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# Cloning and characterization of a *dronc* homologue in the silkworm, *Bombyx mori*

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ABSTRACT

We cloned and characterized a novel *Bombyx mori* homologue (*bm-dronc*) of *Drosophila melanogaster dronc* (*dm-dronc*), which could encode a polypeptide of 438 amino acid residues. Bm-Dronc shares relatively low amino acid sequence identities of 25% and 26% with Dm-Dronc and *Aedes aegypti* Dronc (Aa-Dronc), respectively. Bm-Dronc has the sequence QACRG surrounding the catalytic site (C), which is consistent with the QAC(R/Q/G)(G/E) consensus sequence in most caspases but distinct from the sequences PFCRG and SICRG of Dm-Dronc and Aa-Dronc, respectively. Bm-Dronc possesses a long N-terminal prodomain containing a caspase recruitment domain (CARD), a p20 domain and a p10 domain, exhibiting cleavage activities on synthetic substrates Ac-VDVAD-AMC, Ac-IETD-AMC and Ac-LEHD-AMC, which are preferred by human initiator caspases-2, -8 and -9, respectively. Bm-Dronc transiently expressed in insect cells and *Escherichia coli* cells underwent spontaneous cleavage and caused apoptosis and stimulation of caspase-3-like protease activity in various lepidopteran cell lines, but not in the dipteran cell line *D. melanogaster* S2. The apoptosis and the stimulation of caspase-3-like protease activity induced by Bm-Dronc overexpression were abrogated upon transfection with either a double-stranded RNA against *bm-dronc* or a plasmid expressing functional anti-apoptotic protein Hycu-IAP3 encoded by the baculovirus *Hyphantria cunea* multiple nucleopolyhedrovirus (MNPV). Apoptosis induction in BM-N cells by infection with a *p35*-defective *Autographa californica* MNPV or exposure to actinomycin D and UV promoted the cleavage of Bm-Dronc. These results indicate that Bm-Dronc serves as the initiator caspase responsible for the induction of caspase-dependent apoptosis.

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## 1. Introduction

Apoptosis is a tightly controlled cell death process leading to elimination of unwanted cells in response to a variety of internal and external stimuli, and it plays a vital role in the development, homeostasis and cellular defence of multicellular organisms (Best, 2008; Duprez et al., 2009). The diverse stimuli for apoptosis induction activate either the intrinsic or extrinsic apoptosis pathway, which converges on the activation of the cascades of cysteine proteases known as caspases (Degterev et al., 2003; Hay and Guo, 2006). Caspases are classified as initiator caspases and effector caspases. Initiator caspases activated through either apoptosis pathway in turn proteolytically activate downstream effector caspases, and activated effector caspases cleave a variety of cellular proteins. The cells triggered to undergo apoptosis exhibit various cellular features characteristic of apoptosis, including chromatin condensation, plasma membrane blebbing, cell shrinkage and nucleosomal DNA fragmentation, which ultimately result in apoptotic cell death (Manji and Friesen, 2001).

A number of viruses, including nucleopolyhedroviruses (NPVs), trigger apoptosis (Clem, 2001, 2005, 2007; Hay and Kannourakis, 2002; Roulston et al., 1999). NPVs are large enveloped insect-pathogenic viruses within the family Baculoviridae, which contain a double-stranded, circular DNA genome of 80–180 kbp (Theilmann et al., 2005). These viruses generally display a high degree of host specificity and establish various types of abortive infections (Castro et al., 1997; Morris and Miller, 1992, 1993; Shirata et al., 1999), as well as productive infection leading to the production of high titres of progeny viruses and host cell lysis. In addition, certain NPV-infected insect cells exhibit anti-viral defences, including apoptosis (Chejanovsky and Gershburg, 1995; Clem et al., 1991; Dai et al., 1999; Ishikawa et al., 2003; Palli et al., 1996; Yanase et al., 1998) and global protein synthesis shutdown, in which both viral and cellular protein syntheses are arrested (Du and Thiem, 1997; Guzo et al., 1991, 1992; Kamita and Maeda, 1993; Lu and Miller, 1996; McClintock et al., 1986; Shirata et al., 2004,

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2010; Thiem and Chejanovsky, 2004). In the NPV-infected cells inducing apoptosis or global protein synthesis shutdown, virus yield is severely restricted or abolished (Ishikawa et al., 2003; McClintock et al., 1986; Clem and Miller, 1993; Hershberger et al., 1992).

