International Journal of Biological Macromolecules 101 (2017) 283–289

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Integrity of XIAP is essential for effective activity recovery of apoptosome and its downstream caspases by Smac/Diablo

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ARTICLE INFO

Article history:
Received 30 December 2016
Received in revised form 15 March 2017
Accepted 16 March 2017
Available online 18 March 2017

Keywords:
Apoptosome
Caspase-3
Caspase-7
Smac
XIAP

ABSTRACT

Contribution of individual BIR domains to Smac antagonism is investigated. Ammonium citrate was used to activate caspase-9 and pro-caspase-9 (D315, D330/A). However, the presence of citrate resulted in autoproteolysis of pro-caspase-9 and its inhibition by XIAP BIR3, which was not observed for apoptosome activated pro-caspase-9 indicating abnormal behavior of pro-caspase-9 in kosmotropic citrate salt. Thus, we used Apaf-1(residues 1-591) to activate caspase-9 through the formation of mini-apoptosome instead. Inhibition of apoptosome by XIAP BIR-1-2-3 was observed to be similar to that of BIR3 indicating that the cleavage of XIAP does not affect its potency. However, BIR1-2-3 was more prone to Smac antagonism due to simultaneous interaction of two BIR domains from XIAP with two N-terminal binding sites of Smac. Therefore, despite the role in caspase-9 activation, Apaf-1 does not influence caspase-9 inhibition by XIAP. In addition, caspase-3, -7 and -9 activity recovery by Smac protein and peptide were more efficient for BIR1-2-3 than for BIR1-2. Consequently, it can be proposed that the presence of multiple BIR domains for XIAP among different species along with dimeric nature of Smac are evolutionary designed to strengthen the antagonistic activity of Smac culminating in efficient induction of cell death.

1. Introduction

Apoptosis is the dominant form of programmed cell death which is initiated from either the cell surface, extrinsic pathway, or through the release of cytochrome c from the mitochondria, intrinsic pathway [1]. The intrinsic or mitochondrial pathway is initiated by stress signals such as DNA damage [2]. Released cytochrome c triggers formation of Apaf-1 heptamer as a platform to dimerize and activate the initiator pro-caspase-9 leading to self-cleavage at Asp315 [3]. Caspase-9 in turn cleaves and activates the executioner caspases, pro-caspase-3 and pro-caspase-7 [4]. Cleaved caspase-9, -3 and -7 can be inhibited by a protein called X-linked Inhibitor of Apoptosis Protein (XIAP) [5]. XIAP is a ubiquitously expressed 57 kDa protein with three Baculoviral IAP Repeats, BIR, domains, Ubiquitin Binding Domain, UBA, and a RING zinc finger [6]. XIAP is believed to be the only member of IAPs capable of directly inhibiting both initiator and executioner caspases through distinct interactions [7] underlining the importance of XIAP in regulating apoptosis. Accordingly, XIAP has been the IAP of choice for biochemical and biophysical studies as a potential target to overcome chemotherapy resistance in cancer therapy.

BIR3 domain of XIAP inhibits caspase-9 by preventing its dimerization while BIR2 domain inhibits caspase-3 as well as caspase-7 by positioning the linker preceding BIR2 in the active site [8]. Caspase-9 inhibition is mediated through interaction of ATPF tetrapeptide, exposed following self-cleavage of pro-caspase-9 at the N-terminus of P12 subunit [9]. Meanwhile, Smac protein, arguably the most important endogenous antagonist of XIAP, which is released along with cytochrome c from the mitochondria, competes with caspase-9 through its N-terminal AVPI sequences for BIR3 interaction. These mostly AXPX type sequences are known as IAP binding motives (IBM) or Hid-Grim-Reaper (HRD) and their binding sites on BIR domains are called IBM grooves [10]. The short tetrapeptide sequence of the IBM motif makes it a valuable tool for designing Smac mimetics capable of targeting IAP proteins, especially XIAP [11], because overexpression of XIAP is one of the mechanisms by which cancer cells escapes apoptosis [12]. In addition to XIAP, the SMAC mimetics bind to cellular Inhibitor of

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http://dx.doi.org/10.1016/j.ijbiomac.2017.03.088
0141-8130/

Apoptosis Proteins, cIAPs, with high affinities. Interaction of Smac protein and its mimetics to cIAPs can induce their ubiquitin ligase activity leading to self-ubiquitination and subsequent proteosomal degradation culminating in the activation of the extrinsic apoptotic pathway [13]. Thus, Smac mimetics have been proposed as adjuvant therapy for cancer [14]. Accordingly, understanding the mechanism of the Smac and Smac based peptides in antagonizing IAPs including XIAP is of paramount importance in designing potent cancer therapy drugs.

