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Intracellular localization of human ZBP1: Differential regulation by the Z-DNA binding domain, Zα, in splice variants

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Abstract

We investigated the subcellular distribution of human ZBP1, which harbors the N-terminal Z-DNA binding domains, Zα and Zβ. ZBP1 was distributed primarily in the cytoplasm and occasionally as nuclear foci in interferon (IFN)-treated primary hepatocellular carcinoma cells, and in several other transfected cell types. In leptomycin B (LMB)-treated cells, endogenous ZBP1 efficiently accumulated in nuclear foci, which overlapped PML oncogenic domains (PODs) or nuclear bodies (NBs). In transfection assays, the unique C-terminal region of ZBP1 was necessary for its typical cytoplasmic localization. Interestingly, the Zα-deleted form displayed an increased association with PODs compared to wild-type and, unlike wild-type, perfectly accumulated in PODs in LMB-treated cells, implying that the presence of Zα domain also facilitates the cytoplasmic localization. Our results demonstrate that ZBP1 is localized primarily in the cytoplasm but also associated with nuclear PODs in IFN or LMB-treated cells. Given that about half of ZBP1 mRNA lacks exon 2 encoding the Zα domain, our data also suggest that the localization of ZBP1 may be differentially regulated by the Z-DNA binding domain, Zα, in splice variants.

Keywords: Z-DNA; ZBP1; Localization; PML oncogenic domains; Interferon; Nuclear localization; Nuclear export; Zα domain

The Z-DNA binding protein 1 (ZBP1), which is also known as DLM-1, was originally identified as a tumor-associated and interferon (IFN)-inducible protein [1]. Although the function of ZBP1 is not understood, structural analysis has demonstrated that it harbors N-terminal Z-DNA binding domains homologous to the Zα and Zβ domains of the RNA editing enzyme adenosine deaminase that acts on RNA (ADAR1) [2,3]. The Zα domain of ADAR1 has a winged-helix motif that recognizes Z-DNA in a structure-specific manner [4]. The ADAR1 Zα domain is also capable of binding left-handed Z-RNA [5]. Two additional proteins have been found to contain Z-DNA binding domains; E3L, the poxvirus virulence factor [6,7], and PKZ, the double-stranded RNA (dsRNA)-dependent protein kinase (PKR)-like eukaryotic initiation factor 2α kinase from zebrafish [8]. In view of the structural features, ZBP1 is expected to play a role in host defense against tumor formation or viral infection via its ability to bind to DNA or dsRNA in the Z configuration.

The Zα domain of ADAR1 has been shown to regulate intracellular protein distribution. Two forms of ADAR1 proteins are synthesized from different promoters [9]. A constitutively expressed short form is localized predominantly in the nucleus, whereas the N-terminally extended form is both induced by IFN and able to shuttle between the nucleus and cytoplasm [10–12]. Interestingly, the N-terminal Leu-rich sequences overlapping the Zα domain have been shown to be important for the nuclear export function

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of ADAR1. A nuclear export receptor CRM1 bound to the Zα domain [10] and a small fragment containing these Leu-rich sequences mediated the nuclear export of a reporter construct [12].

A study of the mRNA products synthesized from the human ZBP1 gene found that the ZBP1 gene spans 17 kb and consists of 10 exons, and that transcripts are highly heterogeneous due to alternative splicing, exon variation, and the use of at least two transcription start sites and 3′-terminal exons [3]. Interestingly, this study demonstrated that about half of ZBP1 cDNA clones lacked exon 2, which encodes the Zα domain. Considering the role of the Zα domain in the intracellular distribution of ADAR1, it is conceivable that two types of ZBP1 proteins, with or without the Zα domain, may also exhibit differential functions in different cellular compartments.

In this study, we investigated the subcellular distribution of ZBP1 and role of the Zα domain. We show that ZBP1 is localized primarily in the cytoplasm but also associated with subnuclear structures referred to as PML oncogenic domains (PODs) or nuclear bodies (NBs) in cells treated with IFNγ or leptomycin B (LMB), an inhibitor of the nuclear export receptor CRM-1. Our data also show that the unique C-terminal region of ZBP1 is necessary for its typical cytoplasmic localization. Moreover, although the Zα domain of ZBP1 did not appear to have the LMB-sensitive nuclear export signal, it was shown to facilitate its cytoplasmic localization, suggesting that the presence or absence of exon 2 encoding the Zα domain in the splice variants may modulate their intracellular distribution.

Materials and methods

Cell culture and transfection. The hepatitis B virus (HBV)-positive human primary hepatocellular carcinoma cells SNU449 [13] were obtained from the Korean Cell Line Bank. SNU449, HepG2, and Vero cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For indirect immunofluorescence assays (IFA), cells were seeded into four-well slide chambers and DNA mixtures were introduced into cells using Metafectene (Biontex) (for HepG2) or FuGene6 (Roche) (for Vero) reagents, according to the manufacturers' instructions. For immunoblot analysis, cells were seeded into six-well plates and DNA mixtures were introduced as above. To block CRM-1-mediated nuclear export, cells were incubated with medium containing 0.5 nM of leptomycin B (LMB) for 20 h.

