VIAF, a Conserved Inhibitor of Apoptosis (IAP)-interacting Factor That Modulates Caspase Activation*

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Inhibitor of apoptosis (IAP) proteins are involved in the suppression of apoptosis, signal transduction, cell cycle control and gene regulation. Here we describe the cloning and characterization of viral IAP-associated factor (VIAF), a highly conserved, ubiquitously expressed phosphoprotein with limited homology to members of the phosducin family that associates with baculovirus Op-IAP. VIAF bound Op-IAP both in vitro and in intact cells, with each protein displaying a predominantly cytoplasmic localization. VIAF lacks a consensus IAP binding motif, and overexpression of VIAF failed to prevent Op-IAP from protecting human cells from a variety of apoptotic stimuli, suggesting that VIAF does not function as an IAP antagonist. VIAF was unable to directly inhibit caspase activation in vitro and a reduction of VIAF protein levels by RNA interference led to a decrease in Bax-mediated caspase activation, suggesting that VIAF functions to co-regulate the apoptotic cascade. Finally, VIAF is a substrate for ubiquitination mediated by Op-IAP. Thus, VIAF is a novel IAP-interacting factor that functions in caspase activation during apoptosis.

The iap family comprises a group of genes that were first discovered in the genomes of baculoviruses, and homologs have subsequently been identified in a wide range of organisms (1–3). Several members of this family are potent inhibitors of apoptotic cell death, and functional studies have indicated that IAPs exert their protective effects at both proximal and distal stages in the apoptotic cascade, in part through the direct inhibition of caspases, cysteine proteases known to play key effector roles in the cell death pathway (4–7). For example, XIAP (ILP-1, MIHA) has been shown to directly bind to the initiator caspase-9, as well as the effector caspase-3 and -7, with affinities in the subnanomolar range (5). During apoptosis, the caspase inhibitory properties of IAPs can be neutralized by IAP antagonists, such as the mitochondrial proteins Smac/DIABLO, Omi/HtrA2, and GSPT1/eRF3 (8, 9) that bind to IAP proteins in a manner that displaces caspases, as well as the IAP-interacting proteins XAF1 (10) and NLRGE (11), which inhibit IAPs by less well defined mechanisms.

Several distinct properties in addition to caspase inhibition have also been attributed to IAPs. Through interaction with TRAF1 and TRAF2, the mammalian IAP proteins c-IAP1 and c-IAP2 are central components of the type 2 TNF receptor signaling complex (12–15), and play a role in TNF-mediated activation of the transcription factor NF-κB. XIAP has been implicated in multiple signal transduction cascades including the bone morphogenetic protein/TGF-β cascade (16, 17), stimulation of the transcription factor NF-κB (16, 18, 19), and activation of JNK (17, 20). XIAP-mediated JNK activation involves interaction with TAB1, a cofactor that also plays a regulatory role in TGF-β signaling through its activation of the TGF-β-activated kinase TAK1 (21), and has been suggested to be required for the protective effects of XIAP (22), in part through association with ILPIP (22). Additional studies have defined roles for other IAP-like proteins in the regulation of the cell cycle: survivin is expressed only in dividing cells, and cells lacking survivin exhibit defects in cytokinesis (24–27). Similarly, disruption of IAP-like proteins in yeasts and Caenorhabditis elegans has revealed roles for these factors in mitosis (28–31).

