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Boo, a novel negative regulator of cell death, interacts with Apaf-1

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In this report, we describe the cloning and characterization of Boo, a novel anti-apoptotic member of the Bcl-2 family. The expression of Boo was highly restricted to the ovary and epididymis implicating it in the control of ovarian atresia and sperm maturation. Boo contains the conserved BH1 and BH2 domains, but lacks the BH3 motif. Like Bcl-2, Boo possesses a hydrophobic C-terminus and localizes to intracellular membranes. Boo also has an N-terminal region with strong homology to the BH4 domain found to be important for the function of some anti-apoptotic Bcl-2 homologues. Chromosomal localization analysis assigned Boo to murine chromosome 9 at band d9. Boo inhibits apoptosis, homodimerizes or heterodimerizes with some death-promoting and -suppressing Bcl-2 family members. More importantly, Boo interacts with Apaf-1 and forms a multimeric protein complex with Apaf-1 and caspase-9. Bak and Bik, two pro-apoptotic homologues disrupt the association of Boo and Apaf-1. Furthermore, Boo binds to three distinct regions of Apaf-1. These results demonstrate the evolutionarily conserved nature of the mechanisms of apoptosis. Like Ced-9, the mammalian homologues Boo and Bcl-x_L interact with the human counterpart of Ced-4, Apaf-1, and thereby regulate apoptosis.

Keywords: Apaf-1/apoptosis/Boo/caspase-9/ovary

Introduction

Apoptosis, a morphologically distinct form of cell death, is important for normal development, tissue homeostasis and defense against pathogenic microorganisms (Raff, 1992; Vaux *et al*., 1994). Perturbations of apoptosis can lead to a number of human pathologies such as cancer, auto-immune disease and neurodegenerative disorders (Thompson, 1995).

Despite the diversity of stimuli triggering apoptosis and the wide range of cell types involved, the apoptotic death machinery is evolutionarily conserved from nematodes to mammals. Genetic studies in the nematode *Caenorhabditis elegans* have identified three core components of the cell-death machine: *ced-3*, *ced-4* and *ced-9* (Hengartner and Horvitz, 1994c). *ced-3* and *ced-4* are required for the execution of the cell-death program and loss-of-function mutations in either gene result in the survival of all 131 somatic cells that normally die (Ellis and Horvitz, 1986). *ced-9*, in contrast, is a negative regulator of apoptosis and loss-of-function mutations in *ced-9* cause embryonic lethality as a consequence of ectopic cell death (Hengartner *et al*., 1992). *ced-3* encodes a cysteine protease with aspartic acid specificity and is a relative of a family of caspases (Yuan *et al*., 1993; Kumar and Harvey, 1995) that appear to act as the effectors of the cell-death pathway (Chinnaiyan and Dixit, 1996; Henkart, 1996). These death proteases exist as zymogens which are activated when the regulatory prodomain is removed and they assemble into active heteromeric proteases (Cohen, 1997). The caspase family now comprises 13 known members which can be divided into two classes based on the lengths of their N-terminal prodomains (Humke *et al*., 1998). Accumulating evidence suggests that caspases with long domains, such as caspase-8, and -9, function upstream in the caspase cascade while caspases with short domains operate at the downstream end of the cascade (Cohen, 1997; Golstein, 1997). Activation of these distal caspases leads to proteolytic cleavage of a limited number of key protein substrates and execution of the death program (Cohen, 1997).

The death-repressor gene *ced-9*, like *ced-3*, also has multiple mammalian homologues and its protein product is structurally and functionally homologous to Bcl-2, the prototype member of a family of cell-death regulators (Hengartner and Horvitz, 1994b). First identified for its role in B-cell malignancies, Bcl-2 when overexpressed inhibits apoptotic cell death in diverse biological systems (Reed, 1994). Two functional classes of Bcl-2-related proteins constitute the family: anti-apoptotic members (Bcl-2, Bcl-x_L, Bcl-w, NR-13, A1 and Mcl-1), which inhibit cell death, and pro-apoptotic members (Bax, Bak, Bad, Bik, Bid, Hrk, Bim and Bok/Mtd), which promote apoptosis (Hsu *et al*., 1997; Kroemer, 1997; Inohara *et al*., 1998a; O'Connor *et al*., 1998). Sequence alignments of Bcl-2 family proteins have identified four conserved domains, designated Bcl-2 homology regions, (BH1 to BH4) (Chittenden *et al*., 1995a; Gibson *et al*., 1996; Zha *et al*., 1996). The BH1 and BH2 motifs of the death antagonists (such as Bcl-2 and Bcl-x_L) and the BH3 domain of the death agonists (such as Bax and Bak) are important for homo- or heterodimerization between family members (Yin *et al*., 1994; Chittenden *et al*., 1995b; Simonian *et al*., 1996; Zha *et al*., 1996). The BH4 domain, which is restricted to several anti-apoptotic homologues, appears to be essential for the death-repressing activity (Huang *et al*., 1998). An early model proposed that the balance between pro- and anti-apoptotic Bcl-2 proteins determines cell fate (Oltvai *et al*., 1993; Boise *et al*., 1995; White, 1996; Kroemer, 1997). Contrary to this model, suppression or induction of cell death in some systems is independent of their interactions (Cheng *et al*., 1996, 1997; Simonian *et al*., 1996).

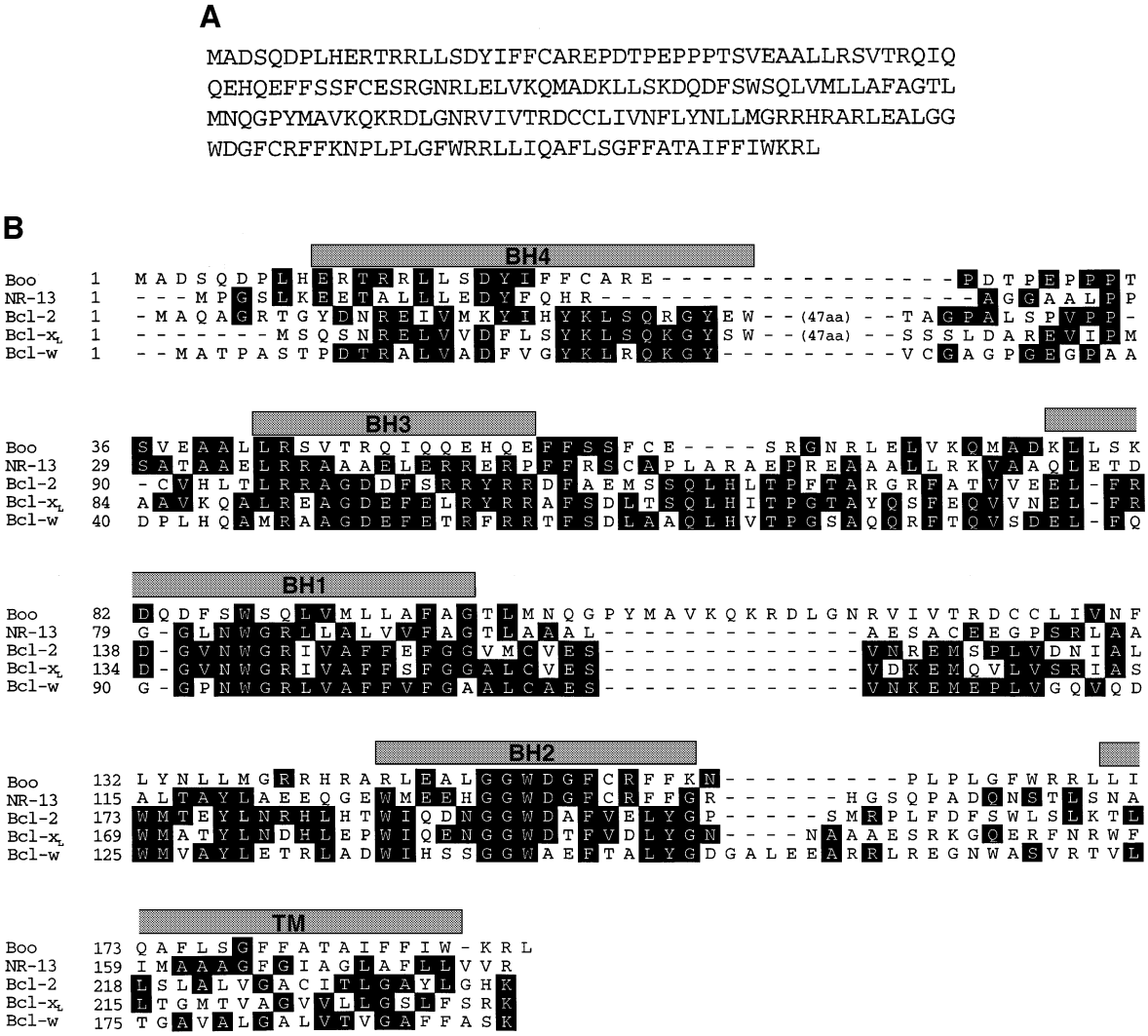

Fig. 1. Sequence characterization of Boo. (**A**) Deduced amino acid sequence of Boo. (**B**) Alignment of Boo amino acid sequence with those of mouse Bcl-2, Bcl-x_L, Bcl-w and chicken NR-13. The numbers on the left indicate the positions of the amino acid in each sequence. The conserved BH domains (1–4) and the C-terminal hydrophobic region (TM, transmembrane) are indicated. Shading marks identical residues. The DDBJ/EMBL/GenBank accession number for Boo is AF102501.