Plasmid construction. Expression plasmids for 5′ flag-tagged human ZBP1 (1–429 amino acids) and the deletion mutants, flag-ZBP1 (81–429), flag-ZBP1 (178–429), and flag-ZBP1 (1–177), were generated in a background of pSG5 [14] using Gateway technology (Invitrogen). Expression plasmids for intact or mutant ZBP1 as HA-tagged forms were also generated in pSG5 using Gateway technology. The Δexon-2 mutant, which had a deletion of amino acids at positions 12–86, was generated by PCR. pGFP-ZBP1 (178–491) was generated in a background of pEGFP-C1 (Clontech).

Antibodies. Rabbit polyclonal antibody (PAb) against ZBP1 was raised using the purified N-terminal fragment of ZBP1 (amino acids 8–166) containing its Zα and Zβ domains (TaKaRa-Korea Biomedical Inc.). Mouse monoclonal antibodies (MAb) M2 against flag epitope and PG-M3 against PML were purchased from Sigma and Santa Cruz, respectively. Anti-HA rat MAb 3F10 conjugated with peroxidase or labeled with fluorescein isothiocyanate (FITC) was purchased from Roche. The mouse MAb for GFP was purchased from Santa Cruz. Rabbit PAb against the C-terminal peptide of PML was previously described [15].

IFA. Cells were fixed with 1% paraformaldehyde, permeabilized with 0.2% Triton X-100, incubated with appropriate Abs in phosphate-buffered saline (PBS) at 37 °C for 1 h, and then incubated with FITC-labeled donkey anti-mouse IgG or with rhodamine/red X-coupled donkey anti-rabbit IgG at a 1:100 dilution at 37 °C for 45 min. For double-labeling, two Abs were incubated together. To stain cell nuclei, mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA) was used. All slides were examined and photographed using a Zeiss Axiophot or a confocal microscope.

Immunoblot analysis. Cells were washed with PBS and lysed with ice-cold RIPA buffer (50 mM Tris–HCl (pH 8. 0), 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] supplemented with protease inhibitor cocktail (Santa Cruz Biotechnology). Clarified cell extracts were separated on a SDS–8% polyacrylamide gel and electroblotted onto nitrocellulose membranes. The blots were blocked by incubation with PBS plus 0.1% Tween 20 (PBST) containing 5% nonfat dried milk for at room temperature for 30 min, washed with PBST twice, and incubated with appropriate Abs at room temperature for 1 h. After 10-min washing with PBST, the blots were incubated with horseradish peroxidase-conjugated secondary Ab at room temperature for 45 min. The blots were then washed three times with PBST, and the protein bands were visualized using an enhanced chemiluminescence (ECL) system (Amersham).

Results

The localization pattern of endogenous ZBP1 and the effect of IFN or LMB treatment

Transcript analyses by Northern blotting or RT-PCR have shown that human and mouse ZBP1 is expressed in several tissues such as lymphatic tissues, spleen, lung, liver, kidney, heart, and testis [1,3]. To investigate the subcellular distribution pattern of endogenous ZBP1, we performed IFA using a rabbit PAb raised against the purified N-terminal fragment of ZBP1, which contained the Zα and Zβ domains. Of the several cell types tested, endogenous ZBP1 was weakly detectable primarily in the cytoplasm in SNU449 cells (a primary human hepatocellular carcinoma cell line) (Fig. 1a). When SNU449 were treated with IFNγ, ZBP1 was easily detectable mostly in the cytoplasm, with being occasionally localized as nuclear foci (Fig. 1b). These nuclear foci resembled the subnuclear structures known as PODs or PML NBs. Indeed, double-labeled IFA revealed that these nuclear ZBP1 proteins were colocalized with PML in PODs (Fig. 1b, arrow). We also found that, like ADAR1, the distribution of ZBP1 is affected by the treatment of LMB. In cells treated with LMB, endogenous ZBP1 was found to efficiently accumulate in PODs in both IFNγ-untreated (Fig. 1c) and treated cells (Fig. 1d and e). In transfected HepG2 or Vero cells, exogenous ZBP1 displayed the similar localization pattern, which was primarily cytoplasmic (Fig. 1f and h) and occasionally associated with nuclear PODs (Fig. 1g and i). Our results suggest that ZBP1 is localized primarily in the cytoplasm but also associated with PODs in the nucleus in IFN or LMB-treated cells.

