 10 µM 2,4-dichlorophenoxyacetic acid, 3 µM thiamine-HCl, 5 µM pyridoxine-HCl, 80 µM nicotinic acid, and 0.56 mM myo-inositol) [21,36]. The medium was reciprocally shaken (140 rpm) in the dark for 14 d [21,36]. Purified Ku protein from human placenta was routinely prepared according to the methods described previously [3,4,19]. The purified human placenta Ku was >95% pure and did not contain DNA-PKcs, as determined by western analysis and silver stain (data not shown, [4,19]).

4.2. Cell extracts

Cell extracts were prepared from rice cell line by lysis with sonication, and from hypocotyls of etiolated mung bean seedlings by a glass grinder. Approximate 1 ml rice cells (0.4 g fresh-weight cells) or 1 g hypocotyl tissue was added to 1 ml of high salt extraction buffer (20 mM HEPES (pH 7.5), 0.5 M KCl, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 M dithiothreitol (DTT), 40% (w/v) glycerol, 1% (w/v) NP-40) for sonication or grinding, respectively. The mixture was centrifuged at 17,200 × g for 30 min and the pellet was discarded. All procedures were carried out on ice or in a cold room, and the whole cell extract was used immediately or stored at −70 °C for further studies.

4.3. Radioactively labeled duplex substrate and competitor DNA

The synthetic oligonucleotides were as follows with the polarity always written as 5′–3′:

IUU, 5′-catGGAAGACTTGGCGCAATACAT-3′;
BCSA, 5′-tgtcATGTATTGCCGCAAGTCTTCC-3′.

(Lower case letters represent non-base-paired nucleotides.) IUU and BCSA were then annealed in TE buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA) by heating at 65 °C for 5 min and slow cooling to room temperature. The duplex DNA probe was end-labeled with T4 polydeoxynucleotide kinase (New England Biolah, Beverly, MA, USA) in the presence of 1.85 × 10⁶ Bq [γ-³²P]ATP (Amersham Biosciences Ltd, Uppsala, Sweden) at 37 °C for 30 min, as suggested by manufacturer. Free [γ-³²P]ATP was removed by chromatography on Sephadex G-50 spin column (Amersham Biosciences Ltd, Uppsala, Sweden).

Plasmids pGEMI and pBR322 were utilized as the competitor DNA either in intact supercoiled or in restriction fragment forms. The restriction fragments were obtained by digesting pGEMI or pBR322 with different restriction enzymes, such as HindIII, AvaII, PvuII, and BglII (New England Biolah, Beverly, MA, USA), respectively.

4.4. EMSA

DEB activities of plant cell extracts and human placenta Ku protein were determined by EMSA [4,19,27]. Purified human Ku protein (5 µg) or plant cell extracts (18 µg) were incubated with labeled probe (1 fmol µl⁻¹) at 4 °C for 20 min in a 25 µl solution containing 40 mM HEPES (pH 7.5), 5 mM DTT, 0.5 mM EDTA, 200 mM KCl, 10% (w/v) glycerol, 0.1% (w/v) NP-40, and 1.2 mM ammonium molybdate. After incubation, the samples were subjected to electrophoresis in a 6% (w/v) non-denaturing polyacrylamide gel at 4 °C and 150 V for 180 min. Gels were dried on Whatman 3 M paper and then autoradiographed at −70 °C. In competitive EMSA, unlabeled competitors, such as supercoiled pGEMI DNA, IUU/BCSA, and single-stranded DNA, were added to the reaction mixture in the presence of the labeled probe. Single-stranded DNA used were oligonucleotides

S₁ (5′-GTCATCACCATAATAGAT-3′),
S₂ (5′-TGCGGTTCTCTTTTTGAG-3′),
S₃ (5′-CTCGGTGGCTCTTCTATG-3′), and
S₄ (5′-CTGAAAAAGACCACCCCA-3′), respectively.
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