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Cloning and functional characterization of the *Lymantria dispar* initiator caspase *dronc*

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ABSTRACT

Ld652Y cells from the gypsy moth, *Lymantria dispar*, are extremely sensitive to various apoptotic stimuli, whereas BM-N cells from the silkworm, *Bombyx mori*, are relatively resistant to apoptotic stimuli. We previously cloned and characterized a *B. mori* homologue (*bm-dronc*) of *Drosophila melanogaster dronc*. In the present study, we cloned and characterized an *L. dispar* homologue of *dronc* (*ld-dronc*) comparatively with Bm-Dronc. The open reading frame of *ld-dronc* consisted of 1329 bp that was predicted to encode a 443 amino-acid polypeptide with a molecular mass of 50,706 Da and 54–57% amino acid sequence identity with Dronc homologues from other lepidopteran insects identified to date. Ld-Dronc had a long prodomain, large p20 domain, and small p10 domain, and a catalytic site composed of ^{308}QTCRG^{312}, which was distinct from the sites QACRG in Bm-Dronc and QMCRG in Dronc homologues of several other lepidopteran insects. Transiently expressed Ld-Dronc underwent proteolytic processing in the lepidopteran cell lines *L. dispar* Ld652Y, *Spodoptera frugiperda* Sf9, and *B. mori* BM-N, and dipteran *D. melanogaster* S2, but only triggered apoptosis in the lepidopteran cell lines. Endogenous Ld-Dronc underwent processing in Ld652Y cells upon infection with vAcΔp35, but not in mock-infected Ld652Y cells, supporting the involvement of Ld-Dronc in apoptosis induction. In vAcΔp35-infected apoptotic cells, Ld-Dronc underwent proteolytic processing more rapidly and extensively than Bm-Dronc. Similar results were obtained for Ld-Dronc and Bm-Dronc expressed transiently in S2, Ld652Y, Sf9, and BM-N cells. Taken together, these findings suggest that the intrinsic properties of Dronc proteinsare responsible, at least in part, for the differing sensitivity of Ld652Y and BM-N to apoptosis induction upon NPV infection.

1. Introduction

Diverse stimuli trigger apoptosis through intrinsic or extrinsic pathways, which converge at activation of the cysteine protease (caspase) cascade [1,2]. Caspases are classified into initiator and effector caspases; the former are activated by both apoptotic pathways and function to activate downstream effector caspases. The activated effector caspases cleave various cellular substrates [3–5], leading to apoptosis. Phylogenetic analyses have revealed that caspases of lepidopteran insects can be classified into six groups, designated as Lep-Caspases-1 to -6 [6]. Lep-Caspases-1, -2, and -3 are effector caspases, whereas Lep-Caspases-5 and -6 are initiator caspases and are homologues of *Drosophila melanogaster* Dronc and Dredd, respectively. Lep-Caspase-4 appears to be a unique caspase with unusual structural features and unknown function [6]. Lepidopteran Dronc homologues have been characterized in the silkworm, *Bombyx mori*, and the fall armyworm, *Spodoptera frugiperda* [7,8].

Caspases exist in non-apoptotic cells as inactive zymogens, called pro-caspases, which consist of an N-terminal prodomain, large internal p20 domain, and small C-terminal p10 domain [1,9]. Initiator caspases harbor 80–100-amino acid (aa) prodomains that contain death effector domains (DEDs) and caspase activation and recruitment domains (CARDs), whereas effector caspases have short prodomains of approximately 30 aa residues. Pro-caspases are activated and proteolytically processed in response to apoptotic signaling, resulting in the generation of activated mature heterotetrameric caspases consisting of two p20 and two p10 subunits [1,9].

Nucleopolyhedroviruses (NPVs), members of the family Baculoviridae, are large enveloped insect-pathogenic viruses with double-stranded circular DNA genomes ranging from 80 to 180 kbp [10]. In cell cultures, NPV infections trigger apoptosis in a number of cell lines that are not permissive for productive infection [11]. In lepidopteran insects, apoptosis serves as one of the major innate antiviral defense mechanisms against baculovirus infections [12–14].

Ld652Y cells from *Lymantria dispar* undergo apoptosis upon infection with NPVs from *B. mori* (BmNPV), *Hyphantria cunea* (HycuMNPV), *Orygia pseudotsugata* (OpMNPV), *Spodoptera exigua* (SeMNPV) and *Spodoptera litura* (SpltMNPV), suggesting that this

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cell line is extremely sensitive to apoptotic stimuli [15]. In contrast, BM-N cells from *B. mori* are relatively resistant to apoptotic stimuli [16]. Comparative functional analysis of cellular factors involved in apoptosis induction and suppression in sensitive and resistant cell lines is expected to facilitate understanding of the regulatory mechanisms of apoptosis.

