Interaction of Drosophila Inhibitors of Apoptosis with Thick Veins, a Type I Serine/Thrreonine Kinase Receptor for Decapentaplegic*

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Decapentaplegic (Dpp) is a Drosophila member of bone morphogenetic proteins, which belong to the transforming growth factor-β superfamily. Members of this family regulate a variety of biological processes such as cell proliferation, morphogenesis, immune response, and apoptosis. Dpp plays a critical role in many aspects of Drosophila development. Members of the transforming growth factor-β superfamily bind to two different types of serine/threonine kinase receptors, termed type I and type II. Type I receptors act as downstream components of type II receptors in the receptor complexes. Therefore, intracellular proteins that interact with the type I receptors are likely to play important roles in signaling. Several proteins have been identified through protein-protein interaction screenings. We identified Drosophila inhibitor of apoptosis (DIAP) I as an interacting protein of a Dpp type I receptor, Thick veins (Tkv). DIAP1 associates with Tkv in vivo. The binding region in DIAP1 is mapped to its C-terminal RING finger region. DIAP2, another Drosophila member of the inhibitor of apoptosis protein family, also interacts with Tkv in vivo. These data suggest that DIAP1 and DIAP2 may be involved, possibly as negative regulators, in the Dpp signaling pathway, which leads to cell apoptosis.

Transforming growth factor-βs (TGF-βs),* bone morpho-

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† The abbreviations used are: TGF, transforming growth factor; BMP, bone morphogenetic protein; TβR, TGF-β receptor; IAP, inhibitor of apoptosis; DIAP, Drosophila IAP; BIR, baculovirus IAP repeat; ICE, interleukin-1β converting enzyme; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.
EXPERIMENTAL PROCEDURES

Plasmid Construction—The bait plasmid, pEG-Tkv, encoding the fusion protein of the LexA DNA-binding domain and the cytoplasmic region of Tkv was constructed as follows. The cytoplasmic region of Tkv was amplified by polymerase chain reaction (PCR) from the full-length clone, Brk25D2 (20), and inserted between the EcoRI and XhoI sites of pEG202 (21). The prey plasmid of Tkv, pQ-Tkv, was constructed by inserting the EcoRI-XhoI fragment into pG4-5 (21). Tkv mutants were constructed by site-directed mutagenesis using the Chameleon mutagenesis kit (Stratagene). Tkv (ΔJM) lacks the juxtamembrane region (amino acids 205–254) of the wild type Tkv. Tkv (Q253D) and Tkv (K281R) have aspartic acid instead of glutamine 253 and arginine instead of lysine 281, respectively. pcDNA3-HA was made by inserting an annealed oligonucleotide between the XhoI and XhoI sites of pcDNA3 (Invitrogen). pcDNA3-FLAG was described (11). The whole coding region of Tkv was amplified by PCR with an EcoRI and an XhoI site added before the starting codon and in place of the stop codon, respectively, followed by insertion between the EcoRI and XhoI sites of pcDNA-HA. The internal EcoRI site of Tkv was removed by site-directed mutagenesis. The yeast expression plasmids of DIAP1 were made by subcloning the EcoRI-XhoI fragment amplified by PCR into pEG202 and pJG4-5. The coding region of DIAP1 without the stop codon was subcloned between the EcoRI and XhoI sites of pcDNA3-FLAG, yielding FLAG-tagged DIAP1. The DIAP2 plasmids were constructed in a similar manner to DIAP1. The details of the plasmid construction including the oligonucleotide sequences are available upon request. All of the PCR products were sequenced.

Screening and Interaction Assay—A Drosophila imaginal disc cDNA expression library (gift of R. Brent) was screened using the interaction trap (21) exactly as described (14). Briefly, the yeast strain, EGY48, was transformed with the reporter, pSH18–34 (21), and pEG-Tkv. The cDNA library was then introduced into EGY48. The transformants were grown on appropriate selection medium, and positive clones were selected depending on β-galactosidase activity and leucine prototrophy. Library plasmids were rescued from EGY48, amplified in bacteria, and sequenced. Interaction assays using the interaction trap were done as described before (14).

Cloning of the Full-length DIAP1—One of the positive clones contained a partial C-terminal region of DIAP1 (see Fig. 1A). PCR was performed under a standard condition to amplify the missing N-terminal region from a Drosophila 4–8-h-old embryo cdNA library in the pNB40 vector (gift of Y. Nishida). The full coding region of DIAP1 was made by ligating the EcoRI-BanII fragment obtained from PCR and the BanII-XhoI fragment obtained from the interaction trap screen.