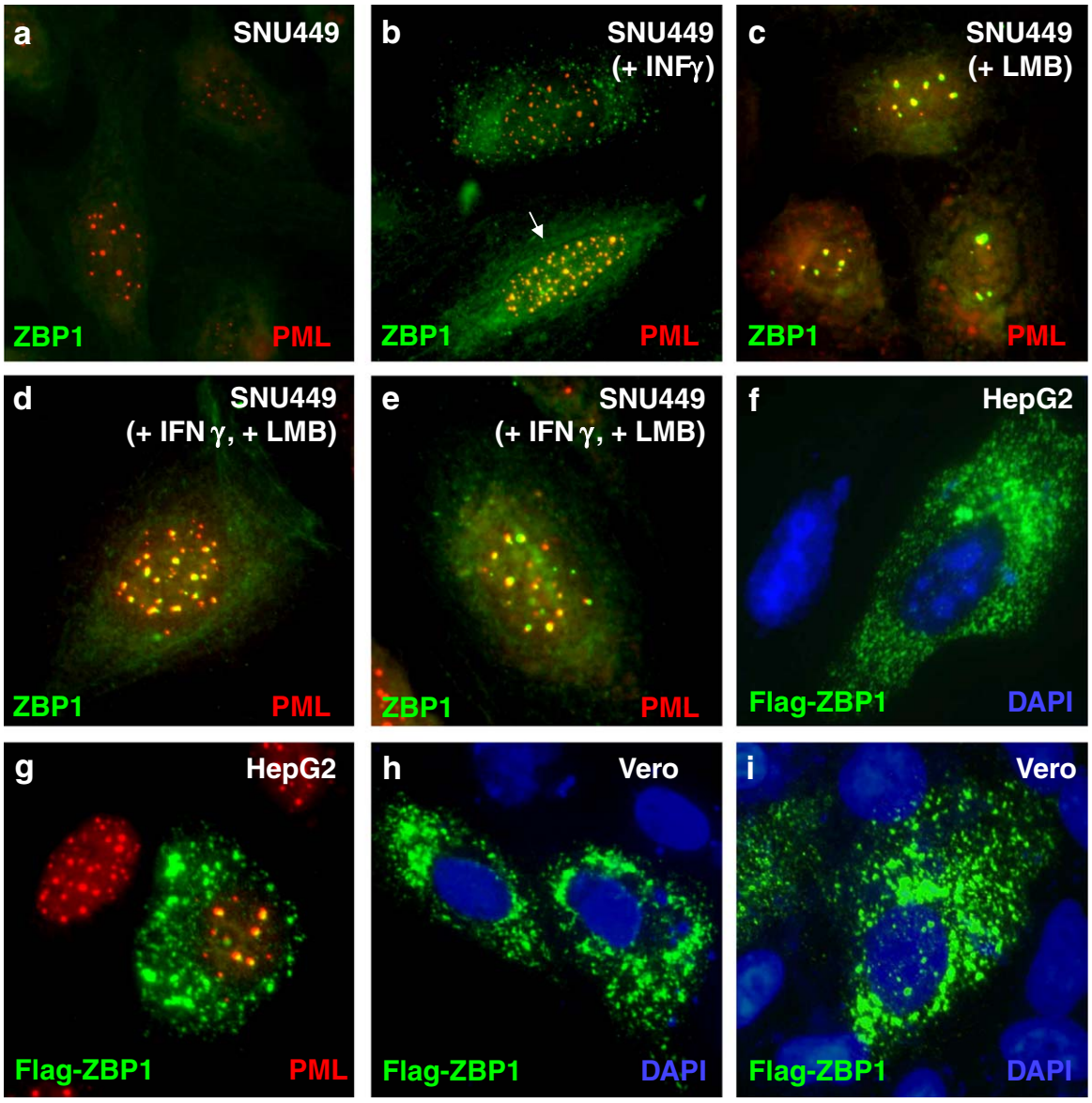

Fig. 1. Localization patterns of ZBP1. (a–e) SNU449 cells were untreated (a) or treated with 100 ng/ml human IFNγ (b), 0.5 nM LMB (c), or both IFNγ and LMB (d,e) for 20 h. Cells were fixed with paraformaldehyde and double-label IFA was carried out using anti-ZBP1 rabbit PAb and anti-PML mouse MAb PG-M3. (f–i) HepG2 (f,g) or Vero (h,i) cells were transfected with 5′ flag-ZBP1 (429 amino acid version). At 24 h, cells were fixed with paraformaldehyde and stained with anti-flag mouse MAb M2 and anti-PML rabbit PAb. FITC-labeled donkey anti-mouse IgG and rhodamine/red X-coupled donkey anti-rabbit IgG were used for visualization. A mounting solution containing DAPI was used to stain cell nuclei.