Two functional domains have been identified in IAP proteins (32). The defining IAP motif is an ~65 residue motif termed the BIR, and from one to three repeats of this domain are found in all IAP proteins. The BIR domain can interact directly with caspases, and this is particularly evident in XIAP, where the third (most C-terminal) BIR binds directly to caspase-9 (33–36). A region immediately N-terminal to the second BIR binds directly to caspase-3 and -7 (37–39). The second major motif found in some, but not all, IAP proteins is the RING domain (40). Where present, the RING domain is positioned at the C terminus of the protein, and provides E3 ubiquitin ligase activity (41–44). The functional significance of IAP RING domains remains unclear, but this domain does not appear to be required for the caspase inhibitory function of IAP proteins (45).
The prototype iap gene (Op-iap) from the baculovirus OpM-NPV was originally identified through its ability to inhibit apoptotic cell death in insect cells (2, 46–48). Subsequent studies revealed that expression of Op-iap in mammalian cells can confer protection from a variety of apoptotic stimuli including Fas/Apo-1/CD95 (49–52). However, reports have described an inability of Op-iap to inhibit mammalian caspases (52, 78), suggesting that Op-iap exerts its anti-apoptotic effects through an evolutionarily conserved mechanism distinct from caspase inhibition. In insect cells, IAPs have been shown to bind to the IAP antagonists Reaper, Hid, Grim, and Doom, (53–58), which are the functional homologs of the mammalian IAP antagonists Smac/DIABLO and Omi/HtrA2. This class of IAP interacting factors typically contains a consensus IAP binding motif, known as an IBM, at their N terminus that is required for binding.

In an attempt to further understand the mechanisms by which IAPs can suppress apoptosis in mammalian cells, we screened a human B-cell two-hybrid library using Op-iap as bait. Here we report the identification of viral IAP-associated factor (VIAF), a ubiquitously expressed phosphoprotein with limited homology to members of the phosducin family. VIAF homologs exist in a number of eukaryotic genomes, including mouse, zebrafish, Drosophila, and yeast, suggesting that the function of this gene is conserved throughout evolution. VIAF efficiently binds Op-iap both in vitro and in intact cells, yet VIAF does not appear to contain a consensus IBM within the primary sequence. Indeed, overexpression of VIAF does not alter the ability of Op-iap to prevent Bax-mediated death in human cells, suggesting that VIAF does not function as an IAP antagonist. However, suppression of VIAF by RNA interference markedly inhibited the processing of caspase-3 following the induction of the apoptotic program by Bax. VIAF was found to be a substrate of Op-iap-mediated ubiquitination, suggesting that VIAF may be regulated by IAP proteins in a ubiquitination-dependent manner. Thus VIAF is a novel factor conserved throughout evolution that interacts with IAPs and modulates the activation of caspases during apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following sources: glutathione-Sepharose (Amersham Biosciences); Ni²⁺-NTA-agarose, l-glutamine, and PBS (Invitrogen); FBS (HyClone); DMEM (Mediated); Apo Target protease assay kit (BIOSOURCE); DEVD-AFC (BioMol); protease inhibitor mixture tablets (Complete mini) per 10 ml and equilibrated for 20 min on ice. Cells were purchased in phosphate-free DMEM, 10% FBS supplemented with 500 Ci/ml of [32P]orthophosphate (carrier-free, ICN). Cells were cultured in 10% FBS and 2 mM glutamine at 37 °C in 5% CO₂. All transfections for both plasmids and siRNA oligonucleotides were performed by calcium phosphate precipitation as previously described (49).

Northern blot hybridization and Metabolic labeling—Northern blot analysis of a human multipotent tissue blot (Clontech) was performed under high stringency conditions with a radiolabeled full-length cDNA of human VIAF according to the manufacturer’s instructions. As a control, the blot was incubated with a radioactive probe for β-actin. For metabolic labeling experiments, HEK293 cells transfected with an expression plasmid encoding human VIAF were incubated for 3 h in phosphate-free DMEM, 10% FBS supplemented with 500 μCi/ml of [32P]orthophosphate (carrier-free, ICN). Cells were harvested on ice and solubilized with Triton buffer. Proteins were separated by SDS-PAGE and visualized by autoradiography.