We previously cloned and characterized a *B. mori* homologue of Dronc (Bm-Dronc) and demonstrated that Bm-Dronc is involved in the induction of apoptosis of BM-N cells [7]. In the present study, we cloned and characterized an *L. dispar* homologue (*ld-dronc*) of *dronc* and demonstrate that Ld-Dronc functions as an initiator caspase and is involved in the induction of caspase-dependent apoptosis. Our analyses in transient expression assays and recombinant NPV-infected apoptotic cells revealed that although Ld-Dronc and Bm-Dronc share similar overall characteristics, they differ in the kinetics of proteolytic processing and for the activation of caspases-3-like protease. These results suggest that the intrinsic properties of initiator caspases are involved, at least in part, in the differing capacity of Ld652Y and BM-N cells to induce apoptosis in response to apoptotic stimuli, including NPV infections.

2. Materials and methods

2.1. Cells, viruses, infection, and transfection

Ld652Y cells from the gypsy moth *L. dispar*were grown in TC100 medium (AppliChem) supplemented with 10% fetal bovine serum (FBS) and 0.26% tryptose broth (Sigma). *D. melanogaster* S2 cells were maintained in Schneider's Drosophila Medium (Invitrogen) supplemented with 10% FBS. The vAcΔp35, a recombinant AcMNPV defective in the *p35* gene [17], was infected into target cell lines at a multiplicity of infection of 1 plaque forming unit, as described previously [18,19].

Monolayer cultures of Ld652Y (4×10^5) and S2 cells (2×10^6) in 35-mm culture dishes (Falcon 3001) were transfected with 2 μg expression plasmids as described previously [7,20,21]. Lipofectin (Invitrogen) and Cellfectin II reagents (Invitrogen) were used for Ld652Y and S2 cells, respectively.

2.2. Cloning of L. dispardronc

Monolayer cultures consisting of 1×10^6 Ld652Y cells in 25-cm^2 culture flasks (Falcon 3018) were washed once with PBS (1 mM Na_2HPO_4, 10.5 mM KH_2PO_4, 140 mM NaCl, 40 mM KCl, pH 6.2) and then lysed in 600 μl Buffer RLT containing 1% SDS (Qiagen). Total RNA was isolated from the cell lysate using a QIAcube instrument (Qiagen) and an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. 5′ and 3′ RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was performed using the FirstChoice RLM-RACE kit (Ambion) according to the protocol provided by the manufacturer. Based on the nucleotide sequence of *ld-dronc* determined by a series of 5′ and 3′ RLM-RACE reactions, the primers Ld-Dronc-Full-F and Ld-Dronc-Full-R (Table S1) were designed and used for the amplification of full-length *ld-dronc* using 3′ RACE products. The resulting PCR product was cloned into pCR4-TOPO using the TOPO TA Cloning kit (Invitrogen), generating TOPO/Ld-Dronc.

2.3. Plasmid construction

Construction of expression plasmids pET-47b(+)/LdDroncHis and pIE1-2/CmycLdDronc (Fig. S1B and D) was performed, as described previously [7]. Briefly, the coding region of *ld-dronc* was PCR-amplified from the plasmid TOPO/Ld-Dronc using PrimeSTAR GXL DNA polymerase and primers Ld-Dronc-NcoI-F and Ld-Dronc-PstI-R (Table S1), and the obtained PCR product was subcloned into pCR4-TOPO, generating TOPO/NcoI-LdDronc-PstI. TOPO/NcoI-LdDronc-PstI was double digested with *Nco*I and *Pst*I, and the excised *ld-dronc* coding region was introduced into the *Nco*I/*Pst*I site of pIE1-2/Cmyc-mcs (Fig. S1C) [7], generating pIE1-2/CmycLdDronc.

For construction of pET-47b(+)/LdDroncHis, the coding region of *ld-dronc* was PCR-amplified from pIE1-2/CmycLdDronc using primers Ld-Dronc_InF_F and Ld-Dronc_InF_R (Table S1), and was then introduced into *Nde*I-digested pET-47b(+)/c-His (Fig. S1A) [7] using the In-Fusion Advantage PCR Cloning kit (Clontech), generating pET-47b(+)/LdDroncHis. Plasmids pIE1-2/CmycBmDronc (Fig. S1E) and pIE1-2/Egfp (Fig. S1F) have been described previously [7,18].