The Zα domain of ZBP1 facilitates the cytoplasmic localization in both LMB-treated and untreated cells

The structures of the four cellular and viral proteins containing the Z-DNA binding domains are compared in Fig. 2A. ZBP1 consists of two Z-DNA binding domains (Zα and Zβ) and a unique C-terminal domain. However, hundreds of different ZBP1 transcripts are expected to be produced due to alternative splicing and the usage of different 5′ and 3′ ends [3]. Of these, those that contain the Zα or the Zβ domain can be classified into at least three subgroups according to the presence or absence of exons 9 and 10. Interestingly, each subgroup is also differentiated based on the presence or absence of exon 2, which encodes the Zα domain. To investigate the roles of specific ZBP1 domains in its intracellular targeting, we used a cDNA clone consisting of sequences from exons 1, 2, 3, 4, 5, 6, 8, and 10. This cDNA encodes a 429 amino acid protein, which may be the largest form containing the Zα or Zβ domains (Fig. 2B). Two N-terminal truncation mutants lacking the Zα domain (ΔZα) or lacking both the Zα and Zβ domains (ΔZα + Zβ), and a C-terminal truncation mutant containing only the Zα and β domains (Zα + Zβ) were constructed in a background of this 429 amino acid version of ZBP1 (Fig. 2C), and used for transfection assays.

