



RNA interference mediated knockdown of an inhibitor of apoptosis protein induces apoptosis in *Mythimna separata* (Lepidoptera: Noctuidae)

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Abstract. Coordinated regulation of apoptosis is critical for development, homeostasis, and immunity in larvae of Metazoa. We determined the full nucleotide sequence of an *inhibitor of an apoptosis protein* in a lepidopteran insect *Mythimna separata* (Walker) (*MslAP*) and carried out functional analyses of the *MslAP*. The full-length cDNA of *MslAP* was 1642 bp, which encoded 379 amino acid residues with a calculated molecular mass of 41,834 Da, and two BIR domains and one RING domain revealed using amino acid sequence analysis. In addition, the sequences of these domains were similar to *Drosophila* IAP1 and those of some other lepidopteran insects. We carried out a functional analyses of *MslAP* related to apoptosis regulation using RNA interference. The effects of *MslAP* knockdown on adhering hemocytes and non-adhering hemocytes as controls were examined using Hoechst33342/propidium iodide staining, effector caspase activity and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining. A significantly higher number of propidium iodide and TUNEL-positive cells was recorded in adhering hemocytes from *MslAP* knockdown larvae than from control larvae, but these differences were not recorded for non-adhering hemocytes. However, higher effector caspase activity was detected in both adhering and non-adhering hemocytes from *MslAP* knockdown larvae compared to that in control larvae. These results indicate that the knockdown of *MslAP* induces apoptosis in larval adhering hemocytes, which *MslAP* negatively and non-redundantly regulate apoptosis, and that IAP function is conserved in *M. separata* and other insect species including *Drosophila* and several lepidopteran insects.

INTRODUCTION

Apoptosis is a genetically programmed cellular process that removes unwanted, damaged, or infected cells by inducing “suicide” in these cells (Clarke, 1990). Thus, apoptosis has a crucial role in development, homeostasis and immunity (Vaux & Korsmeyer, 1999; Opferman & Korsmeyer, 2003). The molecular mechanism of apoptosis was initially investigated in the model nematode *Caenorhabditis elegans* (Maupas), which has a protease *ced-3* and nuclease *nuc-1* that function as positive and negative regulators of apoptosis, respectively (Horvitz, 2003; Riedl & Shi, 2004). *Drosophila melanogaster* (Meigen) and mammals have more complex cell death mechanisms. Multiple family *ced-3* protease homologs, which are known as caspases, have central roles in regulating apoptosis. Caspases fall

into two categories: initiator caspases are activated first and then cleave downstream effector caspases, which degrade several distinct substrates leading to cell death. During the cell death process, a series of morphologically recognizable changes, such as cell shrinkage, chromatin condensation, genomic DNA fragmentation, membrane blebbing and the emergence of apoptotic bodies, occur (Wyllie et al., 1980).

Inhibitor of apoptosis proteins (IAPs) are well-studied negative regulators of apoptosis. IAPs bind directly with both initiator and effector caspases and block their activities (Salvesen & Duckett, 2002; Riedl & Shi, 2004; Vaux & Silke, 2005). For example, *Drosophila* IAP1 (DIAP1), an IAP family protein in *D. melanogaster*, is a key negative regulator of apoptosis and inhibits DRONC (*D. mela-*

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nogaster initiator of caspase), drICE and DCP-1 (*D. melanogaster* effector caspases) (Kornbluth & White, 2005; Tenev et al., 2005). Most IAP family proteins have two important domains, a baculoviral IAP repeat (BIR) domain and really interesting new gene (RING) domain (Riedl & Shi, 2004; Vaux & Silke, 2005). The BIR domain is a Zn^{2+} -binding domain and consists of approximately 70 amino acid residues. This domain interacts with effector or initiator caspases. The RING domain is a Zn^{2+} -binding domain with E3 ubiquitin ligase activity and is involved in the negative regulation of apoptosis.

In several species of Lepidoptera, IAP orthologues function as negative regulators of apoptosis, which suggests that roles of IAPs are conserved in mammals, *D. melanogaster* and lepidopteran insects (Huang et al., 2000, 2001; Liao et al., 2002). We investigated whether the function of an IAP orthologue in the lepidopteran insect, *Mythimna separata* (Walker) (*MsIAP*), is conserved. Previously, we examined its sensitivity to RNAi in multiple tissues of *M. separata* (Yokoi et al., 2013). Using a partial sequence of *MsIAP*, we found that *MsIAP* mRNA was ubiquitously expressed and that significant gene silencing of *MsIAP* was achieved only in adhering hemocytes from isolated abdomens treated with *MsIAP* double-stranded RNA (dsRNA). Here, we determined the full cDNA sequence of *MsIAP* and the predicted *MsIAP* amino acid sequence was analyzed. *MsIAP* function was determined in terms of cell viability, effector caspase activity and genomic DNA fragmentation. We found that *MsIAP* functioned as a negative regulator of apoptosis in adhering hemocytes, which indicates that the function of IAP as a negative regulator of apoptosis is conserved in *M. separata*.

MATERIALS AND METHODS

Insect rearing and preparation of isolated abdomens

M. separata was reared as described in Suzuki & Tanaka (2007) and the method of preparing isolated abdomens is described in Yokoi et al. (2013).

First strand cDNA synthesis for RT-PCR

Total hemocyte RNA was used for 1st strand cDNA synthesis using PrimeScript reverse transcriptase (TAKARA) and oligo-(dT) 20 primer. The 1st strand cDNA library was used as a template for RT-PCR with a pair of degenerate primers. A pair of degenerate primers were designed based on the highly conserved regions identified by alignment of several amino acid sequences of published insect IAPs using the Clustal W 1.83 program (Thompson et al., 1994). Sequences of the degenerate primer pair were: IAP-F1d, 5'-ATGAARACNTTYGARAARTGGC-3'; IAP-R1d, 5'-TCYTCNGCRWARCAIATYTTICA-3'. The locations of these degenerate primers in *MsIAP* are shown in Fig. 1. The RT-PCR reaction mixture was first denatured at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min and elongation at 72°C for 30 s. The PCR products were separated on a 1.8% agarose gel. The DNA bands of the expected sizes were excised and recovered using a QIAquick Gel Extraction Kit (QIAGEN). DNA fragments were checked for integrity and amount using gel electrophoresis, subcloned into the pCR2.1 vector (Invitrogen), and sequenced with an ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and a DNA sequencer (Model 3130, Applied Bio-

systems). BLASTx search (URL: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch) confirmed that the obtained DNA fragment encoded an amino acid sequence that is very similar to known insect IAPs.