Genetic analysis in *C.elegans* indicates that Ced-9 prevents Ced-4 from activating the nematode caspase Ced-3 (Shaham and Horvitz, 1996a,b). Consistent with this, Bcl-2 and Bcl-x_L have been shown to function upstream of some of the caspases in the cell-death pathway (Armstrong *et al.*, 1996; Chinnaiyan *et al.*, 1996). However, the precise mechanism by which these death antagonists regulate caspase activation and apoptosis remains unclear.

Recently, a connection has been established between members of the Ced-9/Bcl-2 family and caspases. Ced-4 interacts with and promotes the activation of Ced-3, and this activation is inhibited by Ced-9 through direct interaction with Ced-4 (Chinnaiyan *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997a,b). Likewise, a similar mechanism also exists in mammalian cells. Apaf-1, a mammalian counterpart of Ced-4 (Zou *et al.*, 1997), simultaneously and independently binds to Bcl-x_L and caspase-9 (Hu *et al.*, 1998; Pan *et al.*, 1998). Bcl-x_L interacts with Apaf-1 and blocks Apaf-1-dependent caspase-9 activation (Hu *et al.*, 1998), further confirming the high degree of evolutionary conservation in the cell-death machinery.

In the present study, we report the identification and characterization of Boo, for Bcl-2 homologue of ovary, a novel anti-apoptotic member of the Bcl-2 family. Boo was exclusively expressed in the ovary and the epididymis. Boo inhibited apoptosis and interacted with selective pro- and anti-apoptotic Bcl-2 family proteins. Furthermore, Boo bound to Apaf-1 at multiple sites implicating it in the regulation of this central component of the death pathway.

Results

Isolation of Boo, a novel Bcl-2-related gene

In an attempt to identify novel apoptosis-regulatory proteins, we searched the GenBank™ expressed-sequence tag (EST) database for cDNAs encoding proteins with homology to chicken neuroretina (NR-13). Several overlapping clones were identified as having statistically significant homology to NR-13. A clone AA623872 (IMAGE Consortium Clone ID 1108004) was characterized further

and analysis of its nucleotide sequence revealed an open reading frame that encoded a protein of 191 amino acids with a predicted relative molecular mass of 22 300 Da (Figure 1A). We designated this gene as *Boo*. Sequence analysis revealed that Boo was a novel member of the Bcl-2 family with conserved BH1 and BH2 domains (Figure 1B). Several residues conserved in the BH domains of NR-13, Bcl-2 and Bcl-x_L are different in the Boo sequence. For example, Boo has substituted Ser88 and Arg144 for Gly and Trp found at the equivalent position in other anti-apoptotic Bcl-2 family members (Figure 1B). Hydrophilicity analysis indicated that the protein has a C-terminal transmembrane region (Figure 1B) which is expected to mediate intracellular membrane localization (Kroemer, 1997). Close inspection of its sequence revealed a stretch of 17 amino acids sharing significant homology with the BH4 domain known to be important for the anti-apoptotic function of mammalian Bcl-2 proteins (Figure 1B). However, Boo lacks a recognizable BH3 domain found in all pro-apoptotic members of the Bcl-2 family.

Boo displays a highly restricted expression in mouse tissues

To characterize Boo further, its expression in various mouse adult and embryonic tissues was examined by Northern blot analysis. Surprisingly, Boo mRNA was undetectable or at very low levels in heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis (data not shown). Moreover, no expression was found during embryonic development from E7–E17 (data not shown). Given that all the EST clones were derived from unfertilized, fertilized and 2-cell stage mouse eggs, we reasoned that the expression of Boo may be restricted to the ovary and other reproductive organs. Indeed, further analysis of a mouse RNA master blot, which contains poly(A)$^+$ RNAs from different tissues and developmental stages, showed that Boo mRNA was predominantly expressed in the ovary and weakly in the epididymis but not in the other tissues examined (Figure 2).

Boo is a non-nuclear intracellular protein

To assess subcellular localization, we expressed Boo in 293T cells as a fusion protein with an N-terminal Flag epitope tag. Immuno-staining with anti-Flag antibodies and visualization by fluorescence microscopy was carried out 24 h after transfection. Analysis of labeled cells with an anti-Flag monoclonal antibody revealed that Flag-Boo displayed a compact, granular and extranuclear staining pattern, consistent with an association with membranes of intracellular organelles and the perinuclear region (Figure 3A). The pattern of Boo staining was strikingly similar to that reported for Ced-9, Bcl-2 and Bcl-x_L (Krajewski *et al*., 1993; Gonzalez-Garcia *et al*., 1994; Wu *et al*., 1997b), suggesting that Boo, Bcl-2 and Bcl-x_L localize to similar intracellular compartments in mammalian cells. The specificity of the labeling was confirmed by comparing the staining pattern with that of cells transfected with a control Flag-tagged GATA-1 expression plasmid. Staining of GATA-1 protein with an anti-Flag antibody revealed the nuclear labeling pattern expected for a transcription factor (Figure 3B).

Localization was also assayed independently by subcellular fractionation studies. Transfected 293T cells were lysed and fractionated into a nuclear, cytosolic and a membrane fraction. Boo was found predominantly in the membrane fraction and some in the low-speed nuclear pellet (Figure 3C). The transcription factor GATA-1 was found exclusively in the nuclear pellet (Figure 3D). These results are consistent with the immunolocalization data and further establish the similarities in subcellular localization between Boo and Bcl-2 (O'Connor *et al*., 1998).

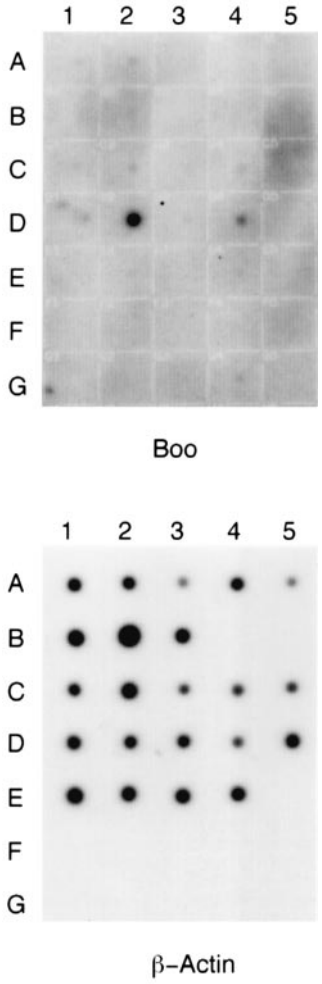

Fig. 2. Distribution of Boo in mouse tissues. Boo was hybridized to poly(A)$^+$ RNA derived from the following mouse tissues: brain (A1), eye (A2), liver (A3), lung (A4), kidney (A5), heart (B1), skeletal muscle (B2), smooth muscle (B3), pancreas (C1), thyroid (C2), thymus (C3), submaxillary gland (C4), spleen (C5), testis (D1), ovary (D2), prostate (D3), epididymis (D4), uterus (D5), embryo 7 days (E1), embryo 11 days (E2), embryo 15 days (E3), embryo 17 days (E4), yeast total RNA (F1), yeast tRNA (F2), *E.coli* rRNA (F3), *E.coli* DNA (F4), poly r(A) (G1), C_0t1 DNA (G2), mouse DNA (G3) and mouse DNA (G4). The same filter was reprobed with β-actin for calibration.

