FEBS Letters 586 (2012) 3142–3149

journal homepage: www.FEBSLetters.org

Bcl-rambo induces apoptosis via interaction with the adenine nucleotide translocator

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ARTICLE INFO

Article history:
Received 23 April 2012
Revised 6 August 2012
Accepted 10 August 2012
Available online 23 August 2012

Edited by Quan Chen

Keywords:
Bcl-rambo
Bcl-2
Permeability transition
ANT
Apoptosis

ABSTRACT

The Bcl-2 family proteins plays a central role in apoptosis. The pro- or anti-apoptotic activities of Bcl-2 family are dependent on the Bcl-2 homology (BH) regions. Bcl-rambo, a new pro-apoptotic member, is unusual in that its pro-apoptotic activity is independent of its BH domains. However, the mechanism underlying Bcl-rambo-induced apoptosis is largely unknown. Mitochondrial localization is indispensable for the pro-apoptotic function of Bcl-rambo. Bcl-rambo interacts physically with the adenine nucleotide translocator (ANT), suppresses the ADT/ATP-dependent translocation activity of ANT. Collectively, our data indicate Bcl-rambo is a pro-apoptotic member of the Bcl-2 family, induces the permeability transition via interaction with ANT.

Structured summary of protein interactions:
Bcl-Rambo and **HSP60** colocalize by fluorescence microscopy (View interaction)
Bcl-rambo binds to **ANT1** by pull down (View interaction)

1. Introduction

Bcl-2 family members are key protein regulators of cell death. This family of proteins contains both pro-apoptotic and anti-apoptotic members that decide the fates of cells at the mitochondrial level [1,2]. Bcl-rambo is Bcl-2 protein family member with a pro-apoptotic function [3,4]. However, unlike other Bcl-2 family protein members, Bcl-rambo possesses a unique c-terminal extension (BHNo domain) with 2 repeated tandem domains A and B (RTA and RTB) [3]. In addition, the conserved BH domains of Bcl-rambo are not essential for triggering cell death [3], obscuring the mechanism by which this protein causes cell death.

Mitochondria, and the mitochondrial permeability transition (MPT) in particular, are known to play a central role in apoptotic cell death [4,5]. The cause of the MPT is the opening of a non-specific pore, known as the mitochondrial permeability transition pore (PTP), which is a protein aggregate composed of Cyclophilin D (Cyp-D), a voltage-dependent anion channel (VDAC), and the adenine nucleotide translocator (ANT) [6]. Members of the Bcl-2 family of proteins interact with VDAC or ANT to regulate PT. For examples, Bax and Bcl-2 physically bind to ANT to modulate its activity [7], and Bcl-XL prevents VDAC from forming large non-specific channels [8,9]. However, the precise molecular mechanisms underlying the pro-apoptotic or anti-apoptotic activity of Bcl-2 family proteins with regard to PTP regulation are largely unknown. In this work, we report how Bcl-rambo facilitates apoptotic cell death and identify signaling molecules involved in the MPT using site-directed mutant plasmids and recombinant proteins.

2. Materials and methods

2.1. Cells and reagents

PC-3 prostatic cancer cells, purchased from the American Type Culture Collection (Rockville, MD), were cultured in RPMI 1640 medium. Anti-VDAC and -ANT antibodies were obtained from Calbiochem (La Jolla, CA). Anti-Cyclophilin D and anti-V5 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Invitrogen (Carlsbad, CA), respectively. Cyclosporin A (CsA) and bonkrekic acid (BA) were obtained from Calbiochem. Mitotracker was from Molecular Probes, Inc. (Eugene, OR). All other chemicals or reagents were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Cloning and plasmid construction

DNA encoding Bcl-2 or Bcl-rambo cloned into pcDNA3.1/V5 (Invitrogen, Carlsbad, CA). Deletion mutants of Bcl-rambo or

Abbreviations: ANT, adenine nucleotide translocator; BA, bonkrekic acid; BH, Bcl-2 homology; CsA, cyclosporin A; MMP, mitochondrial membrane potential; MPT, mitochondrial permeability transition; VDAC, voltage-dependent anion channel

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.08.015

chimeric TM mutants were generated by a splice overlap extension method as described previously [10].

2.3. Apoptosis detection and caspase-3 assay

After DAPI (1 μg/ml in PBS) staining, apoptotic cells with fragmented nuclei or chromatin condensation were counted manually under a immunofluorescence microscope. Caspase-3 activity was measured using the Colorimetric Caspase-3 Assay Kit (Calbiochem, CA) as described previously [11]. Absorbance at 405 nm was determined ~6 h after initiation of the reaction.

2.4. Transfection, immunoprecipitation and immunoblotting

PC-3 cells (3×10^5/10 cm culture dish) were transiently transfected with 2 μg of each plasmid using Lipofectamine 2000 (Invitrogen). For immunoprecipitation, cells were lysed in ice cold NP40 lysis buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% NP-40 and complete protease inhibitor cocktail], incubated with the indicated antibodies, and immunoprecipitated with protein A (Sigma–Aldrich). The precipitates were washed four times, subjected to SDS–PAGE, and analyzed by western blotting.

