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Pore-forming activity of BAD is regulated by specific phosphorylation and structural transitions of the C-terminal part

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ABSTRACT

Background: BAD protein (Bcl-2 antagonist of cell death) belongs to the BH3-only subfamily of proapoptotic proteins and is proposed to function as the sentinel of the cellular health status. Physiological activity of BAD is regulated by phosphorylation, association with 14-3-3 proteins, binding to membrane lipids and pore formation. Since the functional role of the BAD C-terminal part has not been considered so far, we have investigated here the interplay of the structure and function of this region.
Methods: The structure of the regulatory C-terminal part of human BAD was analyzed by CD spectroscopy. The channel-forming activity of full-length BAD and BAD peptides was carried out by lipid bilayer measurements. Interactions between proteins and peptides were monitored by the surface plasmon resonance technique.
In aqueous solution, C-terminal part of BAD exhibits a well-ordered structure and stable conformation. In a lipid environment, the helical propensity considerably increases. The interaction of the C-terminal segment of BAD with the isolated BH3 domain results in the formation of permanently open pores whereby the phosphorylation of serine 118 within the BH3 domain is necessary for effective pore formation. In contrast, phosphorylation of serine 99 in combination with 14-3-3 association suppresses formation of channels. C-terminal part of BAD controls BAD function by structural transitions, lipid binding and phosphorylation. Conformational changes of this region upon membrane interaction in conjunction with phosphorylation of the BH3 domain suggest a novel mechanism for regulation of BAD.
General significance: Multiple signaling pathways mediate inhibition and activation of cell death via BAD.

1. Introduction

Apoptosis or programmed cell death is an evolutionary widely conserved suicidal process by which multicellular organisms remove or replace undesirable cells and it is essential for normal embryonic development. This form of coordinated cell death is widely observed in nature and is not only critical for proper execution of developmental processes, immune responses, and tissue homeostasis but disruptions of this process also have far-reaching effects causing diseases such as tumor development and autoimmune disorders [1–3].

Mitochondria constitute a convergence point in apoptosis progression. Proteins of the Bcl-2 family are crucial regulators of the onset of apoptosis at the level of mitochondria [3,4]. There, apoptosis proceeds through a complex interplay between anti- and proapoptotic proteins of Bcl-2 proteins. However, the exact modes and the mechanisms of the pathways involved in this process are still not completely understood [4]. In addition, several proteins of the Bcl-2 family contribute to the regulation of various other physiological processes beyond regulation of apoptosis, such as autophagy, mitochondrial respiration and glucose metabolism [5]. All Bcl-2 proteins contain at least one of the four so-called Bcl-2 homology domains: BH1-BH4. Several Bcl-2 family proteins contain a carboxy-terminal membrane anchor that may be involved in their binding to plasma- or different intracellular membranes. On the basis of various structural and functional characteristics, the Bcl-2 family of proteins is divided into three subfamilies, including proteins which either inhibit

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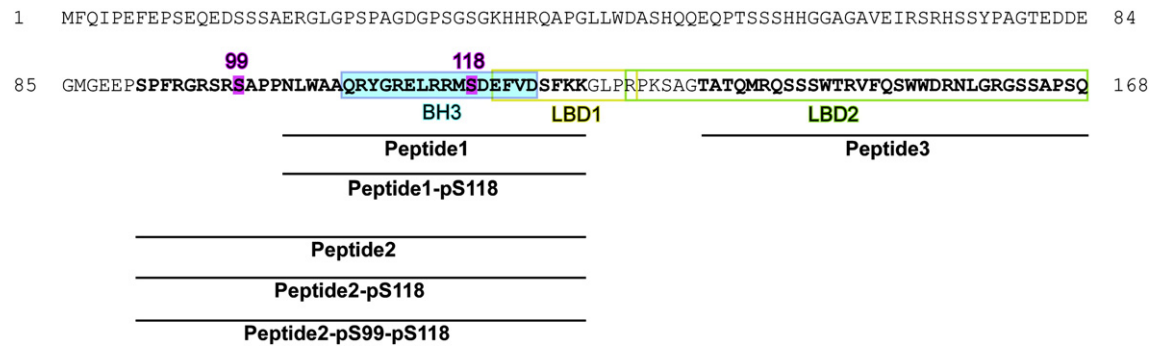

Fig. 1. Amino acid sequence of human BAD protein and peptides covering the BH3 domain, the 14-3-3 binding motif surrounding serine 99 and the C-terminal part of the protein. The BH3 domain is shown in blue, and the putative lipid binding domains (LBD1 and LBD2) are indicated by yellow and green rectangles, respectively. The sequences of Peptide1, -2 and -3 are depicted in bold, and the regulatory serines 99 and 118 are highlighted in magenta.

(e.g., Bcl-2, Bcl-X_L or Bcl-w) or promote programmed cell death (e.g., Bax, Bak or Bok) [4,6,7].

A second sub-class of proapoptotic Bcl-2 family members, the BH3-only proteins, comprises BAD, Bik, Bmf, Hrk, Noxa, tBid, Bim and Puma [4]. BH3-only proteins share sequence homology only at the BH3 domain. The amphipathic helix formed by the BH3 domain (and flanking residues) associates with a hydrophobic groove of several members of the Bcl-2 family proteins [8,9].