Overexpression of Boo inhibits apoptosis

To assess its role in regulation of apoptosis, the Flag-tagged Boo expression plasmid was transfected into FL5.12, an interleukin-3 (IL-3)-dependent prolymphocytic cell line and Baf-3, an IL-3-dependent bone marrow-derived cell line. For comparison, cell lines were also transfected with Flag-Bcl-2 and Flag-Bcl-x_L expression plasmids, respectively. Stable clones expressing comparably high

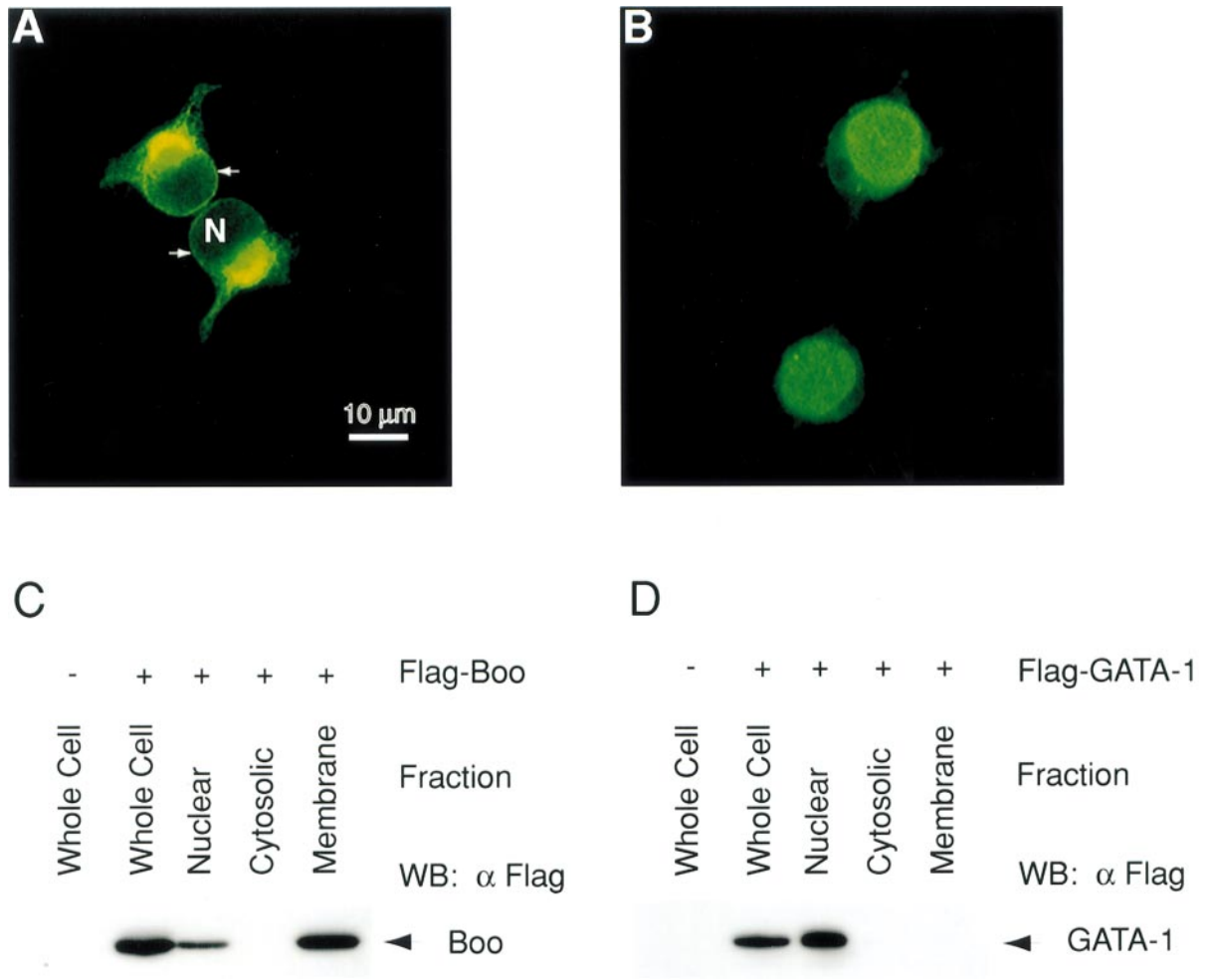

Fig. 3. Subcellular localization of Boo in 293T cells. Cells were transiently transfected with expression plasmids encoding Flag-Boo (**A**) or Flag-GATA-1 as a control (**B**). Shown are images after labeling with anti-Flag and secondary fluorescein-conjugated antibody. Samples were prepared at 24 h after transfection. Arrow, perinuclear region; N, nucleus. Subcellular fractionation of lysates from 293T cells expressing Flag-Boo (**C**) or Flag-GATA-1 (**D**). Lysates from equivalent numbers of unfractionated cells (whole) and of subcellular fractions (nuclear, cytoplasmic or membrane), were resolved by SDS–PAGE and immunoblotted using anti-Flag monoclonal antibody.

levels of Boo, Bcl-2 and Bcl-x_L proteins were selected for further study.

To ascertain whether Boo enhanced or antagonized cell survival, the transfected cell lines were subjected to various cytotoxic conditions. Cells transfected with expression vectors containing only the drug resistance gene served as controls. As shown in Figure 4, both FL5.12 and Baf-3 cells transfected with the neomycin construct alone died rapidly upon IL-3 withdrawal. In contrast, when Boo transfectants were subjected to IL-3 deprivation, they displayed dramatic resistance to cell death and the kinetics of their survival were comparable with that of cells overexpressing either Bcl-2 or Bcl-x_L (Figure 4A and B, left panels). To further compare the anti-apoptotic spectrum of Boo with its homologues, we also exposed FL5.12 and Baf-3 clones to γ-irradiation and cyclosporin A, respectively. Anti-apoptotic Bcl-2 family members have been shown to protect effectively against ionizing radiation-induced cell death as well as the immunosuppressant cyclosporin A (Huang *et al.*, 1997). Boo effectively antagonized both γ-irradiation and cyclosporin A-induced apoptosis (Figure 4A and B, right panels). No significant difference could be detected in the level of protection against these two cytotoxic agents conferred by Boo, Bcl-2 and Bcl-x_L. Collectively, these results clearly place Boo in the sub-family of Bcl-2-related proteins that inhibit cell death.

Localization of mouse Boo gene

Fluorescence *in situ* hybridization analysis using a mouse *Boo* genomic probe clearly assigned *Boo* to mouse chromosome 9 at band d9 (Figure 5). Of the 80 metaphases scored for fluorescent signal using the intron probe, 73 exhibited specific labeling at 9d9.

Boo forms homodimers and heterodimerizes with other Bcl-2 members

To determine whether Boo could form homodimers, we co-transfected 293T cells with expression plasmids producing Flag-Boo and Boo-Myc or a control plasmid. Immunoprecipitates were prepared using an anti-Flag monoclonal antibody, and separated by SDS–PAGE. Immunoblotting with anti-Myc antibody revealed co-precipitated Boo-Myc, indicating the presence of Boo homodimers (Figure 6A). To assess whether Boo interacts with Bcl-2 and Bcl-x_L in mammalian cells, we performed co-immunoprecipitation in 293T cells. As shown in Figure 6B, Boo bound strongly to Bcl-x_L and weakly to