Given the inactivity of caspase-9 due to its extremely low tendency for dimerization, numerous methods have been employed to activate the enzyme including the use of enzyme concentrations orders of magnitude above the physiological concentrations [15,16], employing kosmotropic salts [17] that force dimerization and oligomerization, fusing an N-terminal tag capable of dimerizing [18], and the natural way of activation through Apaf-1 [19]. However, none of these studies have addressed Smac mediated XIAP antagonism of caspase-9 inhibition when Apaf-1 was the mean of caspase-9 activation. This is especially important given the recent discovery that the CARD domain of Apaf-1 makes important contributions to caspase-9 activation [20], which is known as induced conformation model. In addition, studies on XIAP antagonism in relieving executioner caspase activities have been performed using different XIAP and Smac variants under various experimental conditions leading to opposing conclusions. This has hindered proper conclusions with respect to the contribution of SMAC protein moiety and IBM motif in neutralizing XIAP. These prompted us to conduct a comprehensive study of XIAP antagonism by Smac for the entire intrinsic apoptosis pathway with the specific aim of exploring the role of IBM motives and the Smac protein moiety in XIAP antagonism.

2. Materials and methods

2.1. Protein expression and purification

BL21(DE3) cells were transformed with pET21 encoding WD40-deleted Apaf-1 (residues 1-591) plasmid, a generous gift from Dr. Guy Salvesen, and grown in LB medium at 37 °C until OD_{600} reached 0.8–1.2. Using 0.2 mM IPTG, expression of the protein was induced at 23 °C for 7 h. The cells were spun and washed with LB, and stored at −70 °C. Afterwards, the expressed His-tagged protein was purified using Ni-NTA agarose (QIAGENE) as follows. Cell pellet was suspended in lysis buffer (50 mM Tris-HCl pH 8, 100 mM KCl, 15 mM imidazole, 5 mM 2-Mercaptoethanol, 1% Triton X-100) then sonicated, spun and supernatant was shaken with Ni-NTA agarose for an hour at 4 °C. It was further passed through chromatography column by gravity. Following washes with buffer 1 (50 mM Tris-HCl pH 7, 0.5 M KCl, 20 mM imidazole, 5 mM 2-Mercaptoethanol, 20% Glycerol) and buffer 2 (50 mM Tris-HCl pH 7, 100 mM KCl, 40 mM imidazole, 5 mM 2-Mercaptoethanol, 20% Glycerol) the protein was eluted from the resin with 50 mM HEPES pH 7.4, 50 mM KCl, 200 mM imidazole, 5 mM 2-Mercaptoethanol. The pET23b plasmids harboring the wild type caspase-9 as well as single chain caspase-9 (D315, D330/A, to be called pro-caspase-9), gifts from Guy Salvesen, were introduced into BL21(DE3) cells. These cells were grown at 37 °C to an OD_{600} of 0.4–0.7 and then induced by 0.1 mM IPTG for 6 h at 30 °C. The cell pellets were washed with LB, and stored at −70 °C for purification of the enzymes. The His-tagged proteins were purified using Ni-NTA agarose (QIAGENE) according to the procedure used for the Apaf-1 with the following exceptions. The lysis buffer contained 50 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM 2-Mercaptoethanol, 5 mM imidazole, 1% TritonX-100. The washing buffer was made up of 50 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM 2-Mercaptoethanol, 10 mM imidazole, 20% Glycerol and the elution buffer comprised of 50 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM 2-Mercaptoethanol, 300 mM imidazole, 20% Glycerol. pET21a-XIAP BIR1-2-3 [21] construct was used to transform BL21(DE3) cells. Protein expression was induced at OD_{600} ranging from 0.4 to 0.7 using 0.1 mM of IPTG for 15 h at 18 °C. Coding sequence of XIAP BIR1-2 domains, which consists of