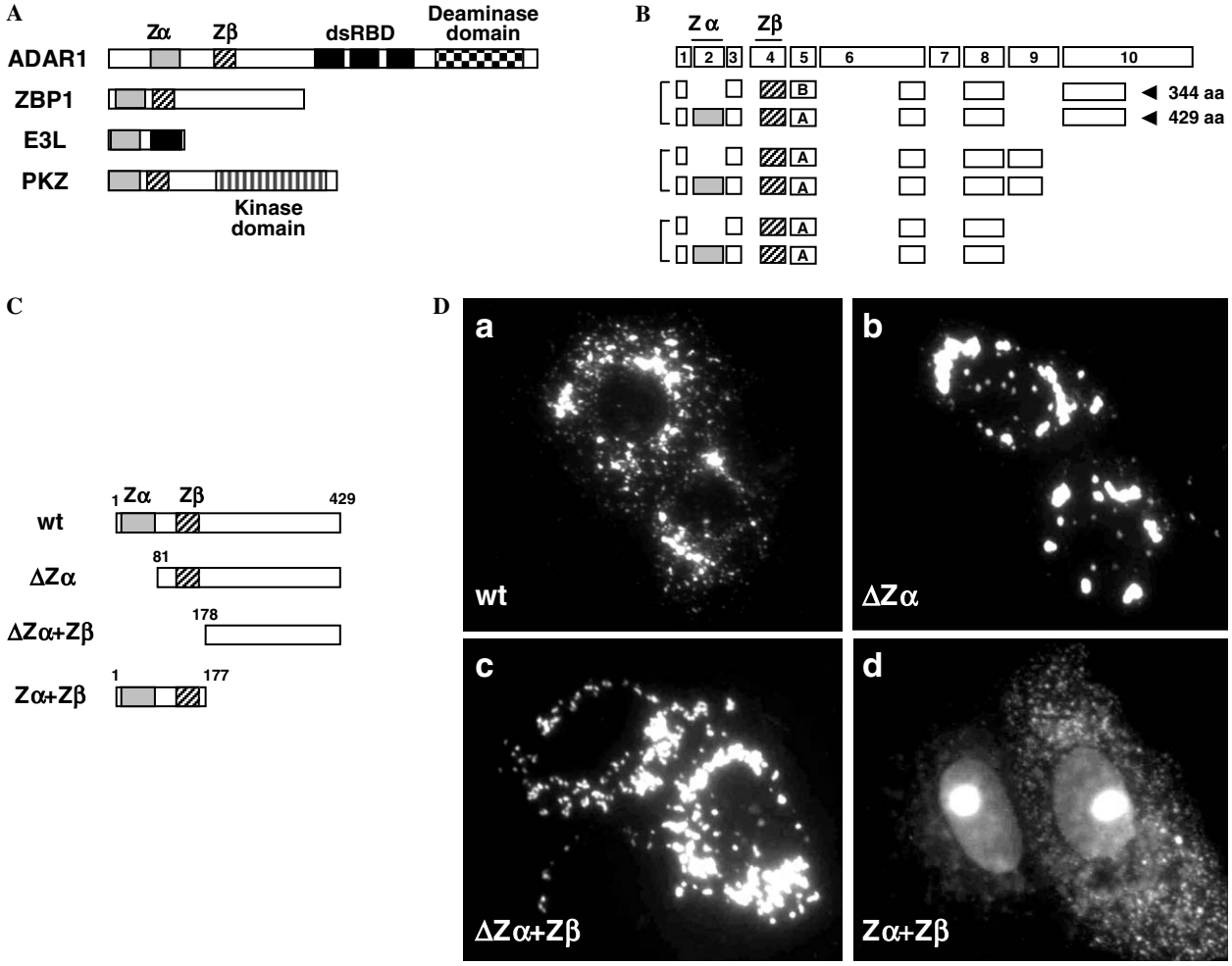

Fig. 2. Exon composition of ZBP1 and localization of mutant ZBP1. (A) Schematic representation of cellular or viral proteins containing the Z-DNA binding domains. ADAR1 contains Zα and Zβ domains, dsRNA binding domains, and the catalytic deaminase domain. ZBP1 possesses Zα and Zβ domains, whereas E3L contains the Zα domain and the dsRNA binding domain. PKZ has the Zα domain and the kinase domain. (B) Exon composition of ZBP1 splice variants containing the Zα or Zβ domains. The exons and their sizes are shown on top of the figure. Exons are numbered, and the exons encoding Zα (exon 2) and Zβ (exon4) are denoted as gray and hatched boxes, respectively. Variants of exon 5 are marked as A and B. The largest cDNA encoding the 429 amino acid version and its variant lacking exon 2 (Zα) encoding 344 amino acids are indicated. (C) ZBP1 constructs used in this study. Full-length ZBP1 consists of 429 amino acids. The ΔZα mutant lacked 80 N-terminal amino acid residues, the ΔZα + Zβ mutant lacked 177 N-terminal amino acid residues, and the Zα + Zβ mutant contained only the 177 N-terminal amino acid residues. (D) Localization of the mutant ZBP1 proteins in transfected cells. Vero cells were transfected with plasmids encoding flag-ZBP1 (wild-type) protein (a), or ΔZα (b), ΔZα + Zβ (c), or Zα + Zβ (d) mutant proteins. At 24 h, cells were fixed with paraformaldehyde, followed by IFA with anti-flag mouse MAb. FITC-labeled donkey anti-mouse IgG was used for visualization.

We expressed intact or mutant ZBP1 as flag-tagged forms in Vero cells by transfection and performed IFA with anti-flag Ab. The result showed that the ΔZα mutant was mainly cytoplasmic, with forming somewhat aggregated forms near the nuclear periphery, and also showed an increased association with PODs in the nucleus compared to wild-type protein (Fig. 2D, a and b). Interestingly, the ΔZα + Zβ mutant was distributed exclusively in the cytoplasm, with showing a mitochondria-like pattern (Fig. 2D, c). When the Zα + Zβ mutant was expressed, it was localized as a mixture of nuclear and nucleolar diffuse forms as well as some cytoplasmic forms (Fig. 2D, d), suggesting that this mutant either lost a region responsible for cytoplasmic retention or diffused into the nucleus and the nucleolus due to its small size.

We further investigated the localization patterns of mutant ZBP1 proteins in LMB-treated cells. Consistent with the results obtained with endogenous ZBP1, LMB treatment resulted in the marked nuclear accumulation of exogenous ZBP1 in PODs, although still the significant amounts of the protein remained in the cytoplasm (Fig. 3A, a–d). Interestingly, unlike the wild-type protein, the ΔZα mutant was localized exclusively as a POD-associated form in LMB-treated cells (Fig. 3A, e–h). These findings suggest that the cytoplasmic localization of the ΔZα mutant may be fully regulated by the CRM-1-dependent nuclear export event, and that the presence of Zα domain