3' and 5' RACE

For 3' RACE, the 1st strand cDNA synthesis was primed with a tagged oligo-(dT) primer (5'-CTACAGTCTGCTCACAGCATAGTATTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'). The 3' region of *MsIAP* was amplified with gene-specific primers (5'-ACGGAGGCTTGAAAGACTGGGAG-3') and a 3' anchor primer (5'-CTACAGTCTGCTCACAGCATAGTA-3') using thermal cycling conditions of initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s and elongation at 72°C for 1 min 30 s. For 5' RACE, a RNA oligo (5'-AAGCAGUGGUAU-CAACGCAGAGUGGG-3') was included in the reverse transcription reaction and the 3'-tagged 1st strand cDNA pool was subjected to RT-PCR with a 5' anchor primer (AAGCAGTGTATCAACGCAGAGT-3') and gene-specific primer (5'-GC-GAGCTTGAACCTCGGCAAT-3'). The locations of the two gene-specific PCR primers used for the RACE procedures and degenerate primers are shown in Fig. 1. Thermal cycling conditions used were an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s and elongation at 72°C for 1 min 30 s. The amplification products were fractionated by agarose gel electrophoresis and specifically-amplified DNA fragments were recovered and sequenced directly. In some cases, the DNA fragments were sequenced directly with the same reagents and device described previously and the sequences assembled into the full *MsIAP* nucleotide sequence. The full *MsIAP* nucleotide sequence was deposited in GenBank (accession no. AB778567).

RNA interference

The materials and methods related to RNAi were the same as in Yokoi et al. (2013). Briefly, the DNA templates for dsRNA synthesis were prepared using conventional PCR with the pairs of T7-tagged PCR primers for the respective factors, for which the locations in *MsIAP* are shown in Fig. 1. The sequences of the primers were: 5'-taatacgactcactataggagaACTTTCCTTTCTG-GAGATGAACT-3' (sense) and 5'-taatacgactcactataggagaT-CATCACCTTCTGGACGTACTC-3' (anti-sense). Synthesis of dsRNA was carried out using a MEGAscript RNAi kit (Ambion) with these templates. The dsRNA fragment was purified according to the manufacturer's instructions, and the integrity and amount were assessed using agarose gel electrophoresis and spectrophotometry, respectively. For the production of dsRNA with unrelated sequences, enhanced green fluorescent protein (EGFP) sequence-derived dsRNA was prepared in the same manner using the pEGFP-N1 plasmid (Clontech) as a PCR template.

For IAP gene function analysis, we prepared isolated abdomens using day 1 6th instar larvae. The detailed procedures for preparing and isolating abdomens and dsRNA injection are described by Yokoi et al. (2013). Briefly, the larvae were constricted between the third thoracic segment and the first abdominal segment with a nylon thread, and the posterior part minus two pairs of haematopoietic organs was used as an isolated abdomen. dsRNA was injected into isolated abdomens 24 h post ligation. We performed a preliminary assay for IAP knockdown effects on isolated abdomens. We found that when 3 µg dsRNA *MsIAP* was injected into an isolated abdomen and the isolated abdomen was incubated for 48 h, there was approximately a 60% reduction in *MsIAP* mRNA in adhering hemocytes compared to that in control hemocytes, but no significant effects of *MsIAP* knockdown was recorded in