Molecular mechanisms underlying the regulation of apoptosis and global protein synthesis shutdown in NPV-infected cells are largely unknown. In *Spodoptera frugiperda* Sf9 and Sf21 cells infected with *Autographa californica* multiple NPV (AcMNPV), viral DNA replication resulting from the viral-encoded functional replicative late expression factors (LEFs: LEF1, LEF2, LEF3, LEF11, P143, DNA polymerase and IE1/IE0) is sufficient to induce apoptosis (Prikhod'ko and Miller, 1996; Schultz and Friesen, 2009; Schultz et al., 2009), while the binding of budded viruses to host cell receptors alone is not sufficient to trigger apoptosis (LaCount and Friesen, 1997). AcMNPV-induced apoptosis is suppressed by viral-encoded apoptotic suppressor P35, P49 or certain members of IAP (inhibitor of apoptosis) (Clem, 2001, 2007; Birnbaum et al., 1994; Crook et al., 1993; Du et al., 1999). Although the exact trigger for global protein synthesis shutdown of AcMNPV-infected Ld652Y cells has not been defined, apoptotic suppressors, including P35, Op-IAP3 from *Orygia pseudotsugata* MNPV (OpMNPV) and Cp-IAP3 from *Cydia pomonella* granulovirus (CpGV), eliminate the global protein synthesis shutdown (Thiem and Chejanovsky, 2004; Thiem, 2009). In addition, silencing of replicative *lef*s abrogates not only apoptosis but also protein synthesis shutdown of Sf21 cells induced by AcMNPV infection (Schultz and Friesen, 2009). These results suggest functional interconnections between apoptosis pathways and pathways mediating global protein synthesis shutdown in response to NPV infections.

In *Drosophila melanogaster*, seven different caspases have been identified. Dronc, Dredd and Strica with long N-terminal prodomains are classified as the initiator caspases, whereas DrICE, DCP-1, Damm and Decay containing short N-terminal prodomains are the effector caspases. Dronc plays a critical role in developmental and stress-induced apoptotic cell death in *Drosophila*, by activating the effector caspase DrICE (Hay and Guo, 2006; Cooper et al., 2009; Kumar and Doumanis, 2000). In lepidopteran insects, while effector caspases have been identified from several insects, including *S. frugiperda*, *Trichoplusia ni* and *Spodoptera littoralis* (Ahmad et al., 1997; Hebert et al., 2009; Liu et al., 2005), initiator caspases have not been characterized, although their presence has been suggested in *S. frugiperda* and *Bombyx mori* cells (Manji and Friesen, 2001; Huang et al., 2001; LaCount et al., 2000; Tanaka et al., 2008; Zhang et al., 2010; Zoog et al., 2002).

We have previously shown that *Lymantria dispar* Ld652Y cells undergo apoptosis upon infection with various NPVs (Ishikawa et al., 2003), while *B. mori* BM-N cells are relatively resistant to NPV infections and other diverse apoptotic stimuli. In addition, both Ld652Y cells infected with AcMNPV and BM-N cells infected with *Hyphantria cunea* MNPV (HycuMNPV) exhibit global protein synthesis shutdown, although the mechanisms likely differ between AcMNPV-infected Ld652Y cells and HycuMNPV-infected BM-N cells (Shirata et al., 2004, 2010). To provide insights into mechanisms for anti-viral responses of NPV-infected insect cells, we have been searching for cellular and viral regulatory factors that are involved in the induction and suppression of apoptosis and global protein synthesis shutdown (Ishikawa et al., 2003, 2004, 2006; Shirata et al., 2004, 2010; Ikeda et al., 2004; Yamada et al., 2011).