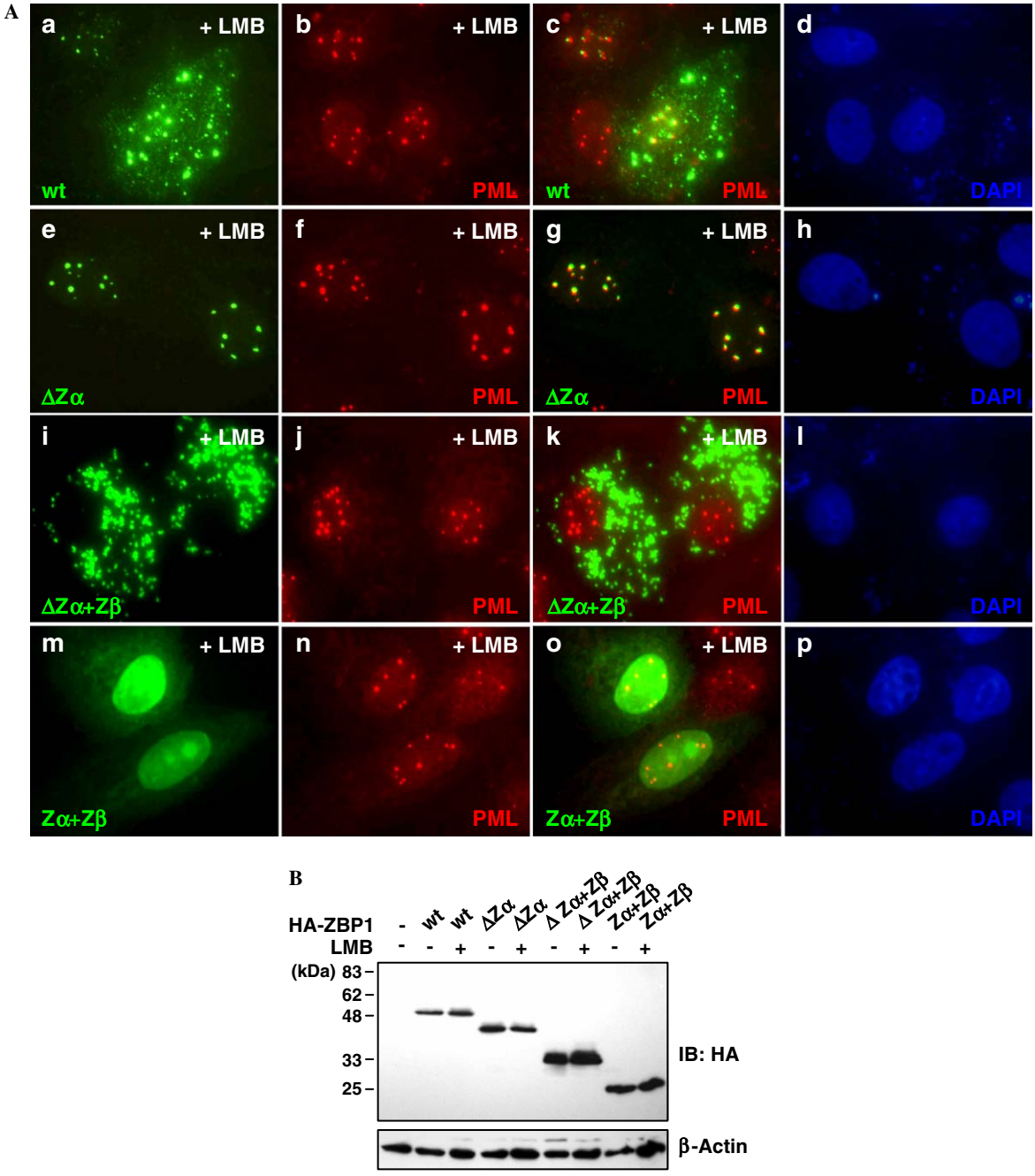

Fig. 3. Effects of LMB treatment on localization of mutant ZBP1. (A) Localization of the wild-type or mutant ZBP1 proteins in transfected cells upon LMB treatment. Vero cells were transfected with plasmids encoding flag-ZBP1 (wild-type) (a–d), or ΔZα (e–h), ΔZα + Zβ (i–l), or Zα + Zβ (m–p) mutant proteins. At 24 h, the culture medium was replaced with fresh medium containing 0.5 nM LMB. After further incubation for 24 h, cells were fixed with paraformaldehyde, followed by double-labeled IFA with anti-flag mouse MAb and anti-PML rabbit PAb as Fig. 1. (B) The expression levels of the wild-type or mutant ZBP proteins. Vero cells were transfected with plasmid expressing HA-tagged ZBP1 proteins and treated or untreated with 0.5 nM LMB. Total cell extracts were prepared as described in Materials and methods. Equal amounts of each extract were subjected to SDS–10% PAGE for immunoblot analysis using anti-HA Ab. β-Actin levels were used as loading controls.

in wild-type protein facilitates its cytoplasmic localization even in the LMB-treated cells. When the ΔZα + Zβ and Zα + Zβ mutants were tested, their localization patterns were not affected by LMB (Fig. 3A, i–p). This result also suggests that the region encompassing amino acids 81–177 containing the Zβ domain may be necessary for nuclear accumulation in LMB-treated cells. The similar localization patterns and effects of LMB were observed with HA-tagged ZBP proteins (data not shown). The expression levels of wild-type or mutant ZBP1 were not significantly affected by LMB treatment in immunoblot assays (Fig. 3B).

The unique C-terminal region of ZBP1 is necessary for its typical cytoplasmic localization

To investigate whether the ΔZα + Zβ mutant form, which contains only the unique C-terminal region of ZBP1 (from amino acids 178 to 491), is indeed associated with mitochondria, cells were cotransfected with plasmids encoding the ΔZα + Zβ mutant and a mitochondria marker, DsRed2-Mito, which is a fusion of *Discosoma* sp. red fluorescent protein and the mitochondrial targeting sequence from subunit VIII of human cytochrome *c* oxidase (Clontech). The result of double-label IFA showed that the ΔZα + Zβ mutant was associated with mitochondria (Fig. 4A).

To further investigate role of the C-terminal region in ZBP1 localization, we generated the GFP-ZBP1 (178–491) fusion protein (Fig. 4B). Interestingly, this fusion protein was localized as cytoplasmic forms, which are similar to those of wild-type ZBP1 (Fig. 4C). This result suggests that the unique C-terminal region of ZBP1 is necessary for the typical cytoplasmic localization of ZBP1. The distribution of this fusion protein was not affected by LMB treatment (data not shown), suggesting that the C-terminal region of ZBP1 does not contain the signals required for nuclear accumulation but may be responsible for the cytoplasmic retention.

