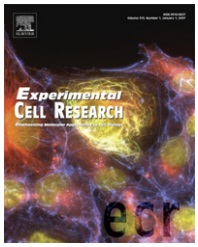
EXPERIMENTAL CELL RESEARCH 313 (2007) 3767–3778

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

In vitro analysis of Bcl-2 proteins in mitochondria and endoplasmic reticulum: Similarities in anti-apoptotic functions and differences in regulation

M. Yano, K. Terada, T. Gotoh, M. Mori*[1]

Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan

ARTICLE INFORMATION

Article Chronology:
Received 30 March 2007
Revised version received
10 July 2007
Accepted 31 July 2007
Available online 7 August 2007

Keywords:
Apoptosis
Bcl-2
Endoplasmic reticulum (ER)
Mitochondria
Bid

ABSTRACT

Anti-apoptotic Bcl-2 localizes in the membranes of mitochondria and endoplasmic reticulum (ER) and resists a broad range of apoptotic stimuli. However, the precise function of Bcl-2 in ER is still unclear. We herein examined the anti-apoptotic potencies of Bcl-2 in mitochondria and ER *in vitro*. The mitochondria isolated from HeLa cells, which have little or practically no Bcl-2, were apoptosis-competent. That is, membrane-bound Bax was activated and cytochrome *c* was released when the isolated mitochondria were incubated at 35 °C. Cytochrome *c* release from the apoptosis-competent mitochondria was suppressed by co-incubation with the mitochondria with overexpressed Bcl-2 (Bcl-2 mitochondria), suggesting that Bcl-2 anchored in one mitochondrion can suppress cytochrome *c* release from another mitochondrion. Similar results were obtained when microsomes with overexpressed Bcl-2 (Bcl-2 microsomes) were co-incubated with apoptosis-competent mitochondria. A quantitative titration analysis showed that Bcl-2 in the ER suppresses cytochrome *c* release as efficiently as that in the mitochondria. An immunoprecipitation assay showed that Bcl-2 in both mitochondria and ER binds to Bax at almost the same degree. However, in the presence of tBid, co-incubation of apoptosis-competent mitochondria with Bcl-2 microsomes, but not with Bcl-2 mitochondria, diminished the Bax-binding to Bcl-2 significantly, suggesting that Bcl-2 in ER is readily inactivated by tBid. Co-incubation assay further confirmed that Bcl-2 in the ER, but not Bcl-2 in the mitochondria, is potentially inactivated by tBid. Our quantitative *in vitro* studies indicate that Bcl-2 in mitochondria and ER are similarly potent in inhibiting Bax-associated apoptosis of other mitochondria, but are regulated by tBid differently.

Introduction

Apoptosis plays an essential role in the embryogenesis and tissue homeostasis of multicellular organisms by removing unnecessary or damaged cells. Apoptosis is regulated by complicated series of interactions among Bcl-2 family proteins [1]. These interactions include hetero- and homo-interactions of proteins containing Bcl-2 homology (BH) regions. After receiving certain apoptotic stimuli, pro-apoptotic BH3-only proteins such as tBid trigger the permeabilization of the mitochondrial

* *Corresponding author*. Fax: +81 96 373 5145.
E-mail address: myano@gpo.kumamoto-u.ac.jp (M. Yano).
[1] Department of Molecular Genetics, Schools of Pharmacy, Sojo University, Ikeda 4–22–1, Kumamoto 860–0082, Japan.

0014-4827/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2007.07.031

outer membrane (MOM) by the multi-BH domain proteins Bax and Bak [2,3]. Bax and Bak permeabilize MOM either by forming a pore or by modifying an existing pore, resulting in the release of several apoptotic factors including cytochrome *c*, Smac and HtrA2 from the mitochondrial intermembrane space [4–6].

The pro-apoptotic protein Bax contains a predicted membrane-anchor sequence (helix 9) and a region of helices 5–6, considered as a putative pore-forming domain. Bax remains in the cytosol until the induction of cell death. Bax targeting to the mitochondria during cell death requires its activation *via* transient interaction with a BH3-only protein such as tBid, which induces a conformational change of Bax. Bax integrates into MOM as a multispanning membrane protein with helices 5–6 and 9 in the lipid bilayer, and oligomerization of the membrane-embedded Bax monomers results in MOM permeabilization, which subsequently forms pores [7].

The anti-apoptotic protein Bcl-2 inhibits apoptosis by antagonizing the pro-apoptotic family members, including the proteins Bax and Bak. Bcl-2 is constitutively bound to membranes through a membrane-anchor sequence (helix 9). The mechanism of inhibition is thought to involve interactions mediated by the hydrophobic BH3 binding pocket on the surface of Bcl-2 because many pro-apoptotic proteins share only the BH3 region [8]. Consistent with this model, Bcl-2 binds to BH3-only proteins and BH3 peptides derived from pro-apoptotic proteins. In the case of Bax, the BH3 region is thought to be exposed to the surface by a conformational change, enabling it to bind to Bcl-2. Bcl-2 was also reported to change its conformation in MOM to bind membrane-inserted Bax monomers and prevent productive oligomerization of Bax [9,10].