1 TTTTATAAGCAGTTTAAACCCAGCTGCGAGAAGCGAGCTTAGATCGATCTAA 60
 61 GCAGTGGATCAACGCGAGTTCGGGCTTCGCACATGTTGGAGAGTTGTGTTGTTG 120
 121 TTGTCATCCGTTTACGTTTGACAAAGTGAGAGTGAAGTCTGTAGTACCTAGTCAA 180
 181 AAACAATCAATTTGATGTGCTGCTTTCATTGCTTGTGTAACACGACATCTCGGAT 240
 1 M W S C S L P C V K T A L S G L 16
 241 TACAAATGGAAAAAGAAATGGACCAATCCAAATCCATCCACCTCTGCAATTCGAGGT 300
 247 Q M E K N G P I Q I H P T S A L P R F 36
 301 TCAAGCTCGCGGCTCCGCGCGACGACGAGTAAACCCCTGGCCAGTCTTGTGCTAC 360
 37 K L A A P R D A A K L K P L A S L V L P 56
 361 CGACGTCACACTACGACTCAATAGCCGCTCTCCGCTTCGTGCTTCATCATCTGCTT 420
 57 T S N Y D S N A G S P A S S P S S S S S 76
 421 CTTCCTCTACGTTCTCCGTCGACAGACCGACACCACTTTAGCTTTGGCAGCGAAT 480
 77 S S T F L V D K T D N H D F S F G T E S 96
 481 CAGTTGATATGCGCAGAGAATGACGAATTAACACGTTTCGAAAAATGGCTGTCACTT 540
 97 V D M R T E D A R I K T F E K W F V T F 116
 541 TCCTTTCTGGAGATGAATCGCGCGAAATGGATTTATTACCTCGGCGGTGTGACGAAG 600
 117 L S G D E L A R N G F Y Y L G R G D E V 136
 5 TACGCTGCGGCTTTCGAAGTTGAATATGAATGAGGTGAGGCGACGATCTCTGTA 660
 137 R C A F C K V E I M K W V E G D D P A K 156
 661 AGGACCATCAGGATGGGACCCCAATGCCATTCGTTTCGAAATTTGGGACGAGCGCA 720
 157 D H Q R W A P Q C P F V R K L G S S A S 176
 721 GTTCAGAGACGATAGCAACGGGCGGACGCTGCGGCTCCGCGCTGCCACGACGCA 780
 177 S E R T S N G R D E G S R A T S S T 196
 781 CTACGCCCCAGAATGACCGCCCGTGCACCTCGTACGCTCCGAAACCGCAAGAC 840
 197 T P P R M T G P V H P R Y A S E T A R L 216
 841 TGCAGCTTTCCAAAGTGGCGGAGATGCATGAACAGAACTGAAGAGCTCGCAGAG 900
 217 R S F Q Q D R C M K Q K P E E L A E A 236
 901 CTGGCTTCTTCTACACCGGTCAAGTGACAAAGTAAATGCTTTACTGCGACGAGGT 960
 237 G F F Y T G Q G D K T K C F Y C D G G L 256
 961 TGAAGACTGGGAACGATGACGTGCGCTGGGAGCAGCAGCGCTTGGTTGACCGCT 1020
 257 K D W E N D D V P W E Q H A R W F D R C 276
 1021 GCGCATACGTGCGCTGGTGAAGGACGCGCTGACCTGCGGAGTGAAGGAGCGCT 1080
 277 A Y V Q L V K G E Y V Q K V M T E A C 296
 1081 GCGCAGTGCCTGAGTGAAGCGGCGGACGCTGCGGAGCAGCAGCTTGAAGTGA 1140
 297 A V P A A E A G R D V A P V R T S T S S 316
 1141 GCTGCGCGCTGACTCGCCGAGAACTCCGTTGACGACTCCAAGTTGTAAATTTGTT 1200
 317 S P L D S N G R C M K Q K P E E L A E A 336
 1201 ACGCGAGGAGCGCAACGTGTGCTTCGTACCGTGCAGACAGTGGTGGCTGTGCAAGT 1260
 337 A E E R N V C F V P C G H V V A C A K C 356
 1261 GCGGCTGGCGCTGACAAATGCCAATGTCGCGAGGAGCTTTCAGAACGACGTGGAT 1320
 357 A L A A D K C P M C R R T F Q N A V R L 376
 1321 TATATTCTCTTGAAGAAGCTGCCTAATCGAAGACTCGATTCTAGTCAGGACGCGCCG 1380
 377 Y P S * 379
 1381 ACAGAGCTGGTGTGTAACCCCTAGCTCCTATGTCAAGTTTCCACAGCGGAGAACCT 1440
 1441 GTTGACATTTATCGCTCTGGTAGATAGCAAGTCTGCTGTGTCAGATAGACGAATA 1500
 1501 CGCTTTACAGTCATTGAGATTGAGTCTCAAGTGGGTTTATGTCAGTACGAGTCTGAC 1560
 1561 ACGCCCTCTGCTCCATCTTAAGCGGAGAGTCAATTGATGATTCCCACTTAAACA 1620
 1621 AAAAAAAAAAAAAAAAAAAAA 1642

Fig. 1. cDNA and predicted amino acid sequences of *MsIAP*. The full-length cDNA sequence was assembled from partial sequences obtained using RT-PCR with degenerate primers, 3' RACE and 5' RACE. Arrows with numerals 1 to 6 indicate the positions of primers used: 1 and 2, a pair of degenerate primers for RT-PCR; 3, gene-specific antisense primer for 5' RACE; 4, gene-specific sense primer for 3' RACE; 5 and 6, T7-tagged primer pair for in vitro transcription template preparation. An asterisk denotes the stop codon. Nucleotide and amino acid residue numbers are shown on the left and right sides of each line.

non-adhering hemocytes, which is similar to the results of Yokoi et al. (2013). Therefore, we used these conditions for *MsIAP* knockdown of hemocytes.

Hoechst33342 and propidium iodide staining

Hemocyte viability was assessed by plasma membrane integrity using double staining with Hoechst33342 and propidium iodide (PI). Hemolymph was diluted with 9 volumes of SF900 III medium (Thermo Fisher SCIENTIFIC) with 8% (v/v) saturated phenylthiourea (PTU), which inhibits spontaneous melanization of the hemolymph and incubated on a glass slide (Matsunami, S2441) for 15 min at room temperature. The glass slide was washed three times with the same medium and non-adhering hemocytes, consisting of spherulocytes, prohemocytes and oenocytoids (Lavine & Strand, 2002) were collected into a plastic tube

and centrifuged at 600 g at 4°C for 10 min. Adhering hemocytes, which remained on the glass slide, mainly consisted of granulocytes and plasmatocytes (Lavine & Strand, 2002). The non-adhering hemocyte precipitate was resuspended in the same medium and the hemocyte suspension was put on a Mas-coated glass slide (Matsunami, S9441), which allows *M. separata* non-adhering hemocytes to adhere to the coated surface. Both adhering and non-adhering hemocytes on the glass surface were washed three times with PBS and double-stained with Hoechst33342 (10 mg/mL in PBS) and PI (2 mg/mL in PBS) for 3 min. The samples were washed briefly with PBS, immediately observed under a fluorescent microscope (Olympus, Model BX41) and imaged. The numbers of Hoechst-positive (all) cells and PI-positive (dead or late apoptotic) cells were counted at 200× magnification in at least six randomly selected fields.

Caspase-3/7 activity assay

The activity of effector caspase-3/7 was determined in larval hemocyte preparations using a Caspase-Glo 3/7 kit (Promega). The hemolymph from larvae pretreated with *MsIAP* or *EGFP* dsRNA was mixed with an equal volume of SF900 III with 8% (v/v) PTU and aliquoted into a 96-well plate. Fifteen minutes later, non-adhering hemocytes were transferred to another well. Hemocyte preparations were lysed in the presence of substrate and caspase activity determined using a luminometer (Turner Designs, Model TD 20/20). The caspase activities in the IAP knockdown and control of adhering and non-adhering hemocytes were determined. First, data normality for each category was tested using the Kolmogorov-Smirnov test. If significant normality of the two samples was detected, a Student's t test was used. If significant normality of the two samples was not detected, a Mann-Whitney U test was used.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay

Adhering and non-adhering hemocyte samples on glass slides were prepared as above. Hemocytes were labelled according to the protocol of an In Situ Cell Death Detection Kit, AP (Roche), samples were observed under a fluorescent microscope and imaged. The total numbers of cells and TUNEL-positive cells in both hemocyte samples were counted.