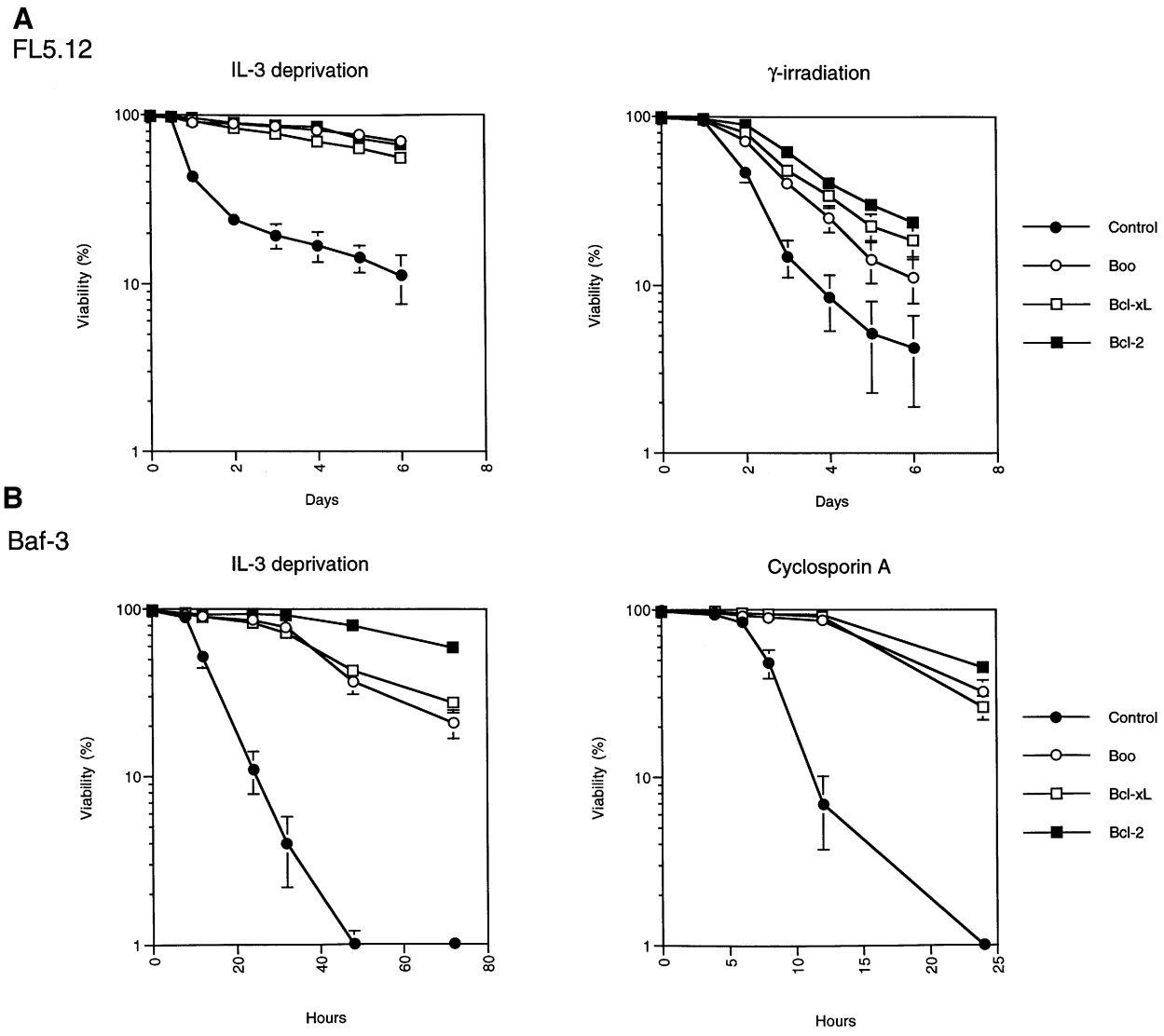

Fig. 4. Effect of Boo, Bcl-2 and Bcl-x_L on apoptosis induced by IL-3 deprivation, γ-irradiation or cyclosporin A. (**A**) FL5.12 cells were either washed three times and cultured in medium lacking IL-3 (left panel) or irradiated (15 Gy) and cultured in conditioned medium (right panel). (**B**) Baf-3 cells were washed three times and cultured in medium lacking IL-3 (left panel) or cultured in non-conditioned medium containing 100 μg/ml cyclosporin A (right panel). Cultures were initiated at 2.5×10^5 cells/ml and cell viability was determined by Trypan blue exclusion. Results are arithmetic means ± SD of at least three experiments and are representative of the results obtained with at least three independent clones.

Bcl-2. Because Boo could inhibit apoptosis induced by different stimuli, we further tested the ability of Boo to interact with Bax, Bak, Bad, Bik and Bid, five pro-apoptotic members of the Bcl-2 family. Associations of Boo with Bak and Bik were readily detected when these proteins were co-expressed *in vivo* (Figure 6C), whereas Bax, Bad and Bid failed to co-precipitate with Boo. These results indicate that Boo interacts only with selective anti- and pro-apoptotic Bcl-2 proteins.

Boo blocks Bak- and Bik-induced apoptosis

In view of the ability of Boo to heterodimerize with Bak and Bik, we next determined whether Boo could block the killing activity of Bak and Bik. In MCF7 cells, overexpression of Bak or Bik induced apoptosis in ~90 and 85% of cells, respectively, but Boo effectively countered Bak- and Bik-mediated killing in this transient assay (Figure 7). On the other hand, Boo did not inhibit Bax-induced cell death (Figure 7). Thus, the anti-apoptotic activity of Boo correlated with its ability to heterodimerize to pro-apoptotic Bcl-2 homologues.

Boo interacts at multiple sites with Apaf-1

Recent reports demonstrate that Bcl-x_L can interact with Apaf-1, the mammalian counterpart of Ced-4 (Hu *et al.*, 1998; Pan *et al.*, 1998). We speculated that an equivalent interaction might exist between Boo and Apaf-1. To test this, 293T cells were transiently co-transfected with Apaf-1-Myc and Boo-Flag or Flag-A20, a control protein. Western blot analysis of protein complexes immunoprecipitated with anti-Flag antibody revealed that Apaf-1-Myc co-precipitated with Boo-Flag, but not Flag-A20 (Figure 8B). To determine the interaction specificity between Boo and Apaf-1, the immunoprecipitation was also performed in reverse using anti-Myc monoclonal antibody. As shown in Figure 8, Boo-Flag but not Flag-A20 co-precipitated with Apaf-1-Myc. To define critical regions in Apaf-1 required for its interaction with Boo,

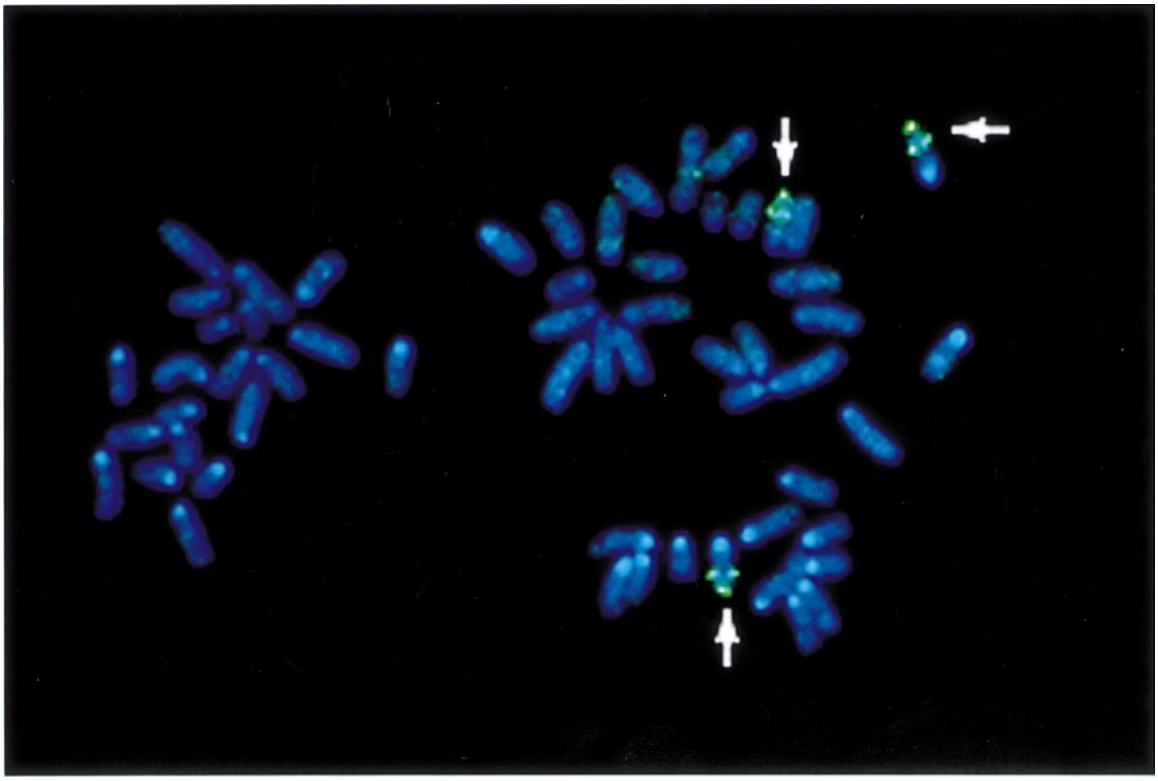

Fig. 5. Localization of Boo on mouse chromosome 9. A Boo genomic probe was used to hybridize to normal metaphase chromosomes derived from mouse embryo fibroblast cells. Hybridization sites on chromosome 9 are indicated by arrows.