BAD (Bcl-2 associated death promoter, Bcl-2 antagonist of cell death) was originally described to promote apoptosis by forming heterodimers with the prosurvival proteins Bcl-2 and Bcl-X_L, thus preventing them from binding to Bax [10]. Phosphorylation plays a crucial role in regulation of BAD function and modulates the association with other proteins as well as its subcellular localization. In the non-phosphorylated state BAD associates with Bcl-2 or Bcl-X_L via its BH3 domain and localizes to mitochondria, representing the active state of BAD. Phosphorylation of specific serine residues, Ser-112 and Ser-136 of murine BAD (mBAD)[1] or the corresponding phosphorylation sites Ser-75 and Ser-99 of human BAD (hBAD), results in association with 14-3-3 proteins and subsequent relocation of BAD [11,12]. Supporting data of Ayllon et al. [13] who showed that BAD segregation from the lipid rafts at the plasma membrane is implicated in the regulation of apoptosis, we provided evidence that the BAD/14-3-3 complex possesses high affinity for cholesterol-rich membranes (lipid rafts) indicating a 14-3-3 driven shuttling of BAD between cholesterol-rich and mitochondrial membranes [11]. Phosphorylation of mBAD at Ser-155 (Ser-118 of hBAD) within its BH3-domain disrupts the association with Bcl-2 or Bcl-X_L promoting cell survival [14]. Therefore, the phosphorylation status of BAD at these serine residues reflects a checkpoint for cell death or survival.

Several members of the Bcl-2 family were shown to form pores in lipid bilayers [15–23]. However, it has not been clarified yet whether this pore formation is an apoptosis- or survival-associated event, since this process was observed for both pro- and antiapoptotic proteins. Keeping in mind that Bcl-2 proteins were demonstrated to be involved in the regulation of other events than apoptosis [5], one cannot exclude a vital function for channel formation. Recently, we identified BAD also as a pore-forming Bcl-2 protein [24,25]. Its pore-forming capacity was dependent on phosphorylation and interaction with 14-3-3 proteins. Although the amino acid sequence of human BAD does not reveal a typical C-terminal transmembrane domain, we found that BAD binds to model membranes with high affinity, predominantly to negative charged phospholipids and cholesterol-rich membranes [11]. Two lipid binding domains (LBD1 and LBD2) with different binding preferences were identified, both located in the C-terminal part of the hBAD protein.

To elucidate the origin of the extraordinarily high affinity of the C-terminal part of BAD for membrane lipids we studied in this report the secondary structure of a 32-residue peptide corresponding to the C-terminus of the proapoptotic protein BAD and containing the lipid binding domain 2 (LBD2) (see Fig. 1). Our results obtained by use of CD spectroscopy indicate a well-defined structure for this region that undergoes structural transitions in the presence of artificial membranes. Importantly, the interaction of the C-terminal part of hBAD with the phosphorylated BH3 region proved to be sufficient for formation of open pores in lipid bilayers. Similar to full-length BAD association with 14-3-3 proteins suppresses channel formation. The binding studies performed with the C-terminus and peptides comprising the BH3 domain of hBAD allow us to conclude that an intermolecular bridge between these two moieties could be established.

2. Materials and methods

2.1. Materials

Benzamidine, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, Nonidet P-40, CHAPS and trifluoroethanol (TFE) were obtained from Sigma. Glutathione–sepharose was purchased from Amersham Biosciences and Ni^{2+}-nitrilotriacetic acid-agarose was from Qiagen. The phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cardiolipin), sphingomyelin and asolectin (soybean lipid extract) were from Sigma. All synthetic peptides used in this study were purified by high pressure liquid chromatography, and the sequences were verified by mass spectrometry analysis. The purity of the preparations was greater than 90%. The sequences of the peptides used are shown in Fig. 1.

2.2. DNA expression plasmids and purification of proteins

The purification of hBAD was carried out as published [24]. GST-hBAD, GST-hBADΔN131, GST-14-3-3 zeta and GST-Bcl-X_L were expressed in *E. coli* using pGEX-2 T vector and purified by glutathione–sepharose affinity chromatography as described [11]. Bcl-X_L was released by thrombin cleavage. His-tagged 14-3-3 zeta was purified from *E. coli* by Ni^{2+}-agarose (Quiagen) affinity chromatography according to the manufacturer's protocol.

2.3. Circular dichroism

CD analyses were conducted at 20 °C using peptide solutions ranging in concentration from 0.1 to 0.2 mg/ml in the presence or absence of unilamellar asolectin vesicles (50 μM total lipid concentration) and/or

[1] *Abbreviations:* BH3, Bcl-2 homology domain 3; mBAD, mouse BAD; hBAD, human BAD; LBD, lipid binding domain; PBS, phosphate-buffered saline; NP-40, Nonidet P-40; TFE, trifluoroethanol; GST, glutathione *S*-transferase; SPR, surface plasmon resonance; RU, response units; CD, circular dicroism; NMR, nuclear magnetic resonance