Sequence analyses

We used GENETYX ver. 9 software (GENETYX corporation) and InterPro (<http://www.ebi.ac.uk/interpro/>) (Hunter et al., 2009) to edit and analyze nucleotide and amino acid sequences. Homology searches were performed using BLAST at DNA Data Bank of Japan (<http://blast.ddbj.nig.ac.jp>).

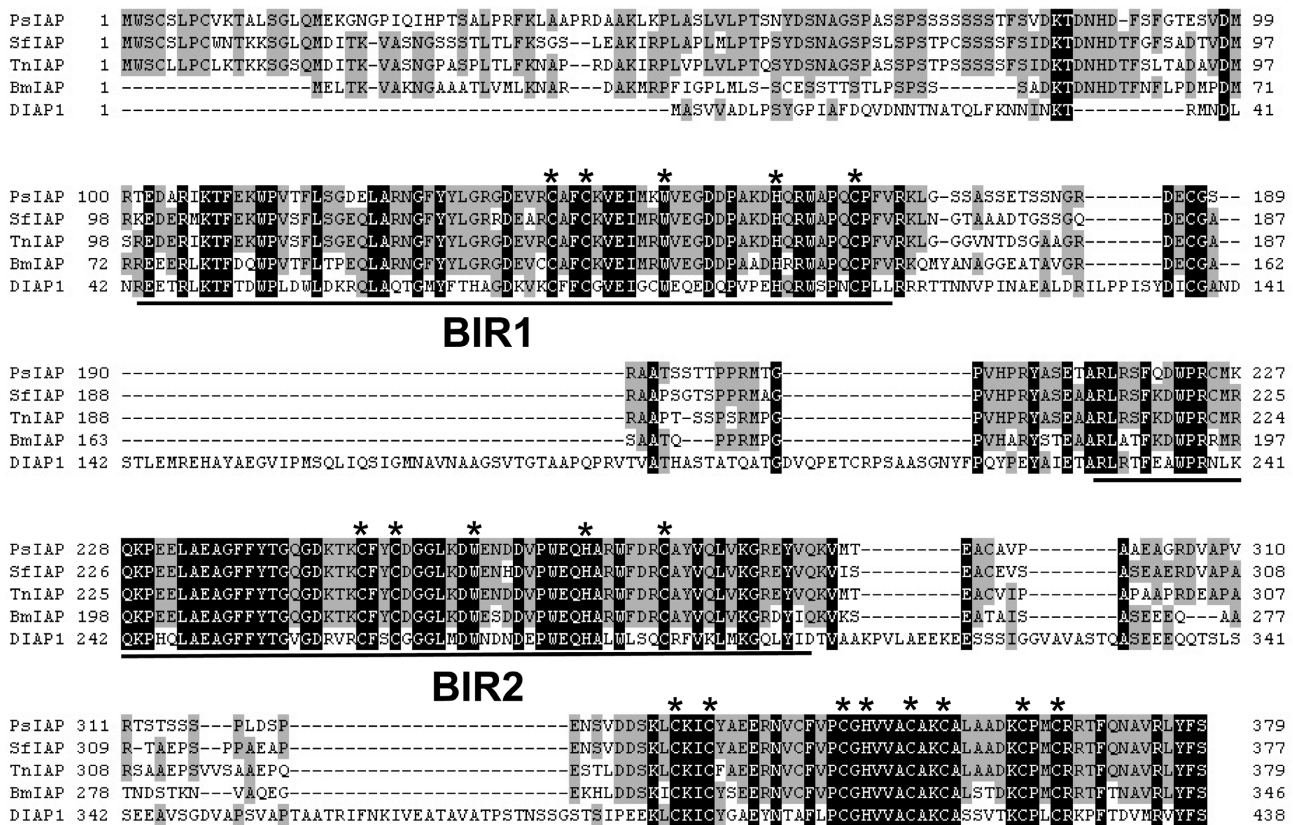
RESULTS

cDNA sequence determination and analyses of *MsIAP*

The full cDNA sequence of *MsIAP* was obtained by a combination of PCR-based approaches. The *MsIAP* cDNA has 1642 nucleotides and encodes a polypeptide of 379 amino acid residues with a calculated molecular mass of 41,834 Da (Fig. 1).

The predicted amino acid sequence of *MsIAP* was aligned with the insect IAP amino acid sequences for *Spodoptera frugiperda* (Smith) IAP (SfIAP, accession no. AAF35285) (Huang et al., 2000), *Tricoplusia ni* (Hübner) IAP (TnIAP, accession no. AF195528) (Liao et al., 2002), *Bombyx mori* (Linnaeus) IAP (BmIAP, accession no. NP001037024) (Huang et al., 2001), and DIAP1 (accession no. Q24306),