2.4. Expression of Ld-Dronc in E. coli and preparation of cell lysate

E. coli strain Rosetta (DE3) pLysS competent cells (Novagen) were transformed with pET-47b(+)/LdDroncHis, and selected transformants were grown to an A_{600nm} of 0.5 at 37 °C in 50 ml LB medium containing 50 μg/ml kanamycin. Ld-Dronc expression was induced by adding IPTG to the cell cultures, which were further grown for an additional 16 h at 37 °C. The *E. coli* cells containing expressed Ld-Dronc protein were processed for preparation of cell lysates as described previously [7].

2.5. Immunoblot analysis

Immunoblot analyses were performed as described previously [7,16]. Briefly, polypeptides were resolved on 12% SDS–polyacrylamide gels, unless otherwise noted, and transferred onto Immobilon-P membranes (Millipore), which were then probed with respective antibodies and visualized by ECL Western blotting detection reagents (GE Healthcare). Anti-Ld-Dronc and anti-Bm-Dronc polyclonal antibodies were raised in rabbits against Ld-Dronc and Bm-Dronc proteins expressed in *E. coli* [7]. Monoclonal antibodies (mAbs) against cMyc- and His-tags were purchased from MBL. The HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG used as secondary antibodies were purchased from Zymed Laboratories. BenchMark pre-stained protein ladder (Invitrogen) was used for protein size markers.

2.6. Caspase activity assay

Caspase activity of Ld-Dronc and Bm-Dronc expressed in *E. coli* cells was fluorometrically determined using a Fluoroskan Ascent Microplate Fluorometer (Thermo Labosystems), as described previously [7,22]. Caspase-3-like protease activity in insect cells was determined as described previously [15,18]. The synthetic substrates were purchased from the Peptide Institute, Inc.

2.7. Nucleotide sequencing

Nucleotide sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit and ABI 3130 Genetic Analyzer (Applied Biosystems), as described previously [7]. The nucleotide sequences obtained were edited and analyzed using Genetyx software (Genetyx Corporation). Deduced amino acid sequence alignments were performed using ClustalW ver 1.83 [23] and motif searches were conducted with InterProScan ver 4.8 [24].

3. Results

3.1. Cloning and sequencing analysis of L. dispar dronc

To identify and determine the nucleotide sequence of the *ld-dronc* open reading frame (ORF), we designed primers based on the nucleotide sequence of *bm-dronc* sequenced previously [7]. The nucleotide sequence of full-length *ld-dronc* cDNA was determined by a series of RLM-RACE reactions using total RNA from Ld652Y cells as template and several specific primers, including those designed based on the nucleotide sequence of *bm-dronc*. Full-length *ld-dronc* cDNA was then PCR-amplified using 3′ RACE products and specific primers designed based on the nucleotide sequence of *ld-dronc* determined by the RLM-RACE reactions. The nucleotide sequence of *ld-dronc* cDNA consisted of a putative ORF of 1329 bp that was predicted to encode a 443-aa polypeptide with a predicted molecular mass of 50,706 Da. The nucleotide sequence of *ld-dronc* cDNA was deposited in the DDBJ/EMBL/GenBank database under accession number AB663140.

A motif search using InterProScan ver 4.8 revealed that the deduced aa sequence of Ld-Dronc contained a CARD (aa 1–78) within the N-terminal prodomain, a caspase family p20 domain (aa 185–314), and a caspase family p10 domain (aa 358–439) (Fig. S2). Secondary structure prediction using PsiPred v3.0 (http://meta.bioinfo.pl/) [25] further revealed that the prodomain contained six α-helices (Fig. S2), as has been reported for Dronc proteins from other insect species [7].

Alignment of the deduced aa sequence of Ld-Dronc with those of Bm-Dronc and human caspase-1 [7,26] demonstrated that the catalytic aa residues C310, H261, and G262 and the aa residues