2.5. Purification of recombinant proteins

Bcl-rambo, Bcl-ramboΔTM, Rambo-Bcl2-TM, Rambo-BNip3-TM, and Rambo-Cyb5-TM cDNA were subcloned into the pGEX-2TK vector (Amersham Pharmacia Biotechnology, NJ) tagged with glutathione S-transferase (GST). Bound GST-fusion proteins were eluted by thrombin protease treatment. The amount of purified recombinant protein was estimated by the Biuret method.

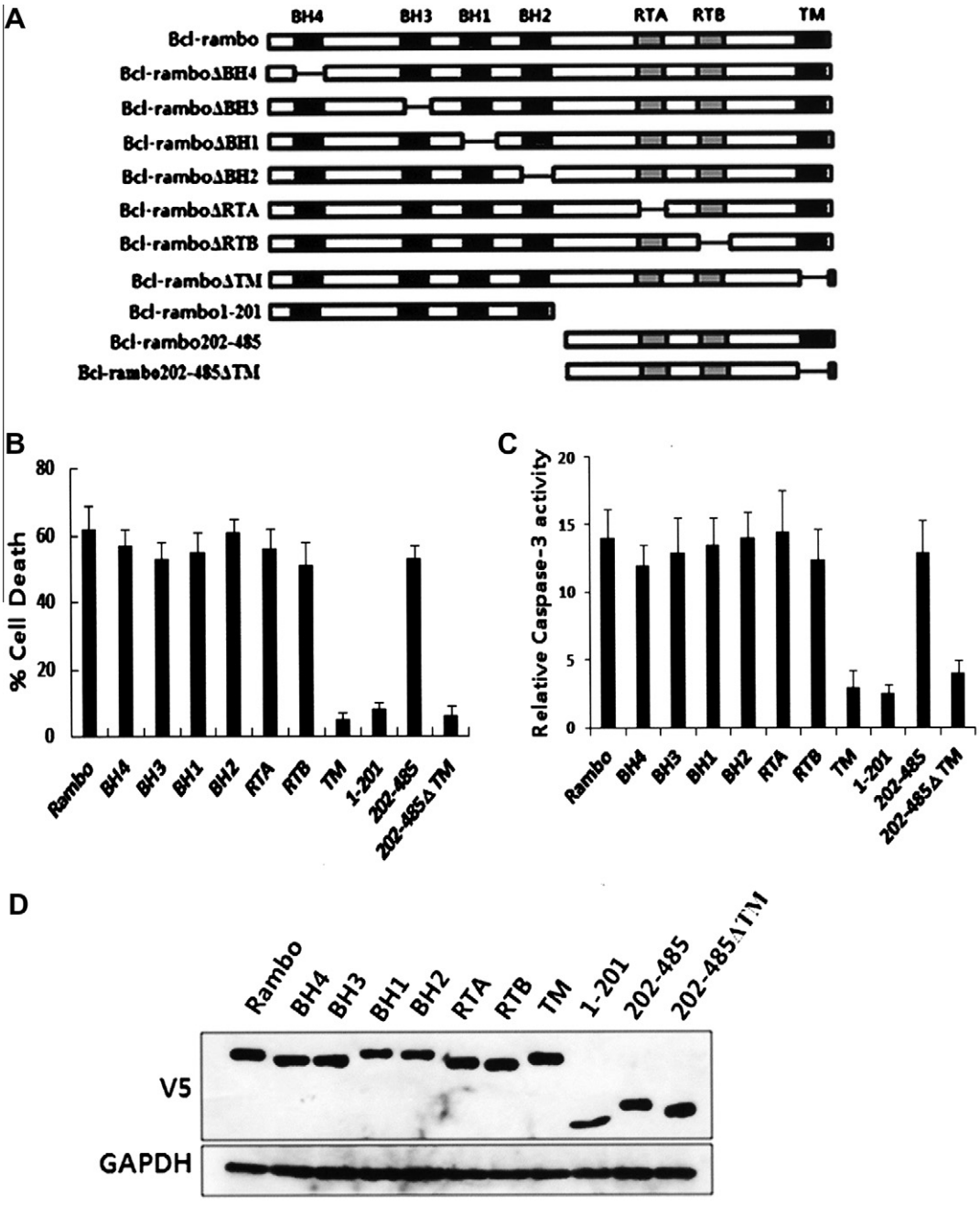

Fig. 1. The TM domain of Bcl-rambo is crucial for induction of apoptotic cell death. (A) Schematic representation of Bcl-rambo deletion mutants. PC-3 cells were transiently transfected with wild-type Bcl-rambo or mutant plasmids for 48 h. Apoptotic cell death was measured by DAPI staining (B) and caspase-3 activity assay (C). The caspase-3 activity of mock-transfected cells was arbitrarily set to 1. (D) Expression levels of V5-tagged pcDNA-Bcl-rambo plasmids determined by performing western blot with anti-V5 antibody.