Differential localization of a splice variant that lacks the Zα domain

Although the results from our transfection assays demonstrate that the ΔZα mutant form of ZBP1 still possesses the region that is required for the complete response to LMB, the data also suggested that the Zα domain may facilitate the cytoplasmic localization. About half of ZBP1 mRNA was shown to lack exon 2 encoding the Zα domain [2,3]. Therefore, we further examined whether the splice variant, which lacks exon 2, is differentially localized compared to that containing exon 2. When wild-type ZBP1 encoding 429 amino acids was expressed in Vero cells, only 27% of transfected cells showed the POD-associated nuclear staining pattern of ZBP1. However, when the Δexon-2 version of ZBP1, which encodes 344 amino acids and lacks the entire Zα domain (Fig. 2B), was tested, 85% of cells showed the nuclear POD-targeting form of ZBP1 (Fig. 5A). A representative three-color image demonstrating the nuclear POD-targeting of Δexon-2 version of ZBP1 is shown (Fig. 5B). These results suggest that the

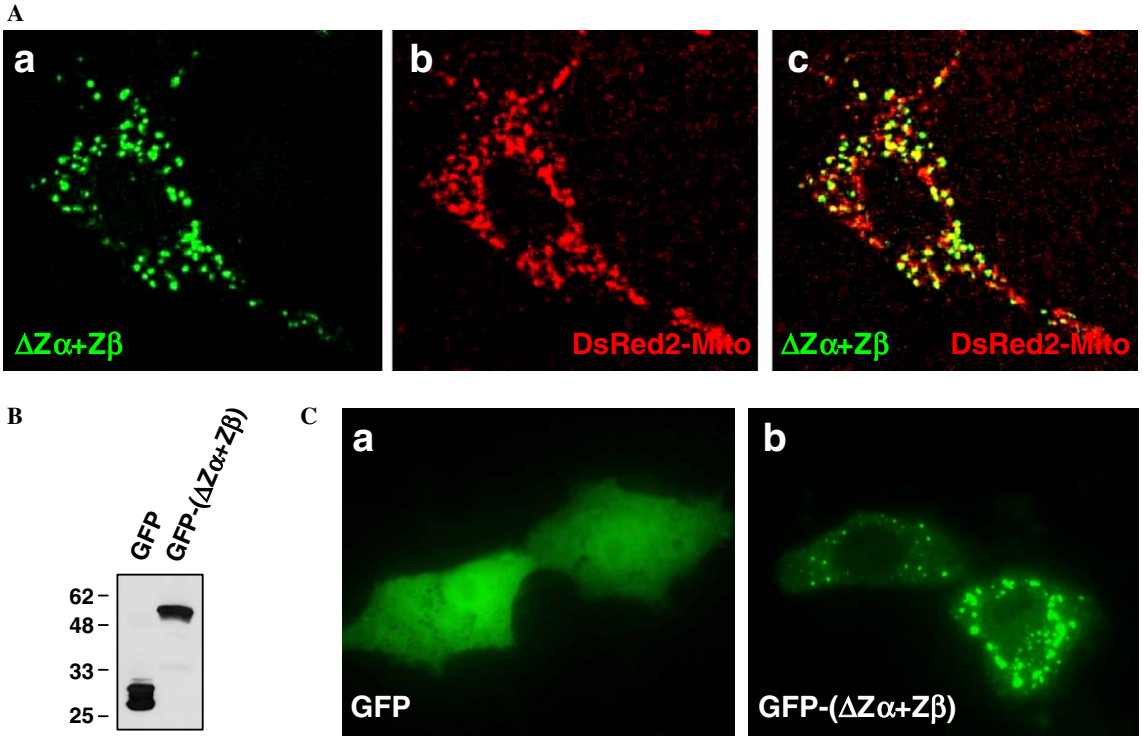

Fig. 4. Localization of ΔZα + Zβ mutant and GFP-ΔZα + Zβ fusion proteins. (A) Confocal microscopy images demonstrating the association of ΔZα + Zβ mutant with mitochondria. Vero cells were cotransfected with plasmids encoding the flag-ZBP1 (ΔZα + Zβ) mutant and DsRed2-mitochondria targeting signal (Mito) fusion protein. At 48 h, cells were fixed and stained with anti-flag Ab. FITC-labeled donkey anti-mouse IgG was used to detect ΔZα + Zβ. Confocal images from each fluorochrome were recorded and superimposed to demonstrate colocalization. (B) The expression levels of pEGFP-C1 or pGFP-ΔZα + Zβ. Vero cells were transfected and followed by immmunoblotting with anti-GFP Ab. (C) Localization of the GFP-ΔZα + Zβ fusion protein. Vero cells were transfected with pEGFP-C1 or pGFP-ZBP1 (178–491) (i.e., pGFP-ΔZα + Zβ). The GFP signals were captured at 24 h using the FITC filter.