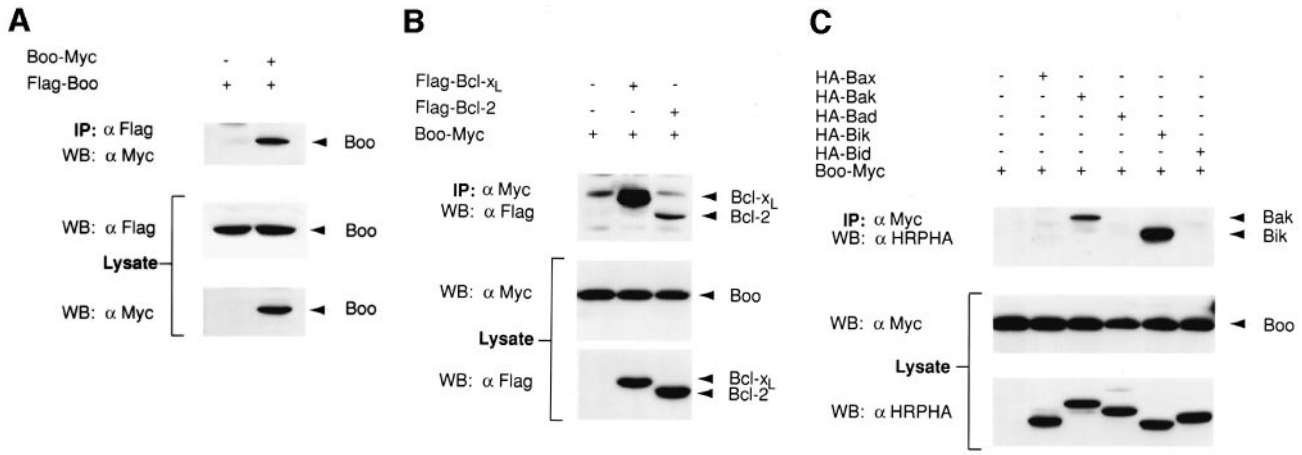

Fig. 6. Boo forms homodimers and interacts with selective Bcl-2 family proteins. (**A**) Homodimerization. 293T cells were transiently transfected with Flag-Boo and Boo-Myc or a control vector. Cell lysates were prepared and immunoprecipitated as described in Materials and methods with the indicated antibodies. Lysates and immunoprecipitates were analyzed by Western blotting as shown. (**B**) Heterodimerization with Bcl-x_L and Bcl-2. 293T cells were transfected with epitope-tagged constructs, and lysates and immunoprecipitates were analyzed by Western blotting as indicated. (**C**) Boo selectively binds to Bak and Bik. Cells were co-transfected with Boo-Myc plus pro-apoptotic Bcl-2 family members. Analysis of lysates and immunoprecipitates was performed by Western blotting as indicated. WB, Western blot analysis; IP, immunoprecipitation.

we engineered five deletion mutants of Apaf-1 (Figure 8A). In initial experiments, an N-terminal deletion mutant (N-Apaf-1; residues 1–601), that lacks the WD-40 repeat region and a C-terminal deletion mutant (C-Apaf-1; residues 602–1194), that contains only the WD-40 repeat domain were assessed for their ability to interact with Boo in a co-transfection assay. Western blot analysis of Boo complexes with anti-Myc antibody revealed that both the N-Apaf-1 and C-Apaf-1 mutants associated with Boo (Figure 8B). Furthermore, Boo also bound to a truncated form of Apaf-1 [Apaf-1(3+4)], that comprises the Ced-3 and Ced-4 homologous regions, and the Apaf-1 unique segment between the Ced-4-like and WD-40 repeat domains (Figure 8B). However, Apaf-1(3), a deletion mutant containing the Ced-3-homologous region, failed to co-precipitate with Boo (Figure 8B). Thus, these results indicate that Boo binds to at least three distinct domains in Apaf-1, the Ced-4-homologous region, the WD-40 repeats, and the Apaf-1 unique domain between the Ced-4-like and WD-40 repeat regions.

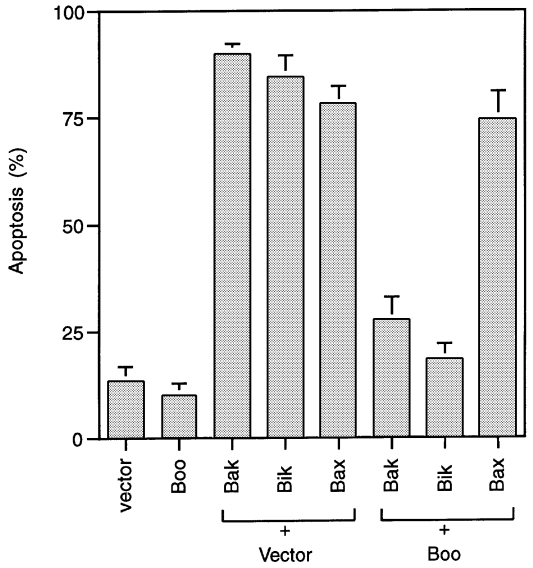

Fig. 7. Boo inhibits apoptosis induced by Bak or Bik. MCF7 cells were co-transfected with Bak, Bik or Bax together with a β-galactosidase-expressing reporter construct in the presence of a 4-fold excess of a control vector or Boo. Cells were stained and examined as described (Duan *et al*., 1996).

Boo and caspase-9 form a ternary complex with Apaf-1

Given that Bcl-x_L and caspase-9 form a ternary complex with Apaf-1 (Pan *et al*., 1998), we investigated whether an analogous biochemical interaction may also occur with Boo. To assess this, 293T cells were transiently co-transfected with Boo-Myc and caspase-9–DN-Flag (DN, dominant negative). Immunoprecipitation of caspase-9–DN-Flag coprecipitated Boo-Myc, but not Myc-TRADD, a control protein (Figure 9A). A recent study has shown that the Ced-3 homologous domain of Apaf-1 interacts with caspase-9 but not Bcl-x_L (Pan *et al*., 1998). Similarly, in the present study, this truncated version of Apaf-1 also failed to bind to Boo. To determine further whether the *in vivo* interaction between caspase-9 and Boo was mediated by an endogenous Apaf-1-like protein, expression plasmids producing caspase-9–DN-Flag, Boo-Myc and Apaf-1(3)-Myc were co-transfected in 293T cells. Expression of Apaf-1(3)-Myc in excess significantly attenuated the ability of caspase-9 to coprecipitate Boo (Figure 9B), suggesting that this deletion mutant of Apaf-1 competitively blocked the interaction of caspase-9 with an endogenous Apaf-1-like molecule, thereby disrupting the association between caspase-9 and Boo. Moreover, ectopic expression of Boo did not affect the binding of caspase-9 to Apaf-1 (Figure 9C), further confirming that Apaf-1 simultaneously interacts with caspase-9 and Boo, forming a ternary complex in mammalian cells. The ability of Bak and Bik to heterodimerize with Boo prompted us to examine whether these two pro-apoptotic members of the Bcl-2 family may function by affecting the association of Boo with Apaf-1. Co-expression of hemagglutinin (HA)-Bak or HA-Bik disrupted the interaction between Boo and Apaf-1 (Figure 9D), indicating that both death promoters may exert their killing activity by abrogating the recruitment of Boo to Apaf-1 and thus favoring Apaf-1-mediated caspase-9 activation.

Discussion

We have identified a novel regulator of apoptosis, Boo, which is homologous to Bcl-2, the prototype of the Bcl-2 family. When stably expressed, Boo greatly enhances the survival of cells exposed to growth factor deprivation, γ-irradiation or an immunosuppressant, cyclosporin A. This wide spectrum of anti-apoptotic activity is characteristic of the Bcl-2 family (Reed, 1994; Huang *et al*., 1997). Boo is also protective in transient assays and effectively inhibits Bik and Bak induced apoptosis. We therefore conclude that Boo, Bcl-2, and Bcl-x_L are functional homologues. Boo clearly falls into the death-repressing rather than the death-promoting sub-group of the Bcl-2 family.