A



B

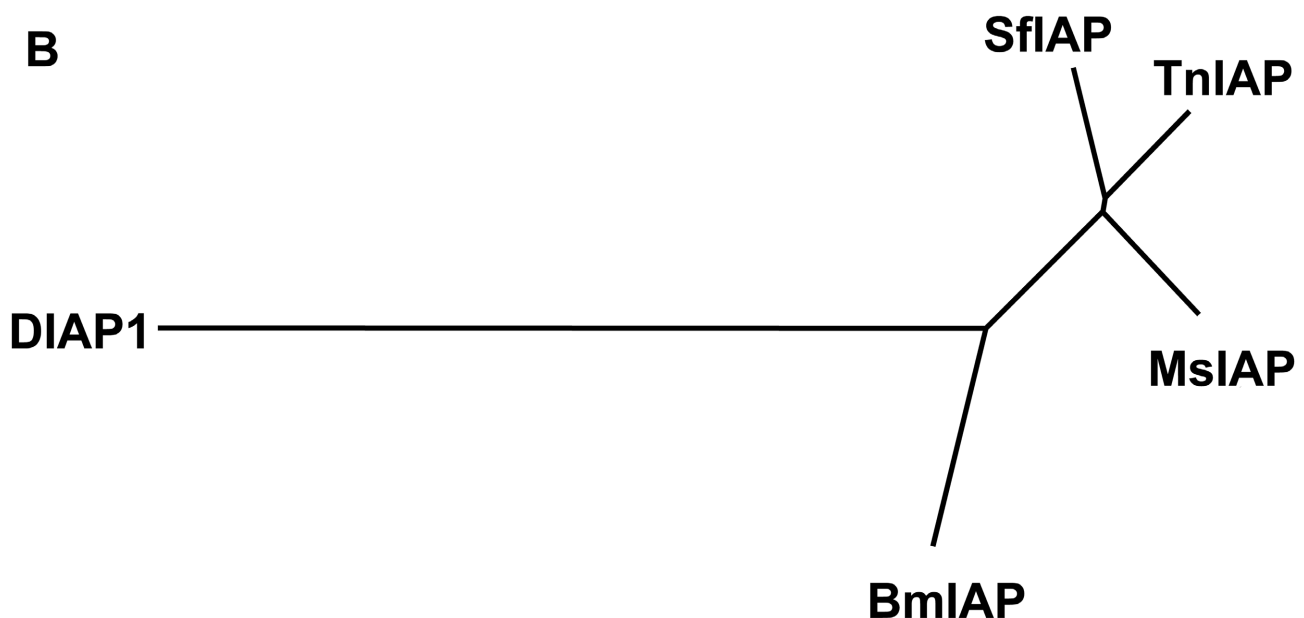


Fig. 2. Amino acid sequence alignment (A) and phylogenetic tree (B) of MsiIAP and known insect IAPs. The *Spodoptera frugiperda* IAP (SfiIAP, accession no. AAF35285), *Tricoplusia ni* IAP (TnIAP, accession no. AF195528), *Bombyx mori* IAP (BmIAP, accession no. NP001037024) and *Drosophila melanogaster* DIAP1 (accession no. Q24306) amino acid sequences were aligned together with MsiIAP sequence using the Clustal W algorithm. In (A), residue numbers are shown on both sides of each line. Gaps introduced are indicated by hyphens. Two BIR domains and one RING domain are marked by thick horizontal bars. The amino acid residues conserved in all (five) sequences are shown in white letters on a black background while those found in three or four sequences are on a grey background. Asterisks indicate important residues in each domain. In (B), is the phylogenetic tree constructed based on sequence alignment data. The length of each branch represents the degree of difference between each sequence.