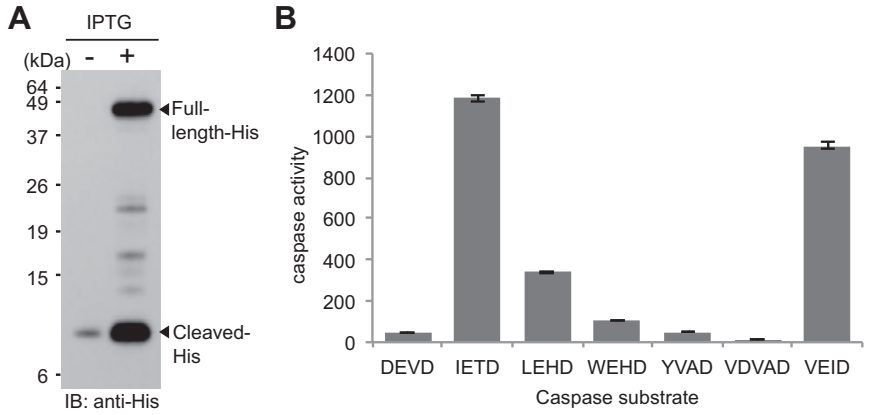

Fig. 1. Caspase activity of Ld-Dronc expressed in *Escherichia coli*. *E. coli* strain Rosetta (DE3) pLysS cells were transformed with pET-47b (+)/LdDroncHis and protein expression was induced by IPTG for 20 h at 25 °C. (A) Immunoblot analysis of Ld-Dronc expression. Polypeptides from transformed *E. coli* cells were resolved on a 15% SDS–polyacrylamide gel and transferred onto an Immobilon-P membrane. Ld-Dronc was probed with anti-His mAb and visualized by ECL Western blotting detection reagents. The numbers to the left of the panel represent the molecular masses (kDa) of marker proteins. The arrowheads to the right of the panel indicate full-length and cleaved Ld-Dronc proteins. (B) Caspaseactivity. Caspase activity was determined fluorometrically using the synthetic substrates Ac-DEVD-AMC (DEVD), Ac-IETD-AMC (IETD), Ac-LEHD-AMC (LEHD), Ac-WEHD-AMC (WEHD), Ac-YVAD-AMC (YVAD), Ac-VDVAD-AMC (VDVAD), and Ac-VEID-AMC (VEID). Caspase activities were normalized to the activity from *E. coli* cells transformed with the empty vector pET-47b (+). The error bars represent standard deviations of the means from three determinations.

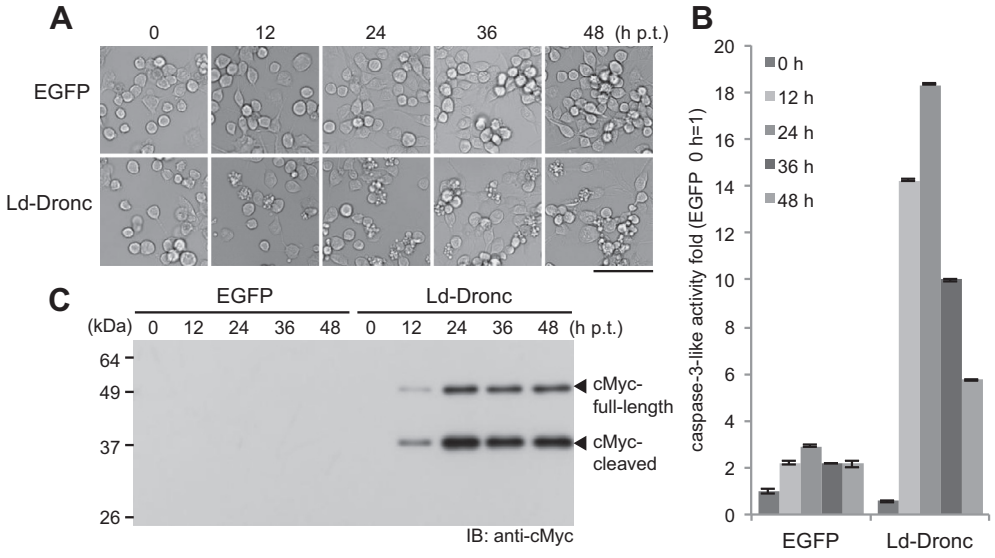

Fig. 2. Transiently expressed Ld-Dronc undergoes proteolytic processing and triggers apoptosis in Ld652Y cells. Monolayer cultures of Ld652Y cells were transfected with pIE1-2/CmycLdDronc (Ld-Dronc) or pIE1-2/Egfp (EGFP) and examined at 0, 12, 24, 36, and 48 h post-transfection. (A) Microscopic examination of apoptosis. The scale bar represents 100 μm. (B) Caspase-3-like protease activity. The caspase-3-like protease activities were determined using Ac-DEVD-AMC as the substrate and are normalized to the activity of pIE1-2/Egfp-transfected cells at 0 h post-transfection. The error bars indicate standard deviations of the means from three determinations. (C) Expression and processing of Ld-Dronc. Ld-Dronc was analyzed by immunoblotting using anti-cMyc mAb. The numbers to the left of the panel represent the molecular masses (kDa) of marker proteins. The arrowheads to the right of the panel indicate full-length and cleaved Ld-Dronc proteins.

involved in coordinating P1 aspartate, R205, Q308, R380, and S386, were conserved in Ld-Dronc (Fig. S2; 26). Ld-Dronc contained a catalytic site composed of ^{308}QTCRG^{312}, which differed from QACRG of Bm-Dronc and QMCRG of the Dronc homologues from *H. armigera*, *S. exigua*, *S. frugiperda* and *S. litura* (Fig. S2). Ld-Dronc exhibited a high degree of aa sequence identity with Dronc homologues from the lepidopteran insects *B. mori* (54%), *H. armigera* (57%), *S. exigua* (55%), *S. frugiperda* (57%), and *S. litura* (57%), but only shared 23% and 25% aa sequence identities with those of the dipteran insects *D. melanogaster* and *Aedes aegypti*, respectively.