Among the anti-apoptotic Bcl-2 proteins, Boo is most similar to chicken NR-13. Alignment analysis reveals that the Boo protein contains conserved BH1 and BH2 domains, but lacks the BH3 motif found in all the death agonists. The presence of a C-terminal transmembrane region is expected to mediate localization of Boo to intracellular membranes. Immunofluorescence and subcellular localization experiments in 293T cells support this view.

Notably, Boo has a serine at position 88 and an arginine at 144. These residues corresponding to G142 in the BH1 and W185 in the BH2 domain of Bcl-2, which are highly conserved among other anti-apoptotic family proteins (Yin *et al*., 1994; Sedlak *et al*., 1995). Previous studies have shown that these positions are important for Bcl-2 function and heterodimerization with Bax (Yin *et al*., 1994). However, replacement of the W residue in Bcl-x_L had minimal effect on their survival function (Cheng *et al*., 1996). Moreover, substitution of the G residue with an E residue in Ced-9 resulted in a gain-of-function mutant (Hengartner and Horvitz, 1994a). These results indicate that the residues necessary for the pro-survival activity of Bcl-2 are not identical to those of Ced-9 and Bcl-x_L. We mutated these positions in Boo and found that expression of the mutant Boo was equally effective at inhibiting Bak and Bik induced apoptosis (unpublished data). Thus, Boo is more similar to Ced-9 and Bcl-x_L in its ability to incorporate silent mutations at these positions.

Recent studies have shown that deletion of the BH4 domain rendered Bcl-2 and Bcl-x_L inactive (Huang *et al*., 1998), indicating that this domain is essential for death-suppressing function of Bcl-2 and Bcl-x_L. Consistent with this finding, Boo also has a recognizable BH4 domain, which may contribute to its inhibitory activity.

The nuclear magnetic resonance (NMR) structural analysis of Bcl-x_L has shown that its BH1, BH2 and BH3 domains form an elongated hydrophobic cleft which can bind the BH3 domains of death-promoting proteins (Sattler *et al*., 1997). Since Boo lacks a BH3 motif, it probably has an interaction-interface distinct from that of Bcl-x_L. In agreement with this hypothesis, Boo shows a selective heterodimerization profile by interacting with some (Bak and Bik) but not other (Bax, Bad and Bid) pro-apoptotic members. Similarly, Bok/Mtd, a pro-apoptotic Bcl-2 homologue, which lacks a BH4 domain and has a less conserved BH3 domain than other Bcl-2 proteins, interacts only with selective anti-apoptotic family molecules (Mcl-1, BHRF1 and Bfl-1) (Hsu *et al*., 1997; Inohara *et al*., 1998a).

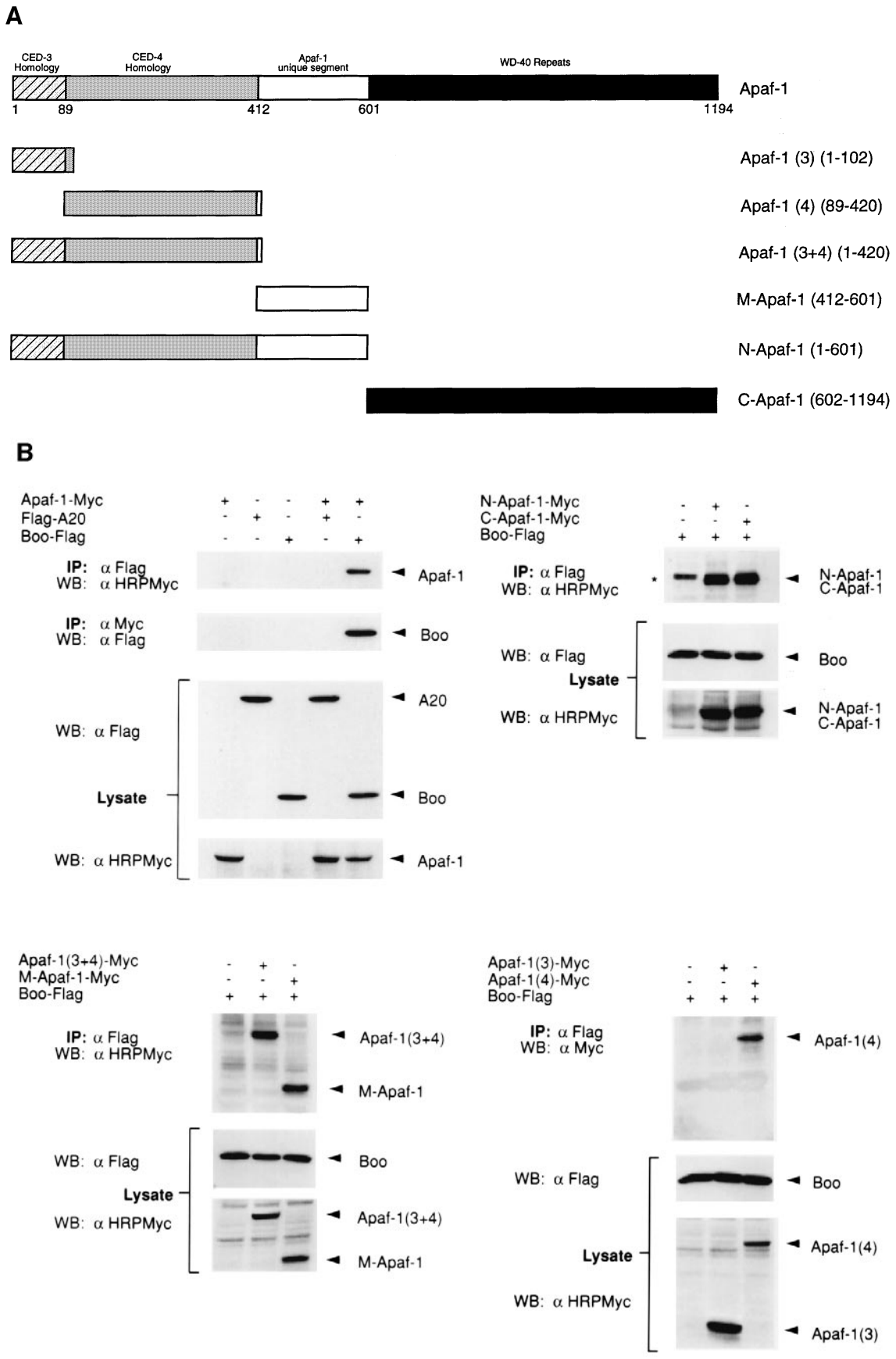

Fig. 8. Boo binds to distinct domains in Apaf-1. (**A**) Schematic drawing of the Apaf-1 constructs used in this study. (**B**) 293T cells were co-transfected with Boo-Flag and the indicated Apaf-1 constructs or a control vector. Flag or Myc immunoprecipitates were analyzed for co-precipitating proteins by immunoblotting with mAb towards Myc, Flag or horseradish peroxidase (HRP)-conjugated anti-Myc. Expression of Boo and the Apaf-1 constructs was visualized by Western blotting of the lysates with αFlag or αHRP-Myc antibodies. Asterisk indicates a non-specific band. WB, Western blot analysis; IP, immunoprecipitation.