Protein Interaction in Vivo—Cos-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 4.5 mM glucose. Cells were transiently transfected using DMRIE-C (Life Technologies, Inc.) with 10 μg of plasmids. After 2 days, cells were labeled with 22.8 mCi/ml [35S]methionine and [35S]cysteine mixture (Amersham Pharmacia Biotech) for 5 h and lysed in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 1% Triton X-100 containing aprotinin. Cleared lysates were divided into two tubes and incubated with anti-FLAG M2 (Eastman Kodak) or anti-HA 12CA5 (Boehringer Mannheim) monoclonal antibodies. Immune complexes were washed and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (8.5% or 10% gel) and analyzed with Fuji BAS 2000 Bio Imaging Analyzer (Fuji Photo Film).

RESULTS

To search for proteins that interact with Tkv, we employed the interaction trap screen developed by Brent and co-workers (21). A Drosophila imaginal disc cDNA library was screened with the cytoplasmic region of Tkv as a bait. 160,000 transformants were screened. Finally, four positive clones were isolated. One clone encoded Drosophila FKB12, a homolog of human FKBP12 that is known as a binding protein for mammalian type I receptors, including TβRI (9, 11, 13) (data not shown). One of the other clones, termed PC1, encoded a partial C-terminal region of DIAP1 (Fig. 1A), which is a homolog of baculovirus IAP (19). The remaining two positive clones were not analyzed. PCR using a Drosophila 4–8-h-old embryo cDNA library was performed to obtain the missing N-terminal region.

We then examined the interaction of the full-length DIAP1 with Tkv using the interaction trap (Fig. 1B). DIAP1 strongly interacted with the wild type Tkv, although its interaction was slightly weaker than that of the partial clone, PC1. Mutants of Tkv with different signaling activities were also tested for the interaction with DIAP1. One Tkv mutant replacing glutamine 253 with aspartic acid (QD), reported to have a constitutively active form of Tkv with different signaling activities, were also tested for the interaction with DIAP1. One Tkv mutant replacing lysine 281 with arginine (KR), which is expected to lack the kinase activity (8), showed weak interaction with DIAP1 and PC1. We also tested a deletion mutant lacking the juxtamembrane region (amino acid positions). PC1 is the original DIAP1 clone obtained from the interaction trap screen. B, various combinations of a bait (receptors) and a prey (DIAPs) with a reporter plasmid were introduced into EGY48. Three independent colonies from each transformation were streaked onto an indicator plate to assay the β-galactosidase activities. Strength of interaction was evaluated by the relative intensity of blue color among colonies. The mutants of Tkv are described in the text. C, interaction of the truncated forms of DIAP1 with Dpp receptors. ND, not done.

FIG. 1. Interaction of DIAPs with Dpp receptors in yeast. A, shown is the schematic representation of DIAPs and the truncated forms of DIAP1 used in the following experiments. Numbers indicate amino acid positions. PC1 is the original DIAP1 clone obtained from the interaction trap screen. B, various combinations of a bait (receptors) and a prey (DIAPs) with a reporter plasmid were introduced into EGY48. Three independent colonies from each transformation were streaked onto an indicator plate to assay the β-galactosidase activities. Strength of interaction was evaluated by the relative intensity of blue color among colonies. The mutants of Tkv are described in the text. C, interaction of the truncated forms of DIAP1 with Dpp receptors. ND, not done.
acids 205–254) where probable transphosphorylation sites by the type II receptor exist (8). The mutant, Tkv (ΔJM), did not interact with PC1 or DIAP1. Contrary to Tkv, Saxophone (Sax), another Dpp type I receptor (20, 23–25) or Punt, a type II receptor for Dpp (26–28), did not interact with PC1 or DIAP1. Thus, DIAP1 specifically interacted with Tkv in the yeast system.

In Drosophila, two types of IAP are known (19). DIAP2, another Drosophila inhibitor of apoptosis, has three baculovirus IAP repeat (BIR) domains, whereas DIAP1 has two BIR domains (Fig. 1A). We tested whether DIAP2 interacts with Tkv. DIAP2 did not show interaction with the wild type or mutants of Tkv, Sax, or Punt in the yeast system (Fig. 1B). To identify the interacting region of DIAP1 with Tkv, we constructed various truncated forms (TF) of DIAP1 (Fig. 1A). The wild type Tkv showed interaction with TF4 that has only the RING finger domain but not with the other truncated forms (Fig. 1C). Thus, the interacting region in DIAP1 is mapped to the RING finger domain. Sax and Punt did not interact with TF4.