3.2. Enzymatic activity and substrate specificity of Ld-Dronc expressed in E. coli

The expression of Ld-Dronc with a C-terminal His-tag in *E. coli* strain Rosetta(DE3)pLysS was examined by immunoblot analysis using anti-His-tag mAb. Upon transformation of cells with pET-47b(+)/LdDroncHis and induction of protein synthesis by IPTG, both full-length (~50 kDa) and a cleaved form of Ld-Dronc (~10 kDa), which corresponded to a polypeptide composed of a single p10 subunit, were detected (Fig. 1A).

To determine whether bacterially expressed Ld-Dronc had enzymatic activity, caspase activity was assayed using synthetic substrates preferred by human caspase-3 (Ac-DEVD-AMC), caspase-8 (Ac-IETD-AMC), caspase-9 (Ac-LEHD-AMC), caspases-1, -4, and -5 (Ac-WEHD-AMC), caspases-1 and -4 (Ac-YVAD-AMC), caspase-2 (Ac-VDVAD-AMC), and caspase-6 (Ac-VEID-AMC). Ld-Dronc exhibited high catalytic activity towards Ac-IETD-AMC and Ac-VEID-AMC and a lower activity on Ac-LEHD-AMC (Fig. 1B), in contrast to that reported previously for Bm-Dronc, which displayed high substrate specificity for Ac-IETD-AMC, Ac-LEHD-AMC, and Ac-VDVAD-AMC, but only negligible specificity for Ac-VEID-AMC [7]. To further examine the differing substrate specificities of Ld-Dronc and Bm-Dronc, we conducted parallel caspase activity

