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Augmentation of drug-induced cell death by ER protein BRI3BP

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Abstract

To determine the contribution of the endoplasmic reticulum (ER) to cell fate decision, we focused on BRI3-binding protein (BRI3BP) residing in this organelle. BRI3BP, when overexpressed, augmented the apoptosis of human embryonic kidney 293T cells challenged with drugs including the anti-cancer agent etoposide. In contrast, the knockdown of BRI3BP reduced the drug-triggered apoptosis. BRI3BP overexpression enhanced both mitochondrial cytochrome *c* release and caspase-3 activity in etoposide-treated cells. In response to etoposide, the ER reorganized into irregularly shaped lamellae in mock-transfected cells, whereas in BRI3BP-overexpressing cells, such reorganization was not observed. These observations suggest that BRI3BP is involved in the structural dynamics of the ER and affects mitochondrial viability. Taken together, BRI3BP, widely expressed in animal cell types, seems to possess a pro-apoptotic property and can potentiate drug-induced apoptosis.

Keywords: Apoptosis; Cytochrome *c*; Endoplasmic reticulum; Etoposide; BRI3BP; Mitochondria

The endoplasmic reticulum (ER) is a multifaceted organelle. It plays a major role in protein synthesis, folding and processing. In addition to its housekeeping functions, the ER emits signals to maintain cellular homeostasis. The accumulation of structurally defective proteins in the ER initiates stress responses, which are collectively referred to as the ''unfolded protein response (UPR)'' [1,2]. By enhancing the ERs capacity to refold and degrade aberrant proteins, the UPR initially operates in favor of cellular survival. In contrast, cell death is induced by the UPR when the cells are exposed to excessive and prolonged ER stress. The importance of the stress response has been demonstrated also in the pathogenesis of various diseases including ischemic/reperfusion injury, neurodegenerative diseases and diabetes [3]. Toward a better understanding of such pathophysiological signals, it is necessary to identify and characterize the signaling proteins transmitting ER information to the cytoplasm.

In this work, we focused on an ER-resident protein, BRI3-binding protein (BRI3BP) [4,5]. On the basis of the results obtained, we propose that BRI3BP contributes to cell fate decision by mediating joint activities between the ER and mitochondria.

Methods

Transfection and pharmacological treatment. Human embryonic kidney 293T (293T) cells were grown in DMEM (WAKO, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) at 37 °C in a 5% CO_2 humidified incubator. For overexpression, the cDNA fragment encoding human BRI3BP or murine calumin was PCR-generated and cloned in frame into the pcDNA4/myc-His vector (Invitrogen). The pcDNA4/myc-His/lacZ vector coding for β-galactosidase was obtained from Clontech Inc. The cells were plated 16 h prior to transfection in 12-well plates at

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1×10^5 cells per well. The expression construct was transfected into the cells using Lipofectamine 2000 (Invitrogen). At 30 h posttransfection, etoposide (Etop), thapsigargin (Tg), and tunicamycin (Tu) (all from WAKO) were added to the culture medium. The cells were incubated for a further 40 h and then examined flow cytometrically. For BRI3BP depletion, the following small interfering RNA (siRNA) duplex obtained from Dharmacon was transfected into 293T cells using X-tremeGENE siRNA transfection reagent (Roche): 5′-gcucuuggauguucuuggauu-3′ and 5′-uccaagaacauccaagagcuu-3′. Depletion of BRI3BP mRNA was confirmed by reverse transcription polymerase chain reaction (RT-PCR) using cDNAs, synthesized with PrimeScript reverse transcriptase (Takara Bio, Shiga, Japan), as templates. The following primers were used: for BRI3BP, 5′-GCGTCGACACCATGGGCGCGCGCGCCTCAGGCGGGC-3′ and 5′-GCGAATTCTACTTGTCCTTGGAGCGGTCCAGGCTC-3′, and for β-actin, 5′-GCATTGCTGACAGGATGCAG-3′ and 5′-CCTGCTTGCTGATCCACATC-3′. The siRNA-transfected cells were split onto another 12-well plate at 72 h posttransfection. After 16 h, the cells were chemically challenged as descried above and analyzed.

Cell viability and caspase-3 activity assay. Cell viability was determined as described previously [6]. To examine caspase-3 activation, the transfected cells were treated with either dimethyl sulfoxide (vehicle) or Etop in the presence or absence of the cell-permeable pan-caspase inhibitor Z-VAD-fmk (BD Bioscience). Subsequently, caspase-3 activity was measured by flow cytometry using the CaspGLOW fluorescein active capase-3 staining kit (Medical & Biological Lab., Nagano, Japan) according to the manufacturer's instructions.

Mitochondrial cytochrome c release and transmembrane potential. Mitochondrial cytochrome *c* content was measured as described previously [7], except that the secondary antibody used was coupled with Alexa Fluor 488 instead of phycoerythrin. For examining the mitochondrial membrane potential, pharmacologically treated 293T cells were incubated for 30 min with DMEM supplemented with 10% FCS and 1 μg/ml rhodamine 123 (Rh123) in a 5% CO_2 humidified incubator, washed with PBS, and subjected to flow cytometry.