In this study, we clone and characterize a *B. mori* homologue (*bm-dronc*) of *Drosophila dronc*. We demonstrate that bacterially expressed Bm-Dronc has cleavage activities on the synthetic substrates preferred by human initiator caspases and that Bm-Dronc overexpressed in lepidopteran insect cells induces apoptosis, stimulating caspase-3-like protease activity. The apoptosis and the stimulated caspase-3-like protease activity upon Bm-Dronc overexpression are abrogated by either RNAi-mediated *bm-dronc* silencing or co-expression of a baculovirus functional IAP. We also show that endogenous Bm-Dronc undergoes spontaneous cleavage in BM-N cells upon baculovirus infection and exposure to actinomycin D and UV. These results indicate that Bm-Dronc serves as the initiator caspase responsible for caspase-dependent apoptosis induction.

## 2. Materials and methods

### 2.1. Cells, viruses and infection

BM-N cells (Volkman and Goldsmith, 1982) and BmN-4 cells from the silkworm, *B. mori* (Maeda, 1989), Sf9 cells from *S. frugiperda* and IPLB-Ld652Y cells from *L. dispar* (Ld652Y cells; Goodwin et al., 1978) were grown at 28 °C in TC100 medium (JRH Biosciences) supplemented with 10% foetal bovine serum (FBS) and 0.26% tryptose broth (Sigma). High Five cells from *T. ni* were maintained in Express Five SFM medium containing L-glutamine (Invitrogen), whereas S2 cells from *D. melanogaster* (Schneider, 1972) were cultured in Schneider's Drosophila Medium (Invitrogen) supplemented with 10% FBS. Viruses used in these experiments were *A. californica* multicapsid nucleopolyhedrovirus (AcMNPV) and vAcΔp35, a recombinant AcMNPV defective in the *p35* gene, provided by Dr. Rollie J. Clem (Kansas State University, Manhattan, KS) (Clarke and Clem, 2003). Insect cells were incubated with various multiplicities of infection (MOIs) of viruses for 60 min at room temperature as described previously (Ikeda et al., 2004).

### 2.2. Cloning of B. mori dronc

Total RNA was extracted from BM-N cells cultured in monolayers in 25-cm$^2$ flasks (Falcon 3018) by using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized by using 1 μg of total RNA by SuperScript III RNase H$^-$ Reverse Transcriptase (Invitrogen), according to the protocol provided by the manufacturer. cDNA of *bm-dronc* was amplified by PCR by using primers BmDronc3R-3 and BmDronc5R-3 (Table 1) in a 50-μl reaction mixture containing 1 μl of template, 1 μl of each 10 mM primer, 4 μl of PrimeSTAR GXL DNA polymerase (Takara), 4 μl of 2.5 mM dNTP mixture (Takara), and 10 μl of 5× PrimeSTAR GXL buffer. PCR was performed for 30 cycles under the conditions of 10 s at 98 °C and 2 min at 68 °C. The PCR products were subcloned by using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions, generating TOPO/Bmdronc.

The 5′- and 3′-RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was performed by using a cDNA library of BM-N cells constructed by a FirstChoice RLM-RACE kit (Ambion) with the primer set BmDronc5R-1 and BmDronc5R-2 for 5′-RACE and the primer set BmDronc3R-1 and BmDronc3R-2 for 3′-RACE. The 5′- and 3′-RACE products were subcloned with a TOPO TA cloning kit, and their sequences were analyzed.

### 2.3. Construction of plasmids

pET-47b(+)/HisBmdronc containing a His-tag at the N-terminus (Fig. 1b) was constructed by cloning the coding region of *bm-dronc* into the *Eco*RI/*Sal*I site of pET-47b(+) (Fig. 1a; Novagen). The *bm-dronc* coding region was amplified by PCR from TOPO/Bmdronc by using primers pET-BmDronc-F and pET-BmDronc-R.

pET-47b(+)/BmdroncHis containing a His-tag at the C-terminus (Fig. 1d) was constructed from pET-47b(+)/c-His (Fig. 1c) and used

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