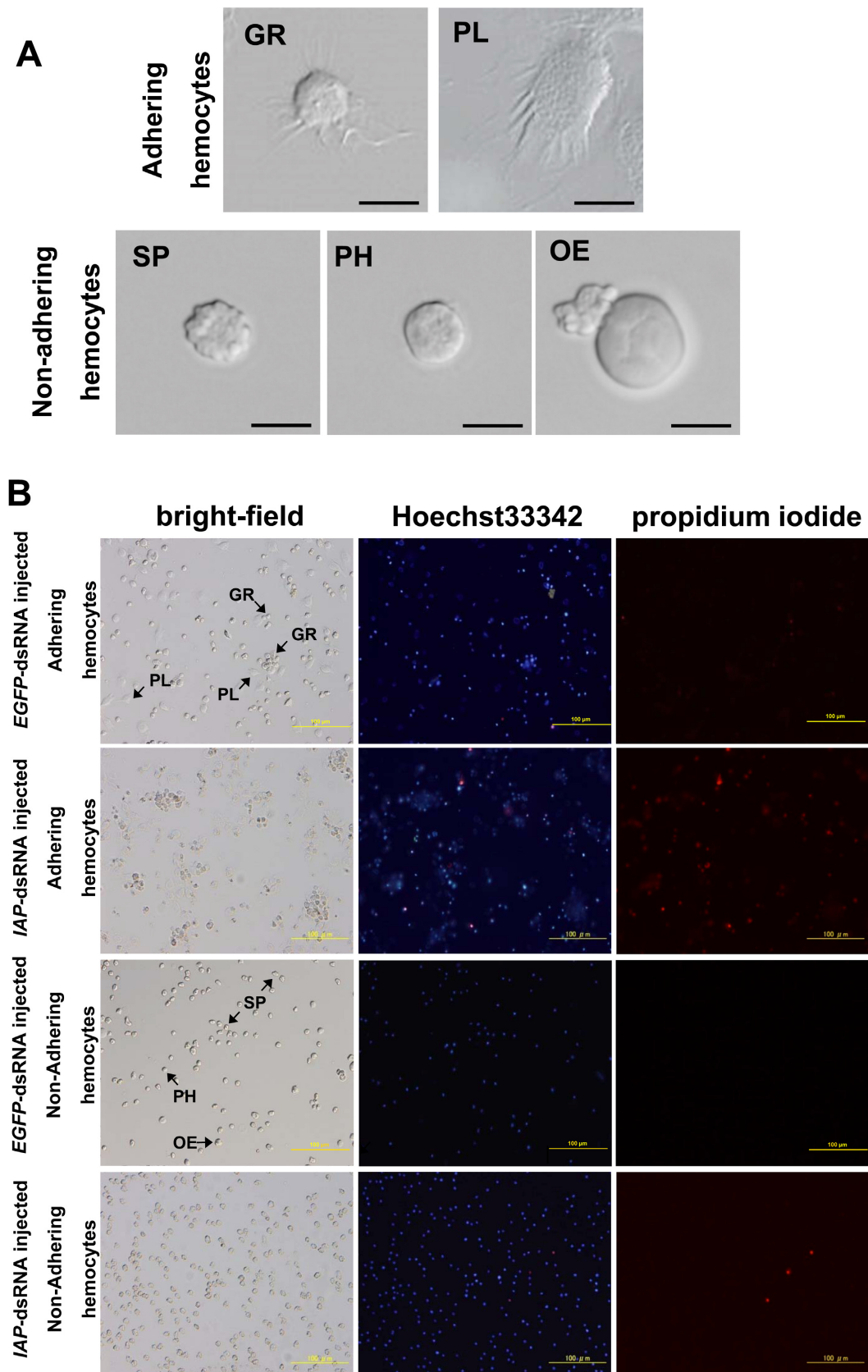


Fig. 3. Hemocyte viability after dsRNA treatment. A – Images of *M. separata* hemocyte species show adhering hemocytes, such as granulocytes (GR) and plasmatocytes (PL), and non-adhering hemocytes, such as spherulocytes (SP), prohemocytes (PH) and oenocytoids (OE), under high magnification with 10- μ m scale bars. An adjacent SP is also present in the OE panel. B – Isolated larval abdomens were injected with dsRNA of either *EGFP* (top and third from top panels) or *MsiAP* (second from top and bottom panels). Forty-eight hours later, adhering and non-adhering hemocyte preparations were double-stained with Hoechst33342 and PI. Adhering hemocyte species with typical shapes are marked in the two upper left panels. Horizontal bars indicate a length of 100 μ m. Note that dense PI staining of adhering hemocytes from *MsiAP* dsRNA-injected larvae was recorded.

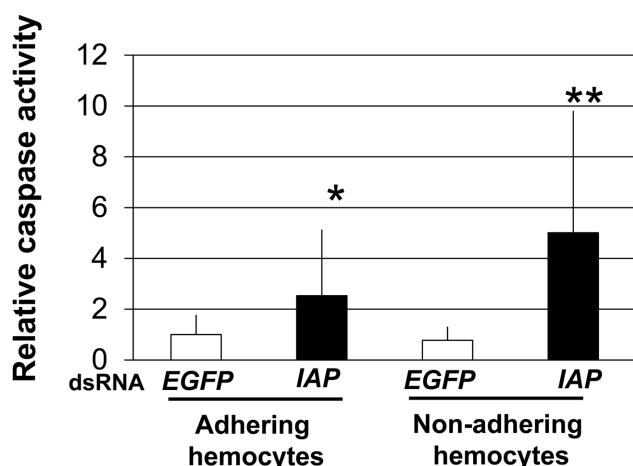


Fig. 4. Caspase activity in hemocytes treated with *MsIAP* or *EGFP* dsRNA. Isolated larval abdomens were treated as in Fig. 3, and caspase-3/7 activity was determined in adhering and non-adhering hemocyte preparations 48 h post-dsRNA injection. There were 6–8 biological replicates in each sample. Each value is calculated relative to the average value of adhering hemocytes from *EGFP* dsRNA-injected larvae, which was set to 1. Each vertical bar is a mean \pm S.D. Asterisk and double asterisks indicate $P = 0.09$ using Student's *t*-test and $P = 0.03$ using Mann-Whitney U test, respectively.

which is the most similar to Lepidopteran IAPs (Hay et al., 1995) (Fig. 2A), and the phylogenetic tree was constructed using sequence alignment (Fig. 2B). The InterPro search of the predicted *MsIAP* revealed two BIR (BIR1 and BIR2 in Fig. 2A) domains and one RING domain (Hunter et al., 2009), and the sequences of these domains of *MsIAP* are similar to that in other insect IAPs. Lepidopteran IAPs formed a cluster in the phylogenetic tree except for *DIAP1*, which indicates that lepidopteran IAP sequences are conserved (Fig. 2B).

Effects of *MsIAP* dsRNA injection on hemocyte viability

Hemocytes were double-stained with Hoechst33342 and PI and observed using fluorescence microscopy. We counted the number of hemocytes double-stained with Hoechst33342 and PI or stained only with Hoechst33342 (Table 1). Typical images of the five hemocyte species are shown in Fig. 3A. Hemocyte spreading was observed in adhering hemocytes, such as granulocytes and plasmatocytes, but not in non-adhering hemocytes such as spherulocytes, prohemocytes and oenocytoids. Typical images of adhering and non-adhering hemocytes from isolated abdomens treated with *EGFP* or *MsIAP* dsRNA are shown in Fig. 3B. Adhering hemocytes consisted mainly of granulocytes and plasmatocytes, and non-adhering hemocytes consisted mainly of spherulocytes, prohemocytes and oenocytoids as indicated in the bright field images in Fig. 3B. For adhering hemocytes from *EGFP* dsRNA injected larvae, only a few hemocytes were PI-positive (dead or late apoptotic) (Fig. 3B, top panels and Table 1). *MsIAP* knockdown increased the number of PI-stained adhering hemocytes but not the number of non-adhering hemocytes (Fig. 3B, the second panels from top and Table 1). The numbers of Hoechst33342-positive and PI-positive hemo-

Table 1. Numbers of Hoechst33342-positive and propidium iodide positive hemocytes in *dsIAP*-injected and *dsEGFP*-injected larvae.

Sample larvae replicate	No. of Hoechst33342-positive hemocytes	No. of propidium iodide-positive hemocytes	Biological replicates
(A) Adhering hemocytes			
<i>dsEGFP</i> -injected	1834	188	8
<i>dsIAP</i> -injected	3632	1586	12
$P < 2.2 \times 10^{-16}$ by Fisher's Exact Test.			
(B) Non-adhering hemocytes			
<i>dsEGFP</i> -injected	455	40	6
<i>dsIAP</i> -injected	800	41	7
$P = 0.9956$ by Fisher's Exact Test.			

cytes in each sample are shown in Table 1. Thus, we confirmed that injection of *MsIAP* dsRNA into isolated larval abdomens caused cell death of adhering hemocyte species. For non-adhering hemocytes there was no significant difference between the *MsIAP* dsRNA treatment and control samples (Fig. 3B, the third from top and bottom panels, and Table 1).