Immunoblotting. For the immunoblotting of whole cell lysates, transfected 293T cells were lyzed at 24 h posttransfection in RIPA buffer (10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 1% sodium deoxycholate, protease inhibitor cocktail (Nacalai Tesque, Tokyo, Japan)). The antibodies against the following proteins were used: BAK, BAX, Bcl-2, Bcl-X_L and actin (Santa Cruz Biotech.), and GRP78 (Abcam). BRI3BP antiserum was produced by injecting rabbits with a glutathione *S*-transferase fusion protein containing amino acids 203–253 of murine BRI3BP.

Ultrastructural analysis. The pharmacologically treated cells were prepared for electron microscopy study as described previously [8].

Statistics. Statistical significance was evaluated using Student's *t* test unless otherwise mentioned.

Results

Facilitation of drug-induced apoptosis by BRI3BP overexpression

The localization of BRI3BP to both the ER and the nuclear membrane prompted us to explore the possibility that BRI3BP is involved in signaling from the ER. To this end, 293T cells transfected with human BRI3BP that was fused to the mycHis tag (Suppl. Fig. 1A and B) were incubated with apoptosis inducers including ER stressors such as Tg (sarcoplasmic/endoplasmic Ca^{2+}–ATPase inhibitor) and Tu (*N*-glycosylation inhibitor), and the chemotherapy drug Etop (topoisomerase II inhibitor). The cells were probed using a combination of fluorescein isothiocyanate-coupled Annexin V (Annexin V-FITC) and the DNA-specific fluorochrome 7-amino-actinomycin D (7-AAD) to simultaneously determine phosphatidyl serine exposure and plasma membrane permeability by flow cytometry [9,10]. Subsequently, cell subsets undergoing early (Annexin V^+/7-AAD^-) and late (Annexin V^+/7-AAD^+) apoptosis were quantified (Suppl. Fig. 1C). BRI3BP transfection led to a 15–30% increase in apoptosis compared with mock transfection. On the other hand, no obvious effects of β-galactosidase (cytosolic protein) and calumin (ER transmembrane protein [6]) on apoptosis were detected (Suppl. Fig. 1D), suggesting that BRI3BP specifically enhances drug-initiated apoptosis. Increased vulnerability to the pharmacological insults was corroborated over a wide range of drug concentrations (Fig. 1A).

Reduction of drug-induced apoptosis by BRI3BP depletion

We further investigated the role of BRI3BP using an siRNA-mediated knockdown approach. Transfection of an siRNA duplex corresponding to the BRI3BP open reading frame, but not to a scrambled (Sc) sequence, resulted in a marked decrease in the levels of BRI3BP mRNA and protein, as shown by RT-PCR and immunoblotting,

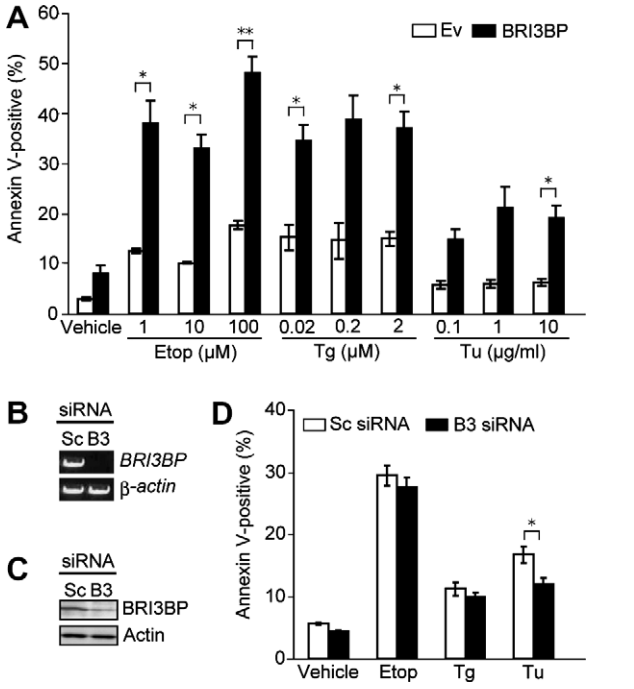

Fig. 1. Enhancement of drug-induced apoptosis by BRI3BP. (A) 293T cells transfected with either an empty vector (Ev) or the plasmid encoding the mycHis-tagged BRI3BP (BRI3BP) were pharmacologically treated for flow cytometric analysis. Annexin V positivity indicates the total percentage of two subpopulations (Annexin V^+/7-AAD^- and Annexin V^+/7-AAD^+). The result represents mean ± SEM of five separate experiments. $^*P < 0.05$, $^{**}P < 0.01$. (B, C) 293T cells were transiently transfected with siRNA duplexes for either BRI3BP (B3) or an irrelevant sequence (Sc), followed by RT-PCR and immunoblotting. (D) The siRNA-transfected cells were challenged with the vehicle, 100 μM Etop, 2 μM Tg or 10 μg/ml Tu for flow cytometry. The result represents mean ± SEM of five independent experiments. $^*P < 0.05$.