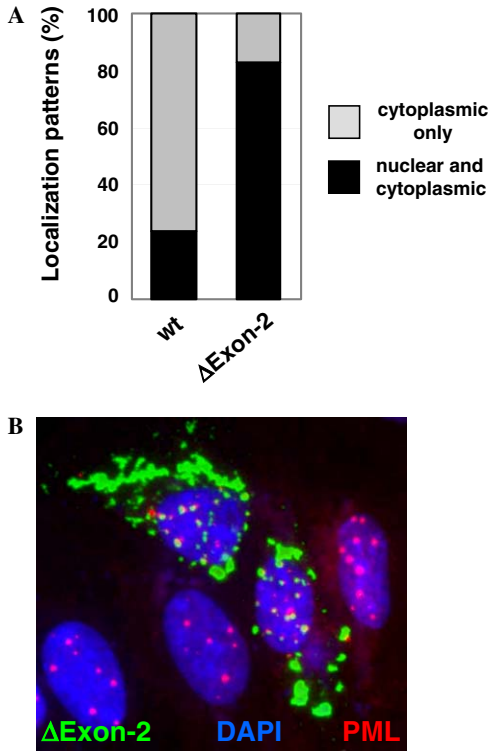

Fig. 5. Localization of a splice variant that lacks exon 2 encoding the Zα domain. (A) Vero cells were transfected with flag-tagged wild-type or Δexon-2 ZBP1. At 48 h, double-label IFA was carried out with anti-flag and anti-PML Abs. The numbers of cells (among about 200 transfected cells for each) showing only cytoplasmic punctate localization (gray bars) or showing both cytoplasmic and nuclear punctate localization (dark bars) were counted under a microscope. (B) A representative triple-label image of Vero cells transfected with the Δexon-2 splice variant showing nuclear accumulation near PODs in (A) is shown. Green, mutant ZBP1; red, endogenous PML; blue, DAPI stained DNA.

presence or absence of exon 2 encoding the Zα domain in the splice variants may regulate the intracellular distribution of ZBP1.

Discussion

The present study demonstrates that the Z-DNA binding protein ZBP1 is distributed primarily in the cytoplasm but also localized as a POD-associated form in the nucleus in IFN or LMB-treated cells. In transfection assays, the unique C-terminal region of ZBP1 was shown to be necessary for its typical cytoplasmic localization. Moreover, although the N-terminal truncated mutant, which lacks the Zα domain, appears to contain either the fully functional CRM-1-dependent nuclear export signal (NES) or a region responsible for the complete nuclear retention in LMB-treated cells, the Zα domain was shown to facilitate the cytoplasmic localization of wild-type protein. Given that about half of ZBP1 mRNA lacks exon 2 encoding the Zα domain [3], our data suggest that the localization of ZBP1 may be differentially regulated by the Z-DNA binding domain, Zα, in splice variants.

In our transfection assays, the Zα (containing from 81 to 491) and ΔZα + Zβ (containing from 178 to 491) mutants differently responded to LMB treatment. In contrast to the Zα mutant, which showed the complete nuclear accumulation in LMB-treated cells, the ΔZα + Zβ mutant was not affected by LMB treatment. These prompted us to speculate that the region encompassing amino acids position 81–177 may contain the signal(s) required for nuclear accumulation. We noticed the basic consecutive basic amino acid sequences Arg-Arg-Ala-Lys at positions between 172 and 175 as a putative NLS signal. However, the deletion of these sequences in intact ZBP1 did not affect nuclear accumulation, implying that these sequences do not act as the NLS or these mutations are not sufficient to block nuclear accumulation (data not shown). Interestingly, when the C-terminal region of ZBP1 was expressed, it was distributed as a mitochondria-associated form. However, when GFP was placed in its N-terminus, this GFP-ZBP1 (178–491) gave the typical cytoplasmic localization pattern of wild-type ZBP1. Therefore, whether the C-terminal region indeed contains a mitochondrial targeting signal is not clear yet. However, this C-terminal region of ZBP1 appears to play a key role in its typical cytoplasmic localization. It is also conceivable that the C-terminal region may contain the NES.

Importantly, for the first time, we show that an IFN-inducible Z-DNA binding protein is associated with PODs. PODs are believed to play roles in transcriptional regulation and in anti-viral IFN response, since many POD-associated cellular proteins, such as PML and Sp100, are induced by IFN and act as transcriptional repressors [16], and because PML overexpression confers resistance to several virus infections [17]. In this regard, our findings provide a new insight into the functions of this Z-DNA binding protein in regulating gene expression and viral replication. Indeed, ZBP1 has been recently identified as an IFN-inducible protein in hepatitis B virus (HBV)-infected cells [18]. Further studies are warranted to determine whether ZBP1 indeed has antiviral functions in association with PODs.

Acknowledgments

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