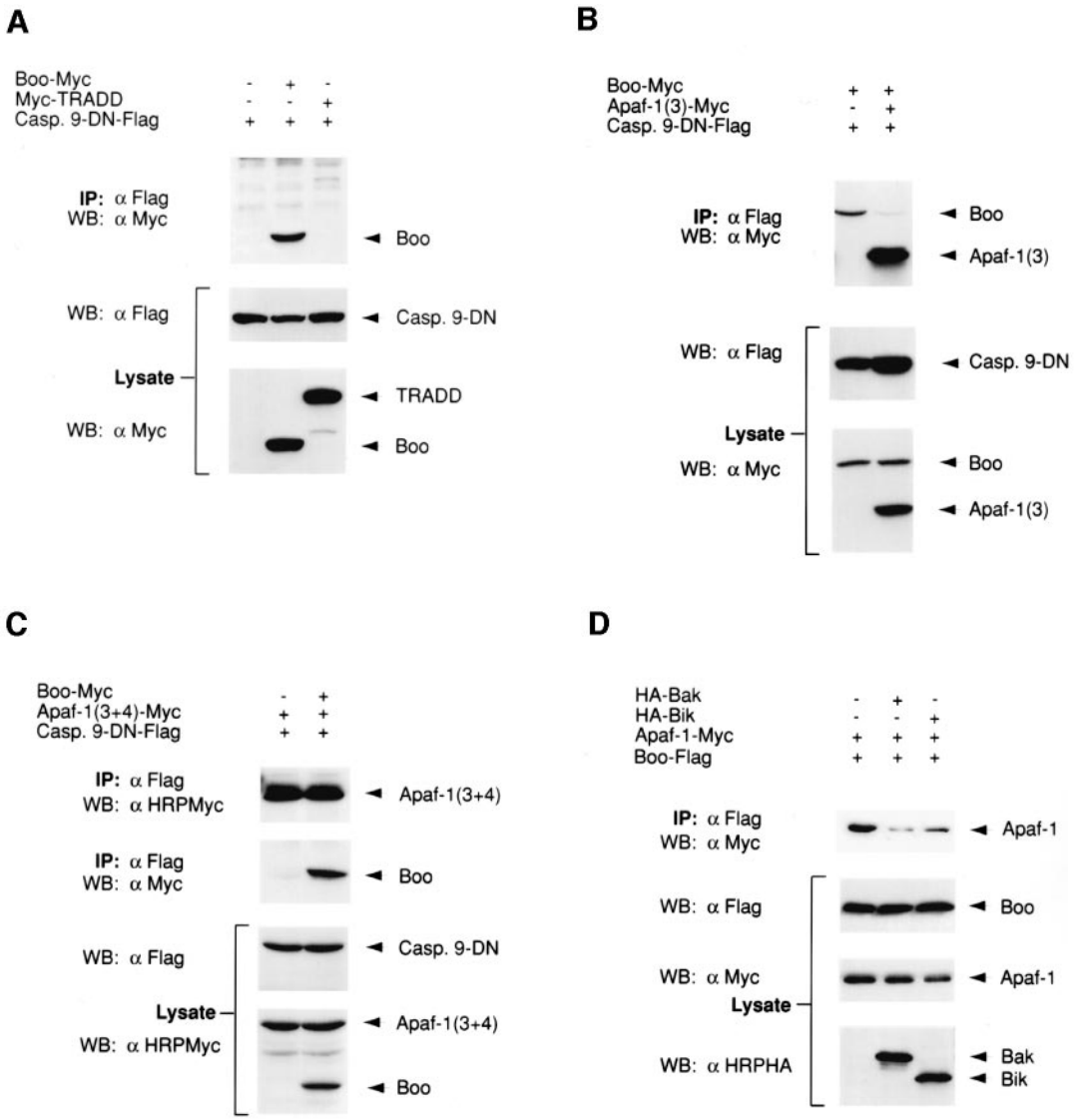

Fig. 9. Boo and caspase-9 form a multimeric complex with Apaf-1. (**A**) Caspase-9 associates with Boo through an endogenous Apaf-1-like molecule. 293T cells were co-transfected with caspase-9–DN-Flag and Boo-Myc, Myc-TRADD or a control vector. Anti-Flag immunoprecipitates were blotted with anti-Myc. Expression of the transfected gene products was assayed in lysates by Western blotting as indicated. (**B**) Co-expression of the Ced-3-homologous region of Apaf-1 disrupts the association of caspase-9 with Boo. 239T cells were co-transfected with caspase-9–DN-Flag and Boo-Myc in the presence of a control vector or a construct expressing Apaf-1(3)-Myc. Immunoprecipitation and Western blot analysis was performed as in (A). (**C**) Boo does not affect the interaction between caspase-9 and Apaf-1. Caspase-9–DN-Flag was co-expressed with Apaf-1(3+4)-Myc in the presence of either a control vector or a Boo-Myc construct. Immunoprecipitation was carried out with anti-Flag affinity gel and blotted with anti-Myc or HRP-conjugated anti-Myc. Expression of the transfected gene products was assayed in lysates by Western blotting as indicated. (**D**) Bak or Bik disrupts the interaction between Apaf-1 and Boo. Boo-Flag and Apaf-1-Myc were co-expressed in the presence of either a control vector, HA-Bak or HA-Bik. Anti-Flag immunoprecipitates were blotted with anti-Myc. Lysates were analyzed for expression of the transfected gene products by Western blotting with anti-epitope antibodies as shown. WB, Western blot analysis; IP, immunoprecipitation.

Moreover, the anti-apoptotic KS-Bcl-2 protein, which contains a poorly conserved BH3 domain and lacks a BH4 region, can bind neither to Bak and Bik nor to Bcl-2 and Bcl-x_L (Cheng *et al*., 1997). These observations suggest that anti-apoptotic proteins have a variety of interaction surfaces for Bcl-2 homologues, and that the binding specificity is determined by multiple domains. However, only detailed structural information will permit full understanding of these protein–protein interactions. Furthermore, restricted heterodimerization between pro- and anti-survival members of the family may have evolved to regulate multiple death and survival signals.

Interestingly, Boo can also interact with Bcl-2 and Bcl-x_L. Consistent with this observation, other death antagonists such as Bcl-x_L, Mcl-1 and BHRF1 have been shown to interact with Bcl-2 (Farrow and Brown, 1996; Brown, 1997). However, the precise role of these dimerizations between pro-survival proteins in regulation of apoptosis remains unclear.

It has been proposed that the ratio of pro- to anti-apoptotic members and their dimerization determine death or survival. For example, Bcl-2 mutants fails to heterodimerize with Bax and no longer can inhibit apoptosis (Yin *et al*., 1994). However, this view was challenged by the identification of Bcl-x_L mutants and a viral homologue, KS-Bcl-2, that do not interact with Bak or Bik but still inhibit apoptosis (Cheng *et al*., 1996, 1997). Surprisingly, a recent study has shown that Bcl-2 and Bcl-x_L mutants

that can not heterodimerize with Bax or Bak still retain the ability to interact with Bad (Ottilie *et al*., 1997), suggesting that it is difficult to draw definite conclusions from these studies because of the redundancy among Bcl-2 family proteins.

How might Boo function given that it interacts only with a subset of Bcl-2 death agonist but protects from a broad range of apoptotic stimuli? The recent discovery that Apaf-1 can form a ternary complex with caspase-9 and Bcl-x_L has raised the possibility that the death-suppressing members of the Bcl-2 family function by regulating Apaf-1-like proteins and thereby controlling activation of procaspases and cell death (Li *et al*., 1997; Hu *et al*., 1998; Pan *et al*., 1998). In keeping with this hypothesis, here we have shown that Boo interacts with Apaf-1. We further demonstrate that Boo associates with caspase-9. The biochemical linkage between Boo and caspase-9 could be attenuated by the presence of a deletion mutant of Apaf-1 that binds caspase-9 but not Boo. This led to the suggestion that the interaction between Boo and caspase-9 is mediated by an endogenous Apaf-1 and that mutant Apaf-1 blocks the recruitment of endogenous Apaf-1 and thus Boo to caspase-9. The observation that overexpressed Boo did not compete for the association between caspase-9 and Apaf-1 provides additional evidence that Apaf-1 simultaneously interacts with caspase-9 and Boo, forming a ternary complex in mammalian cells. This complex can be disrupted by Bak and Bik. These results, taken together with the recent evidence that Bax and Bak can abolish the association of Bcl-x_L with Apaf-1 (Pan *et al*., 1998), indicate that the death-promoting Bcl-2 family homologues may function by binding to death-repressing members of the Bcl-2 family and displacing Apaf-1-like molecules, thereby permitting the activation of procaspases. These data imply that anti-apoptotic Bcl-2 homologues have the dominant function in regulating apoptosis.

We found that Boo binds to three distinct regions in Apaf-1. Since Bcl-x_L could not inhibit caspase-9 maturation mediated by an N-terminal mutant of Apaf-1 which contains only the Ced-4-like domain (Hu *et al*., 1998), it is conceivable that both the WD-40 repeat region and the Apaf-1 unique segment act as regulatory elements controlling the activity of Apaf-1. Binding of Boo to these two domains could induce a conformational change in Apaf-1 that would inactivate Apaf-1 and thus inhibit the processing of caspase-9. We are currently testing these possibilities.