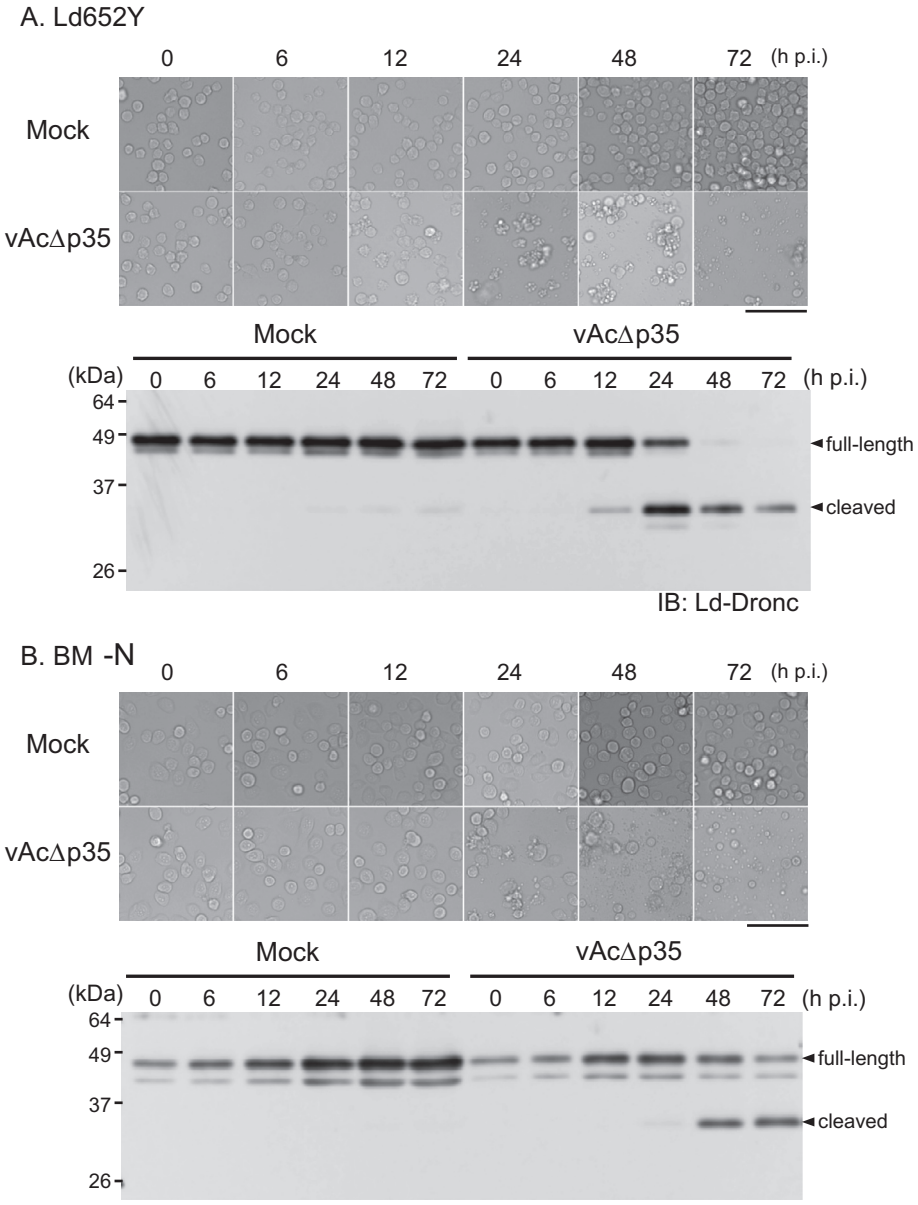

Fig. 3. Kinetics of processing and decay of endogenous Ld- and Bm-Dronc proteins in Ld652Y and BM-N cells, respectively, infected with vAcΔp35. Ld652Y (A) and BM-N cells (B) were mock-infected (Mock) or infected with vAcΔp35 (vAcΔp35) and examined at 0, 6, 12, 24, 48, and 72 h post-infection for apoptosis (upper panels) and expression and processing of Ld-Dronc and Bm-Dronc by immunoblotting using anti-Ld-Dronc and anti-Bm-Dronc polyclonal antibodies, respectively (lower panels). In the upper panels, the scale bars represent 100 μm. In the lower panels, full-length and cleaved Dronc proteins are indicated by the arrowheads to the right of the panels. The numbers to the left of the lower panels represent molecular masses (kDa) of marker proteins.

assays using cell lysates from *E. coli* expressing Ld-Dronc and Bm-Dronc, and found that both enzymes had high caspase activity towards Ac-IETD-AMC, Ac-LEHD-AMC, and Ac-VEID-AMC, but only negligible activity for Ac-VDVAD-AMC (data not shown). In addition, both Ld-Dronc and Bm-Dronc had low, but clearly detectable caspase activity when Ac-WEHD-AMC was used as a substrate.

3.3. Transient expression of Ld-Dronc triggers apoptosis of Ld652Y cells

Ld652Y cells were transfected with plasmids pIE1-2/Egfp or pIE1-2/CmycLdDronc for the expression of EGFP or Ld-Dronc with an N-terminal cMyc-tag, and then examined for apoptosis and caspase-3-like protease activity at 0, 12, 24, 36, and 48 h post-transfection. Transiently expressed Ld-Dronc triggered apoptosis and activation of caspase-3-like protease by 12 h post-transfection (Fig. 2A and B). The induction of apoptosis increased to 48 h post-transfection, whilecaspase-3-like protease activity increased to 24 h post-transfection and then began to gradually decrease from 36 h post-transfection. Immunoblot analysis of transiently expressed Ld-Dronc using anti-cMyc mAb revealed two polypeptides with molecular masses of ~50 and ~38 kDa (Fig. 2C), which corresponded to full-length cMyc-tagged Ld-Dronc and cMyc-tagged Ld-Dronc lacking the p10 domain. The band of cleaved Ld-Dronc was stronger than that of full-length Ld-Dronc. No bands corresponding to cMyc-tagged polypeptides were detected in Ld652Y cells expressing EGFP (Fig. 2C). Both lepidopteran *S. frugiperda* Sf9 and *B. mori*BM-N cells also induced apoptosis, accompanied by the activation of caspase-3-like protease, upon transfection with pIE1-2/CmycLdDronc (unpublished).

3.4. Ld-Dronc is responsible for apoptosis of Ld652Y cells triggered by NPV infection and undergoes processing more rapidly and extensively than Bm-Dronc

To determine whether Ld-Dronc was involved in apoptosis triggered by NPV infection, we first generated a rabbit polyclonal antibody against Ld-Dronc protein from *E. coli* and verified its specificity by immunoblot analysis of lysates from Ld652Y cells transfected with pIE1-2/Egfp and pIE1-2/CmycLdDronc, which express EGFP and Ld-Dronc with an N-terminal cMyc-tag, respectively. At 0 and 24 h post-transfection, only a single major band of ~48 kDa was detected in pIE1-2/Egfp-transfected Ld652Y cells. In contrast, pIE1-2/CmycLdDronc-transfected Ld652Y cells exhibited four major polypeptides with sizes of ~50, ~48, ~38, and ~36 kDa (data not shown), corresponding to full-length cMyc-tagged Ld-Dronc, endogenous full-length Ld-Dronc, cMyc-tagged p10 domain-defective Ld-Dronc, and p10 domain-defective endogenous Ld-Dronc, respectively, indicating that the antibody is reactive to both full-length Ld-Dronc and Ld-Dronc lacking the C-terminal p10 domain.