dsRNA treatment and caspase activity

MsIAP dsRNA treatment disrupted plasma membrane integrity and caused cell death in adhering hemocytes (Fig. 3), which indicates that knockdown of *MsIAP* induced apoptosis in these cells. We determined the activity of two effector caspases, caspase-3 and caspase-7, in the apoptosis of hemocytes. Adhering and non-adhering hemocyte fractions were prepared from isolated larval abdomens treated with either *EGFP* or *MsIAP* dsRNA. Caspase activity in adhering hemocytes from *MsIAP* dsRNA-treated larvae was approximately 2-fold higher. A test of normality for the two samples was performed, and the P values of *MsIAP* dsRNA-treated and control samples were 0.9977 and 0.8562, respectively. Therefore, we tested the difference in caspase activities using a Student's *t*-test. At the 10% significance level, a significant difference in caspase activities was recorded, which is consistent with the results of the Hoechst/PI staining (Fig. 4). Surprisingly, *MsIAP* dsRNA treatment increased caspase activity in non-adhering hemocytes even though these hemocytes were PI-negative and a significant RNAi knockdown was not achieved (Fig. 4). The test of normality of the results for the *MsIAP* and dsRNA-treated and control samples had P values of 0.6296 and 0.9804, respectively. Therefore, a Mann-Whitney U test was used because significant normality in the *MsIAP* dsRNA-treated sample was not detected. A significant difference in caspase activities between the two categories was detected at the 10% significance level.

TUNEL staining

To further characterize the type of cell death caused by *MsIAP* knockdown, hemocytes treated with dsRNAs were subjected to TUNEL staining, which detects genomic DNA fragmentation. Typical images of TUNEL staining for each sample are shown in Fig. 5. The results were similar to those obtained using PI staining (Fig. 3). Only adhering hemocyte samples prepared from *MsIAP* dsRNA-treated

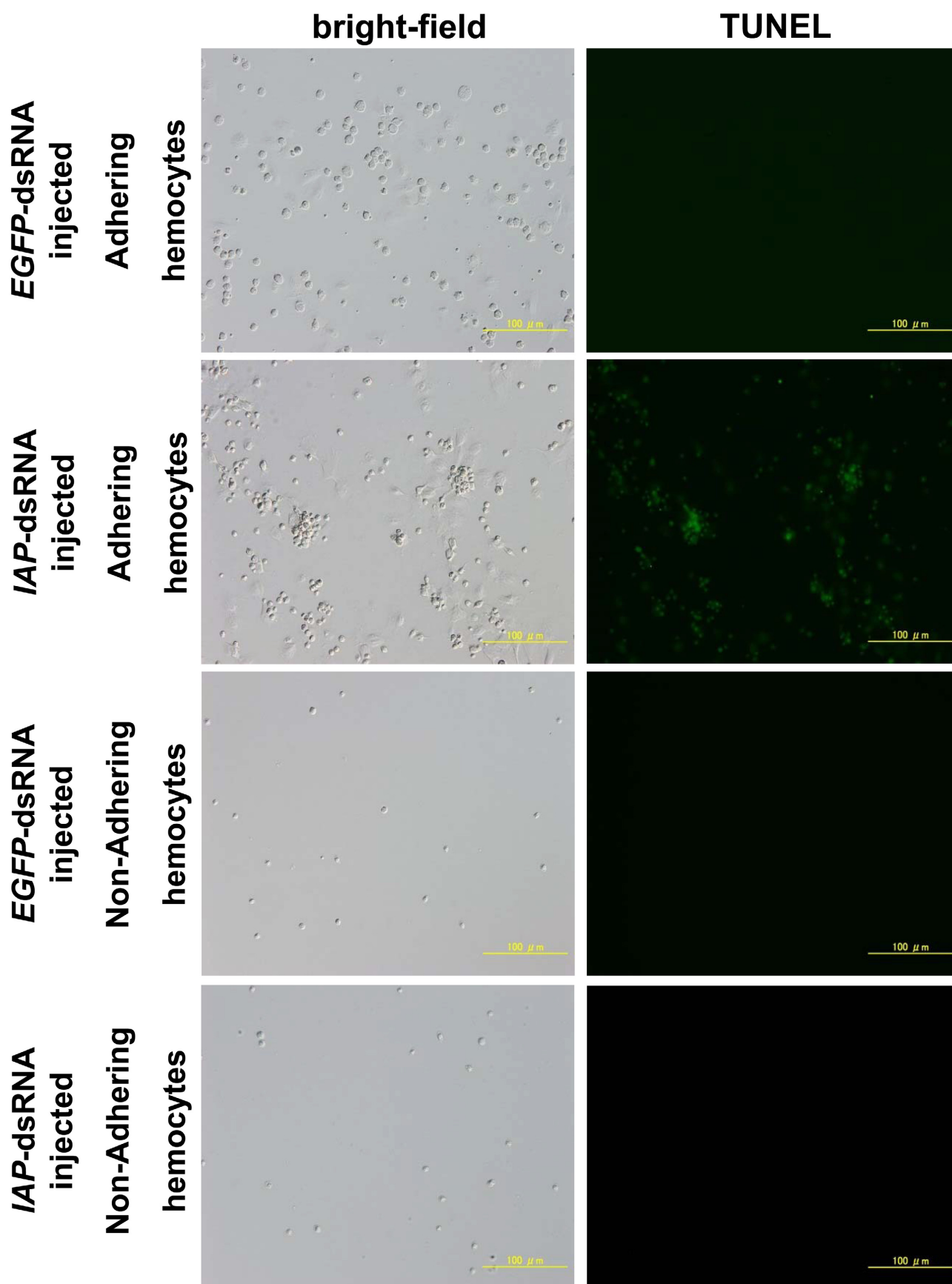


Fig. 5. TUNEL staining of hemocytes treated with *MsIAP* or *EGFP* dsRNA. Isolated abdomens of larvae were injected with either *EGFP* (top and third from top panels) or *MsIAP* dsRNA (second from top and bottom panels). Adhering and non-adhering hemocytes were prepared 48 h later and stained for fragmented genomic DNA using TUNEL.

larvae had a high proportion of TUNEL-positive cells and other samples were TUNEL-negative. In addition, we counted the total and TUNEL-positive hemocyte numbers and present the ratio of TUNEL-positive hemocytes in Fig. 5. The ratio of the number of hemocyte cells to the number of TUNEL-positive cells in adhering hemocytes from ds*EGFP*-injected larvae and adhering hemocytes from ds*MsIAP*-injected larvae were 310/0 and 301/256, respectively. The ratio of the number of hemocyte cells to the number of TUNEL-positive cells in non-adhering hemocytes from ds*EGFP*-injected larvae and non-adhering hemocytes from ds*MsIAP*-injected larvae were 22/0 and 26/0, respectively. Thus, TUNEL-positive cells were recorded only in adhering hemocytes treated with *MsIAP* dsRNA.