residues 1 through 240, was produced in BL21(DE3) cells using pET28a plasmid as described previously [22]. Production and purification of N-terminally His-tagged XIAP BIR1-2 was similar to XIAP BIR1-2-3 as follows. The cell pellets were lysed with 50 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM imidazole, 5 mM 2-Mercaptoethanol, 1% Triton X-100. Then passed through chromatographic column by gravity and the resin was washed with 50 mM Tris-HCl pH 8, 100 mM NaCl, 40 mM imidazole, 5 mM 2-Mercaptoethanol, 10% Glycerol following washing with 50 mM Tris pH 8, 500 mM NaCl, 5 mM imidazole, 5 mM 2-Mercaptoethanol, 10% Glycerol. The proteins were then eluted with 50 mM HEPES pH 7.4, 50 mM KCl, 200 mM imidazole, 5 mM 2-Mercaptoethanol, 10% Glycerol. The pET23b construct expressing Smac was used to produce Smac protein as described [8]. N-terminally His-tagged full length caspase-3 and -7 were expressed in BL21(DE3) cells using pET28a constructs as described previously [23,24]. Induction time at 37 °C for the expression of caspase-3 was 4 h while for caspase-7 at 30 °C was 7 h. GST-tagged XIAP BIR3 protein was produced as described previously [15]. Briefly, pGEX-Kg plasmid encoding BIR3 domain of XIAP protein was introduced into DH5α cells and grown over night at 37 °C. It was then diluted 20 folds in LB medium and grown to OD_{600} of 0.7. Expression of the protein was induced by 0.1 mM IPTG at 25 °C for one hour. The cells were spun, washed with LB, and stored at −70 °C. The cell paste was resuspended in lysis buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM 2-mercaptoethanol, 1% Triton X-100) at 4 °C, sonicated, and centrifuged at 20,000 × *g* for 20 min. Glutathione-Sephadex 4B beads were added to the supernatant and washed with the lysis buffer followed by two washing steps. Ten mM reduced glutathione in 50 mM Tris-HCl pH 8, 10% glycerol, 125 mM NaCl, 5 mM 2-mercaptoethanol was used to elute the protein. Expression of recombinant XIAP BIR2 protein was similar to that of BIR1-2-3. Concentrations of proteins were measured using Thermo Scientific NanoDrop 2000c Spectrophotometer and Bio-Rad protein assay kit. Apaf-1 was freshly prepared on the day of experiment or stored at 4 °C for at most 2 days. Purified caspases and XIAP BIR1-2-3 were aliquoted, flash frozen in liquid nitrogen and stored at −70 °C until use. Smac protein was stored at 4 °C. BIR1-2 variant of XIAP was used on the day of purification due to its reduced response to Smac antagonism upon storage.

2.2. Caspase activity assays

Caspase-9 activity was measured using Ac-LEHD-pNA chromogenic substrate or Ac-LEHD-AFC fluorogenic substrate (Alexis Biochemicals, San Diego, CA) using BioTek Synergy H4 (excitation at 400 nm and emission at 505 nm). For colorimetric assays, 100 μl reactions were setup in the assay buffer (50 mM HEPES, 10 mM DTT, 0.1 mM EDTA, 10% Glycerol) at 30°C. The concentration of capapse-9 was in the range of 500–600 nM and Apaf-1 in the range of 950–1200 nM while keeping the ratio of caspase-9 over Apaf-1 at 50%. Release of pNA was monitored using Bioteck-Power wave SX2 microplate reader at a 405 nm wavelength following the addition of 140 μM Ac-LEHD-pNA. Caspase-3 and caspase-7 assays at concentrations ranging from 35 to 50 nM were performed in 50 mM HEPES pH 7.4, 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 10 mM DTT, and 0.1 μg/μl albumin using 200 μM Ac-DEVD-pNA as a substrate in a 96-well-plate format. The hydrolysis of Ac-DEVD-pNA was monitored at 405 nm at 30 °C for 60 min. The substrate and enzyme concentrations were kept constant while varying the concentration of the XIAP variants and their antagonists. To antagonize XIAP inhi-