To determine whether endogenous Ld-Dronc underwent proteolytic processing upon infection with NPV, Ld652Y cells were mock-infected or infected with vAcΔp35 and examined for endogenous Ld-Dronc by immunoblot analysis using anti-Ld-Dronc polyclonal antibody. Infection of Ld652Y cells by vAcΔp35 triggered apoptosis (Fig. 3A). Here, in vAcΔp35-infected Ld652Y cells, the levels of full-length Ld-Dronc markedly decreased from 24 h post-infection and only a negligible amount was detected at 48 and 72 h post-infection, whereas the levels of cleaved Ld-Dronc decreased only slightly until 72 h post-infection (Fig. 3A), indicating that endogenous Ld-Dronc underwent rapid processing following vAcΔp35 infection. In addition, we performed parallel experiments in vAcΔp35-infected BM-N cells using polyclonal antibody against Bm-Dronc prepared previously [7], and found that substantial proteolytic processing of Bm-Dronc is first observed only at 48 h post-infection, and remarkable amount of full-length Bm-Dronc remained even at 72 h post-infection (Fig. 4B). These results indicate that the processing and decrease of full-length Ld-Dronc occur earlier and more extensively after vAcΔp35 infection than does full-length Bm-Dronc.

3.5. Ld-Dronc transiently expressed in Drosophila S2 cells undergoes processing but does not trigger apoptosis

To determine whether Ld-Dronc triggered apoptosis of dipteran cells, S2 cells were transfected with pIE1-2/Egfp, pIE1–2/CmycBm-Dronc, or pIE1-2/CmycLdDronc. In agreement with the previous results for Bm-Dronc [7], both Ld- and Bm-Dronc did not trigger apoptosis or activate caspase-3-like protease (Fig. 4A and B), despite the fact they were successfully expressed and processed (Fig. 4C). These results suggest that lepidopteran Dronc expressed in dipteran cells is not functional for apoptosis induction. It was also found that the relative level of cleaved Dronc to full-length