DISCUSSION

We determined the full nucleotide sequence of *MsIAP* and whether it functioned as a negative regulator of apoptosis using RNAi. Sequence analysis revealed that *MsIAP* had two BIR domains and one RING domain. A phylogenetic tree indicated that the amino acid sequence of *MsIAP* is similar to that of other lepidopteran IAP amino acid sequences. The zinc-binding BIR domain serves as an interface for protein-protein interactions, especially with caspases (Salvesen & Duckett, 2002). The BIR1 and BIR2 domains of *MsIAP* both had the canonical pattern of three cysteines and one histidine, CX₂CX₆WX₉HX₆C (Deveraux & Reed, 1999), similar to that in other insect IAPs. The RING domain is also found in IAPs, and IAPs with RING domains have ubiquitin protein ligase (E3) activity. The *MsIAP* RING domain has a C3HC4 pattern, which is conserved in other insect IAPs. Binding caspases undergo ubiquitination by the C-terminal RING domain, which has ubiquitin ligase activity (Meier et al., 2000; Riedl & Shi, 2004; Vaux & Silke, 2005). In lepidopteran insects, a few IAPs have been investigated, and all of them have two BIR domains and one RING domain, except for *Bombyx* IAP2 that has three BIR domains (Seshagiri et al., 1999; Huang et al., 2000, 2001; Vilaplana et al., 2007; Zhang et al., 2010). *S. frugiperda* and *B. mori* IAPs inhibit mammalian initiator caspase-9 but not effector caspase-3 or caspase-7 (Huang et al., 2000, 2001). In *Drosophila*, DIAP1 is essential for cell survival because the loss of DIAP1 leads to spontaneous initiation of apoptosis (Wang et al., 1999). DIAP1 also has two BIR domains, which are functionally distinct from each other. BIR1 mediates direct physical interaction with effector caspases, drICE and DCP-1, and BIR2 provides an interface for binding to the initiator caspase DRONC. Our sequence alignment and phylogenetic tree results reveal that *MsIAP* is similar to other lepidopteran IAPs, which indicates that *MsIAP* has the same target specificity as the IAPs of *S. frugiperda* and *B. mori*.

Because of the reduced mRNA levels of *MsIAP* in adhering hemocytes from isolated abdomens treated with *MsIAP* dsRNA (Yokoi et al., 2013), we determined viability using Hoechst/PI staining. Dead cells or cells with damaged plasma membranes were frequent among the ad-

hering hemocytes from *MsIAP* dsRNA-injected isolated larval abdomens, and less so in samples from controls, which indicates that knockdown of *MsIAP* induces cell death in adhering hemocytes. We determined the activity of caspase-3 and caspase-7 using an apoptosis-specific TUNEL assay. Contrary to our expectations, the activities of effector caspase-3 and caspase-7 were higher in both adhering and non-adhering hemocytes from *MsIAP* dsRNA-treated larvae compared to those from controls. Apoptotic pathways in mammals and *Drosophila* are well conserved (Riedl & Shi, 2004) so it is likely that the apoptotic pathways in *M. separata* are also conserved. Initiator caspase activation leads to effector caspase activation in mammals and *Drosophila*. Thus, the higher activities of caspase-3 and caspase-7 in adhering hemocytes from larvae treated with *MsIAP* dsRNA could be due to activation of the *M. separata* caspase-9 homologue, which is a potential target of *MsIAP*. The percentage of TUNEL-stained adhering hemocytes recorded for *MsIAP* dsRNA-injected larvae was comparable to that of PI-positive cells, which clearly indicates that knockdown of *MsIAP* in hemocytes induced apoptosis. These results indicate that *MsIAP* negatively regulates apoptosis and that the function of IAP is conserved in mice, *Drosophila* and lepidopteran insects.

In mice, gene disruption of *XIAP* results in no major cell death phenotypes because its effect is compensated for by the upregulation of the related genes, *cIAP1* and *cIAP2* (Harlin et al., 2001). In mutant DIAP-deficient flies, cell death and death of embryos are reported (Martin, 2002), which indicates that DIAP is indispensable for cell survival. In the case of *M. separata*, knockdown of *MsIAP* induced apoptosis in adhering hemocytes, which indicates that *MsIAP* is a non-redundant factor that prevents unwanted apoptosis in this species.

Although injection of *MsIAP* dsRNA into isolated abdomens did not alter the amount of *MsIAP* mRNA in non-adhering hemocytes (Yokoi et al., 2013), a significantly higher activity of caspase-3 and caspase-7 was recorded. Higher activation of caspases may be due to an immune reaction. Caspases are involved in innate immune reactions in mammals (Meunier et al., 2014). Fig. 5 shows that numerous apoptotic adhering hemocytes from *MsIAP* knockdown occurred in hemolymph from isolated abdomens treated with *MsIAP* dsRNA. The immune reaction in non-adhering hemocytes may be induced by these apoptotic hemocytes, which activates caspases in the immune reaction by an unidentified mechanism.

In conclusion, we found an IAP orthologue in *M. separata* and the amino acid sequence of *MsIAP* is similar to that of other lepidopteran IAPs. In addition, we found that *MsIAP* functioned as a negative and non-redundant regulator of apoptosis, which indicates that the functions of IAP are conserved between *Drosophila* and lepidopteran insects including *M. separata*.

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