We next examined the interaction between DIAP1 and Tkv in vivo. DIAP1 was epitope-tagged with FLAG at the C terminus (DIAP1-FLAG). HA-tagged Tkv and/or DIAP1-FLAG were transiently expressed in COS-7 cells. Labeled lysates were immunoprecipitated with anti-HA or anti-FLAG monoclonal antibodies and then subjected to SDS-PAGE (Fig. 2A). Each antibody specifically recognized Tkv-HA and DIAP1-FLAG, respectively (Fig. 2A, fourth and seventh lanes from the left). Anti-FLAG antibody coprecipitated Tkv only when DIAP1 was expressed, demonstrating that DIAP1 interacts with Tkv in vivo (Fig. 2A, ninth lane from the left). Both constitutively active (QD) and kinase-inactive (KR) mutants interacted with DIAP1 as efficiently as the wild type Tkv (Fig. 2A, eleventh and thirteenth lanes from the left). The reason for the difference between this result in COS-7 cells and that in the yeast assay (Fig. 1B) is not known, but the difference in mammalian cells and in yeast was also observed in the interaction of FKBP12 with TβR-I (11).

We also tested the interaction of DIAP2 with Tkv in vivo. Tkv-HA and/or DIAP2-FLAG were transiently transfected in COS-7 cells (Fig. 2B). Although the interaction of DIAP2 with Tkv was not detected in the yeast assay (Fig. 1B), DIAP2 coprecipitated not only with the wild type Tkv but also with the QD and KR mutants in vivo.

To determine the region of DIAP1 required for the interaction with Tkv in vivo, expression plasmids of the BIR domain (BIR-FLAG) and C-terminal region of DIAP1 (PC1-FLAG) were constructed (Fig. 3A). Tkv-HA was coexpressed with BIR-FLAG or PC1-FLAG (Fig. 3B). Tkv-HA was detected in a stable complex only with PC1-FLAG but not with BIR-FLAG (Fig. 3B, eleventh and thirteenth lanes from the left). These results indicate that the interaction region of DIAP1 is the C terminus, which contains the RING finger domain, consistent with the results in the yeast assay. TF4-FLAG that has only the RING finger domain was tested, but the expression level was too low to detect interaction (data not shown).

DISCUSSION

Members of the TGF-β superfamily regulate growth, differentiation, and apoptosis of various cell types. Recent data have clearly shown that Smad proteins transduce signals for the TGF-β superfamily proteins; however, it is likely that other signaling pathways may exist for the TGF-β superfamily. BMPs and TGF-β have been reported to induce apoptosis both in vitro and in vivo (29, 30). More recently, caspase family proteases, such as interleukin-1β converting enzyme (ICE), are involved in the apoptosis signals for the TGF-β superfamily (30). However, it is not yet known whether Smads are involved in the apoptosis pathway.

DIAP1 is a homolog of the baculovirus IAP that prevents cell apoptosis when virus infects cells. Recently, an increasing number of IAPs have been reported in virus (OpIAP, CpIAP) (31, 32), Drosophila (DIAP1, DIAP2/DIAP/DIHA/dILP) (33–35).
35), mouse (MIHA, mc-IAP-1) (34, 36), and human (XIAP/hILP, MIHB/c-IAP1/hIAP2, MIHC/c-IAP2/hIAP1, neuronal apoptosis inhibitory protein) (33–35). IAPs share conserved regions, i.e., two or three BIR domains at the N-terminal region and one RING finger domain at the C-terminal region, with an exception of neuronal apoptosis inhibitory protein that has three BIR domains but not the RING finger domain. It has been reported that IAPs are able to prevent cell apoptosis induced by ICE (34, 35, 37), although direct interaction of IAPs with ICE has not been shown.

Because IAPs prevent apoptosis of the cells, proteins that interact with IAPs seem to play important roles for cell death signals. Tumor necrosis factor receptor-associated factor 1 and 2 (36), and tumor necrosis factor receptor-1-associated death domain protein, a 34-kDa cytoplasmic protein containing a C-terminal death domain (38) were shown to associate with mammalian IAPs. *Drosophila* Doom, which induces apoptosis in insect cells, interacts with viral IAPs (39). Reaper is a small polypeptide with 65 amino acids that induces apoptosis in *Drosophila*. DIAP2 prevents Reaper-induced cell death by binding to it (40). All of the five proteins above bind to IAPs through the BIR domains, whereas Tkv associates with DIAP1 through the RING finger domain.

Mutations of DIAP1 enhanced apoptosis caused by Reaper, whereas overexpression of DIAPs in *Drosophila* eyes suppressed normally occurring cell death, causing rough eye phenotype (19). Tkv QD mutant induces rough eye (22), suggesting a possible involvement of Tkv in the apoptosis signal pathway. Our results suggest that Tkv may induce apoptosis by suppressing the DIAP1 function. Future studies will be directed to investigate how the interaction between Tkv and DIAP1 is regulated, which molecules are targets of Tkv-DIAP1 pathway, and whether Smad proteins are linked to the signaling activity of DIAP1.

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