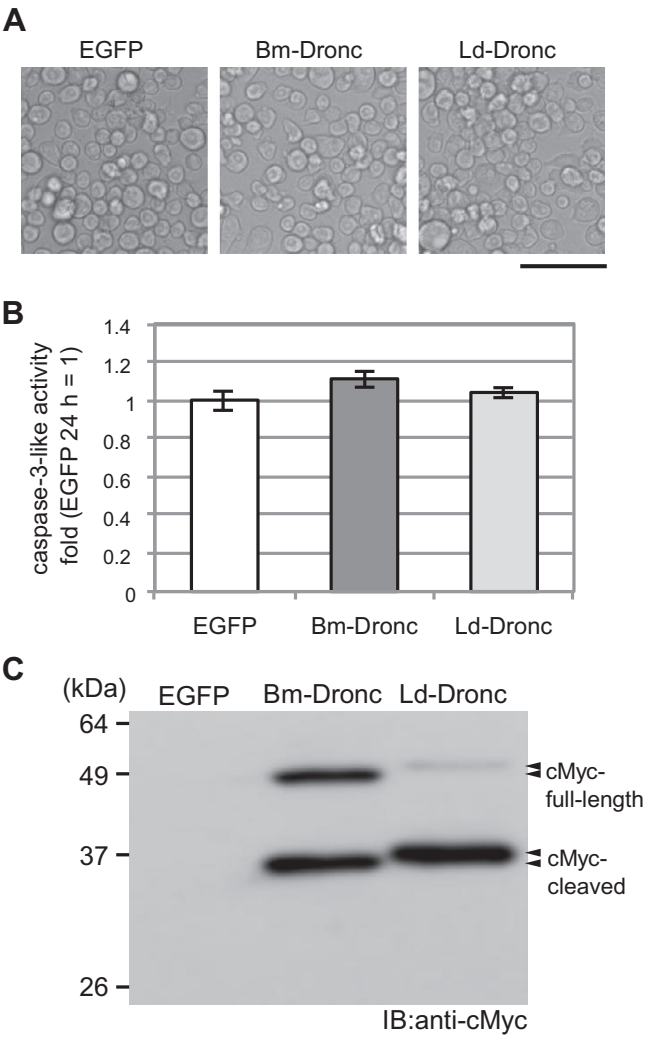

Fig. 4. Ld-Dronc undergoes processing, but does not trigger apoptosis or activation of caspase-3-like protease in S2 cells in a transient expression assay. S2 cells were transfected with pIE1-2/Egfp (EGFP), pIE1-2/CmycBmDronc (Bm-Dronc), or pIE1-2/CmycLdDronc (Ld-Dronc) and examined for apoptosis (A), caspase-3-like protease activity (B), and processing of Bm- and Ld-Dronc proteins (C) at 24 h post-transfection. The scale bar in the panel A represents 50 μm. In the panel B, the caspase-3-like protease activities are normalized to the activity of pIE1-2/Egfp-transfected cells at 24 h post-transfection, and the error bars indicate standard deviations of the means from three determinations. In the panel C, positions of maker proteins and full-length and cleaved Dronc proteins are indicated to the left and right of the panel, respectively.

Dronc was extremely lower for Ld-Dronc than Bm-Dronc (Fig. 4C). As in S2 cells, relative levels of cleaved Dronc to full-length Dronc were also lower for Ld-Dronc than Bm-Dronc in Ld652Y, Sf9, and BM-N cells (unpublished).

4. Discussion

In this study, we cloned and characterized an *L. dispar* homologue (*ld-dronc*) of *D. melanogaster dronc* from Ld652Y cells, which are highly sensitive to apoptotic stimuli and undergo apoptosis upon infection with various NPVs [15,16,21]. Our results demonstrated that Ld-Dronc transiently expressed in Ld652Y cells undergoes proteolytic processing and triggers cellular apoptosis and activation of caspase-3-like protease, suggesting its important role in apoptosis induction. The involvement of Ld-Dronc in apoptosis induction in Ld652Y cells is further supported by the finding that endogenous Ld-Dronc undergoes proteolytic processing in apoptotic Ld652Y cells infected with vAcΔp35 but not in mock-infected Ld652Y cells. These results indicate that Ld-Dronc cloned in this study functions effectively as an initiator caspase and activates effector Ld-caspase-1, which executes apoptosis of Ld652Y cells [21].

Our results also demonstrated that there is a considerable difference between Ld-Dronc and Bm-Dronc in their kinetic properties of proteolytic processing, even though they bear obvious similarities in their structure, substrate specificity, and functional role in apoptosis induction of lepidopteran and dipteran insect cells. Specifically, our data showed that Ld-Dronc undergoes more rapid and extensive proteolytic processing than does Bm-Dronc upon vAcΔp35 infection, and full-length Ld-Dronc in vAcΔp35-infected Ld652Y cells is exhausted earlier after infection than full-length Bm-Dronc in vAcΔp35-infected BM-N cells. A similar difference in the kinetic properties of Dronc processing was also observed in a transient expression assay using S2 cells, in which Ld-Dronc undergoes more rapid and extensive proteolytic processing after plasmid transfections than does Bm-Dronc. Further transient expression assays using Ld652Y, Sf9, and BM-N cells gave results similar to that in S2 cells, and co-expression of Ld-Dronc or Bm-Dronc, together with Apsup, a baculovirus apoptosis suppressor that prevents processing of initiator caspases [[27], unpublished], showed that the amounts of Ld-Dronc and Bm-Dronc expressed in each cell line are similar (unpublished), confirming that processing kinetics differ between Ld-Dronc and Bm-Dronc. These results are consistent with the fact that Ld652Y cells are more sensitive to apoptotic stimuli-induced apoptosis and caspase-3-like protease activation than BM-N cells. Thus, our findings indicate that differences in the intrinsic properties of lepidopteran Dronc proteinsare responsible, at least in part, for the difference in the apoptosis induction capacity between Ld652Y and BM-N cells.

Finally, our results demonstrated that transiently expressed Ld-Dronc undergoes proteolytic processing, but does not trigger apoptosis or activation of caspase-3-like protease in S2 cells, in agreement with the previous results for Bm-Dronc [7]. These findings suggest that Dronc proteins of lepidopteran insects are generally unable to activate dipteran effector caspases that are responsible for apoptosis execution, indicating the occurrence of functional, as well as structural, divergence of Dronc proteins between lepidopteran and dipteran insects. However, transiently expressed *D. melanogaster* Dronc undergoes proteolytic processing and triggers apoptosis in Ld652Y cells (unpublished), as well as S2 cells [28]. Furthermore, in contrast to upstream initiator caspases, downstream effector caspases have been relatively well conserved during evolution [7,21], and transiently expressed *L. dispar* effector caspase Ld-caspase-1 modulates apoptosis of S2 cells [21]. Comparative systematic analyses of lepidopteran and dipteran insect cells is expected to provide important insights into the structural and functional conservation and divergence of apoptosis regulators, in addition to shedding light on the evolution of defense strategies of insects relevant to apoptosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.103.

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