The tissue distribution of Boo is the most restricted of any of the Bcl-2 family members. The expression of Boo in the epipidymis may indicate that it contributes to sperm maturation. A precedent for a specific role of the Bcl-2 family of proteins in the regulation of the male reproductive tract is the ROSA41 mouse strain that carries a disrupted *Bclw* gene. Although *Bclw* is expressed widely, its inactivation leads mainly to reduced size of the testis and seminal vesicles causing sterility (Ross *et al*., 1998). Bcl-2 overexpression in spermatogonia and loss of function of Bax also leads to disruption of spermatogenesis (Knudson *et al*., 1995; Furuchi *et al*., 1996). This implies that several Bcl-2 homologues with very similar functional properties are necessary during different stages of sperm maturation and Boo is likely to be important during the epipididymal phase.

Highest expression of Boo is detected in the ovaries. Cell loss in the ovaries occurs during pre and postnatal ovarian development (Beaumont and Mandl, 1962). Apoptotic cell death plays an important role during this process (Tilly, 1996). In addition, oocytes are particularly sensitive to chemo- and radiotherapy and such treatments often lead to sterility (Familiari *et al*., 1993; Ried and Jaffe, 1994). Therefore, understanding the molecular processes specific for ovarian apoptosis are of considerable interest. Boo is not the only anti-apoptotic Bcl-2 homologue expressed in the ovary. Mcl-1, Bcl-2 and Bcl-x have also been detected in ovarian tissues (Tilly *et al*., 1995; Hsu *et al*., 1997). Multiple pro- and anti-apoptotic proteins have been shown to have an effect on oocyte apoptosis. For example, Bcl-2 knock-out mice contain aberrantly formed primordial follicles (Ratts *et al*., 1995). The oocytes of Bax as well as caspase-2 knock-out mice are remarkably resistant to doxorubicin-induced apoptosis (Perez *et al*., 1997; Bergeron *et al*., 1998). Therefore, more detailed studies need to be carried out to understand the interplay of these molecules in the ovarian context. Targeted gene disruption of *Boo* should be part of such an analysis.

Materials and methods

Cloning Boo and expression vectors

The partial nucleotide sequences of cDNAs encoding peptides with homology to chicken NR-13 were found in EST database of GenBank™ using the TBLASTN program. The nucleotide sequence of EST clones 1108004 (DDBJ/EMBL/GenBank accession No. AA623872) was determined by dideoxy sequencing. The full-length Boo from EST clone 1108004 was cloned into pcDNA3 (Invitrogen) with a N- or C-terminal Flag tag, or a C-terminal Myc tag. The full-length Apaf-1 was cloned into pcDNA3.1(–)/Myc-His B (Invitrogen). The truncated mutants of Apaf-1, N-Apaf-1, C-Apaf-1, M-Apaf-1, Apaf-1(3+4), Apaf-1(3) and Apaf-1(4) were amplified by PCR using the Apaf-1 cDNA as a template and also cloned into pcDNA3.1(–)/Myc-His B. Bad and Bid were cloned into pcDNA3 with a N-terminal HA tag. The constructs encoding Flag-Bcl-2, Flag-Bcl-x_L, HA-Bax, HA-Bak, HA-Bik, caspase-9–DN-Flag and Myc-TRADD have been described previously (Chinnaiyan *et al*., 1996, 1997; Duan *et al*., 1996; Duan and Dixit, 1997).

Northern blot analysis

A fragment (nucleotides 1–1008) of the Boo cDNA was radiolabeled by PCR-labeling method and applied for analysis of mouse adult and embryo multiple-tissue Northern blots, and mouse RNA master blot (Clontech) according to the manufacturer's instructions. The mouse RNA master blot was also hybridized with a β-actin cDNA probe.

Tissue culture, stable transfection and cell-viability assay

MCF7, a human breast carcinoma cell line was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), non-essential amino acids, L-glutamine, and penicillin/streptomycin. FL5.12, an IL-3-dependent murine pro-B lymphocyte line and Baf-3, an IL-3-dependent bone marrow-derived line were cultured in the same medium supplemented with 10% WEHI-3B-conditioned medium as a source of IL-3. Dulbecco's modified Eagle's medium containing 10% FBS, non-essential amino acids, L-glutamine and penicillin/streptomycin was used to maintain the 293T cells.

FL5.12 and Baf-3 cells were transfected using a Gene-pulser (Bio-Rad) with pcDNA3 plasmid containing Flag-Boo, Flag-Bcl-2 or Flag-Bcl-x_L or a control pcDNA3 plasmid (200 V, 960 μF). Transfectants were selected by growth in G418 (1 mg/ml). Individual clones were isolated by limiting dilution of a polyclonal neomycin-resistant population into 96-well plates and then screened for the expression of the transfected gene of interest by immunoblotting. Clones expressing comparably high levels of Flag-Boo, Flag-Bcl-2 and Flag-Bcl-x_L were selected for further study.

To test their sensitivity to apoptosis, FL5.12 cells were cultured in IL-3-free medium, or in IL-3-containing medium after exposure to 15 Gy of γ-irradiation (provided by a ^{60}Co source at a rate of 215 cGy/min). Baf-3 cells were cultured in IL-3-free medium, or in IL-3-free medium supplemented with 100 μg/ml of cyclosporin A (Sigma). Cell viability was determined by Trypan blue exclusion and counting in a hemocytometer. The percentage of survival cells represents the mean value from at least three independent experiments with at least three independent clones.

Immunofluorescence, subcellular fractionation, immunoprecipitation and immunoblotting

To investigate the subcellular localization of Boo, 293T cells were transfected with pcDNA3–Flag-Boo, pcDNA–Flag-GATA-1, or empty vector as described above. Twenty-four hours after transfection, cells were incubated with anti-Flag antibody for 1 h at 23°C and the labeling was visualized with fluorescein-conjugated goat anti-mouse IgG. After washing, the cells were mounted in Vectashield mounting medium (Vector Laboratories, Inc.) and examined with a Leitz orthoplan fluorescence microscope. To test for protein–protein interactions *in vivo*, 293T cells were transiently transfected with indicated plasmids by calcium phosphate precipitation.

For subcellular fractionation, lysates were prepared as described previously (O'Connor *et al*., 1998). Briefly, 2×10^6 cells were homogenized in 1 ml of hypotonic lysis buffer (10 mM Tris–HCl pH 7.4, 0.5 μg/ml Pefabloc, 1 μg/ml each of leupeptin, aprotinin, soybean trypsin inhibitor and pepstatin, 5 mM NaF and 2 mM Na_3VO_4) with a Dounce homogenizer. The lysates were centrifuged at 900 *g* for 10 min to obtain the nuclear pellet and the supernatant centrifuged at 130 000 *g* for 60 min to obtain the soluble cytosolic and the pelleted membrane fractions.

Protein immunoprecipitation and Western blot analysis with relevant antibodies were performed as described previously (Duan and Dixit, 1997). Briefly, cells were lysed in 1 ml of lysis buffer (20 mM Tris pH 8.0, 137 mM NaCI, 10% glycerol, 1% nonident P40, 2 mM EDTA) 24–48 h after transfection and soluble lysates were incubated with anti-Flag, anti-Myc or anti-HA antibodies and protein G–Sepharose (Sigma) overnight at 4°C. Immune complexes were centrifuged, washed with excess cold lysis buffer at least three times, and then boiled in gel-loading buffer. Eluted proteins were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-Flag, -Myc or -HA antibodies.

Apoptosis assay

These were performed essentially as described previously (Duan *et al*., 1996). Briefly, MCF7 cells were plated on a six-well tissue culture plate (2×10^5 cells/well) and transiently transfected with 0.1 μg of the reporter plasmid pCMV-β-galactosidase plus 0.25 μg of pcDNA3–HA-Bak or pcDNA3–HA-Bik in the presence or absence of 1 μg of pcDNA3–Flag-Boo by the Lipofectamine procedure. The total amount of transfected plasmid DNA was adjusted to 1.5 μg/well by adding pcDNA3. Transfected cells were detected by staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as described previously (Duan *et al*., 1996). The percentage of apoptotic cells represents the mean value from three independent experiments.

Fluorescence in situ hybridization

A mouse Boo genomic probe was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblast cells in a solution containing 50% formamide, 10% dextran sulfate and 2 × saline-sodium citrate buffer. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated anti-digoxigenin antibodies followed by counterstaining with 4,6-diamidino-2-phenylindole (DAPI).

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Note added in proof

The gene for Boo has been recently described as DIVA (Inohara *et al*., 1998b).