Bcl-2 localizes not only in mitochondria but also in the endoplasmic reticulum (ER) [11]. This raised the possibility that Bcl-2 acts at an extra-mitochondrial site. One possibility is that Bcl-2 in the ER interferes with intracellular calcium stores and calcium release, which may be relevant for apoptosis induction [12–15]. The interaction between Bcl-2 and inositol 1,4,5-trisphosphate receptor (InsP3R) has been reported to inhibit InsP3R activation and thus regulate InsP3-induced calcium release from the ER [16]. In addition, Bax and Bak were also suggested to be localized in the ER and induce apoptosis by depleting calcium in the ER [17]. Recently, it was suggested that Bax and Bak function at the ER membrane by activating IRE1 signaling and by providing a physical link between the apoptotic pathway and the unfolded protein response [18]. On the other hand, it is also possible that Bcl-2 proteins in the ER protect mitochondria from a distance. An analysis using a Bcl-2 mutant which localizes only in the ER indicated the existence of molecular crosstalk between ER and mitochondria, in which apoptosis was interrupted by Bcl-2 in the ER [19]. It was also reported that BH3-only proteins mediate between Bcl-2 in the ER and Bax on the mitochondria, and thus Bcl-2 in the ER indirectly protects the mitochondria from Bax *via* BH3-only proteins [20]. Therefore, although many potential functions of Bcl-2 family proteins on ER have been proposed, the precise mechanisms and interaction between these Bcl-2 family proteins on ER are still uncertain. Specifically, it remains unclear whether Bcl-2 proteins in the mitochondria and the ER have equivalent potency in inhibiting the apoptotic function of Bax and whether both Bcl-2 proteins in mitochondria and ER are regulated in the same manner by BH3-only proteins.

In this study, we unexpectedly found that the isolated mitochondria from HeLa cells released cytochrome *c* in a temperature-dependent manner. A further analysis indicated that the isolated mitochondria were apoptosis-competent, in which MOM-bound Bax proteins changed into active oligomer form under 35 °C incubation. Therefore, taking advantage of the apoptosis-competent mitochondria, we performed an *in vitro* assay, in which apoptosis-competent mitochondria were co-incubated with mitochondria with overexpressed Bcl-2 (Bcl-2 mitochondria) or microsomes with overexpressed Bcl-2 (Bcl-2 microsomes) at 35 °C, to compare the anti-apoptotic activities of Bcl-2s in the mitochondria and ER. The co-incubation assay indicated that Bcl-2s in the mitochondria and ER can inhibit apoptotic events in the apoptosis-competent mitochondria. A quantitative analysis suggested that both Bcl-2 proteins in mitochondria and ER have almost equivalent potencies to suppress cytochrome *c* release. The combination of immunoprecipitation and co-incubation assays showed that both Bcl-2s in the mitochondria and ER bind to Bax proteins at almost the same degree. However, Bcl-2 in ER, but not that in the mitochondria, was potentially inhibited from binding to Bax by the addition of tBid. Co-incubation assay further indicated that Bcl-2 in the ER, but not Bcl-2 in the mitochondria, is potentially inactivated by tBid. Our quantitative analyses indicate that both Bcl-2s localized in the mitochondria and the ER can protect mitochondria from Bax-associated apoptosis with almost equivalent potency, but their anti-apoptotic activities are regulated by tBid differently.

Materials and methods

Materials

Mouse anti-cytochrome *c* monoclonal antibody, rabbit anti-HtrA2/Omi polyclonal antibody, goat anti-Bcl-2 polyclonal antibody and caspase-8-cleaved recombinant human Bid (tBid) were purchased from R&D Systems, Inc. Mouse anti-Hsp60 monoclonal antibody and rabbit anti-calnexin polyclonal antibody were obtained from Stressgen. Rabbit anti-Smac (DIABLO) polyclonal antibody was obtained from BIOMOL Research Laboratories, Inc. Rabbit anti-AIF polyclonal antibody and rabbit anti-Bax (N-20) polyclonal antibody were obtained from Santa Cruz Biotechnology. Rabbit anti-Bak polyclonal antibody was obtained from Upstate Biotechnology. Anti-human Tom22 antibody was previously prepared [21]. Anti-human Tom40 antibody was raised in rabbit using standard protocols. Bismaleimidohexane (BMH) was purchased from Pierce.

Plasmids

The plasmid pUSEamp(+)-Bcl-2(wt) was purchased from Upstate. Control vector pUSEamp(+)-Control was constructed by deleting Bcl-2 cDNA fragment from pUSEamp(+)-Bcl-2(wt) with *Bam*HI. To construct the plasmid expressing FLAG-tagged Bcl-2, a DNA fragment was amplified by polymerase chain reaction (PCR) using pUSEamp(+)-Bcl-2(wt), as the template. The upstream and downstream primers were 5′-AAAAAAGCTAGCATGGACTACAAAGACGATGACGACAAGGCGGCGCAAGCCGGGAGAACAGG-3′ and 5′-AAAAAAGTTTAAACTCACTTGTGGCCCAGGTA-3′,