Immunofluorescence—HEK293 cells (10⁶) were plated onto coverslip chamber slides and transfected either with a plasmid encoding ViaF-deRRED, or plasmids encoding FLAG-VIAF and Op-IAP. For ViaF-deRRED transfectants, live cells were stained with the nuclear dye Hoechst and immediately examined using a Zeiss Axiosvert 100M confocal microscope equipped with a Zeiss LSM 510 Meta spectrometer. Cells transfected with FLAG-VIAF and Op-IAP were fixed in PBS containing 4% paraformaldehyde and then incubated with mouse anti-FLAG or rabbit anti-IAP antibodies. Following washing, cells were then incubated with Alexa-488-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies for 45 min at room temperature. Blots were visualized by enhanced chemiluminescence using Kodak XAR film.

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Cellular Fractionation—Cell fractions were prepared using 5 × 10⁷ HEK293 cells as described (64) with minor modifications. Cells were resuspended in 1 ml of buffer A (250 mM sucrose, 20 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 protease inhibitor tablet (Complete-mini per 10 ml) and equilibrated for 20 min on ice. Cells were then disrupted with a Wheaton overhead stirrer with Teflon pestle

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RESULTS

Cloning and Expression of the VIAF Gene—To gain insight into the mechanism by which Op-IAP can suppress cell death in mammalian cells, a yeast two-hybrid screen was performed on a human B-cell cDNA two-hybrid library using Op-IAP as bait. Three overlapping, independent groups of clones, whose in-frame coding sequences possessed identical C termini, were isolated. These were found to interact specifically with Op-IAP but not a variety of negative controls (data not shown), including the GAL4 DNA binding domain alone and the cytoplasmic domain of the TNF receptor family member CD30 (60). Interestingly, these Op-IAP-interacting clones also scored negative against Ac-IAP (Autographa californica IAP), a related baculovirus protein, which, despite its high homology to Op-IAP, is unable to suppress cell death (48). Full-length cDNA clones were identified through analysis of EST databases and found to encode a previously undescribed 239 residue protein with a predicted molecular mass of 28 kDa (Fig. 1A), which we designated VIAF. The two larger VIAF clones identified encoded identical amino acid sequences (143 residues each, Fig. 1A), differing only in the 3'-untranslated sequences present in each. The smallest VIAF clone obtained through two-hybrid screening encoded the C-terminal 128 residues (Fig. 1A), indicating that this region is sufficient for interaction with Op-IAP.

The ability of full-length VIAF to interact with Op-IAP was confirmed first by co-precipitation of in vitro translated 35S-labeled Op-IAP with recombinant GST-VIAF protein. Whereas GST alone failed to precipitate Op-IAP, Op-IAP was efficiently precipitated with GST-VIAF (Fig. 1B), suggesting that the interaction between these two proteins is direct. This interaction was subsequently confirmed in intact cells as follows: HEK293 cells were transfected with plasmids encoding HA-tagged (N-terminal) Op-IAP and GST-tagged (C-terminal) VIAF, and cell lysates were precipitated with glutathione-conjugated beads. The presence of Op-IAP was then assessed by immunoblot analysis. As shown in Fig. 1C, Op-IAP was efficiently precipitated by VIAF-GST, whereas XIAP, a closely related mammalian IAP homolog, was not precipitated by VIAF-GST, indicating that the interaction between VIAF and Op-IAP is specific. Furthermore, a physical association between VIAF and other mammalian members of the IAP family, including c-IAP1, c-IAP2, and survivin, could not be detected (data not shown). Hybridization of a Northern blot containing poly(A)-selected RNAs from a panel of tissues with a radiolabeled VIAF cDNA fragment revealed a ~1.2-kb transcript, which was present in all human tissues tested (Fig. 1D).

Furthermore, in vivo labeling studies with HEK293 cells transiently transfected with a VIAF expression vector and incubated in media containing [32P]orthophosphate revealed that VIAF is a phosphoprotein (Fig. 1E). VIAF Homologs Are Present in Lower Organisms—The finding that a baculovirus IAP was able to associate with human VIAF suggested that the VIAF gene might have been conserved throughout evolution. Therefore, homology searches were performed on databases containing non-human sequences. Closely related murine, rat, zebrafish, and Drosophila viaf genes were identified and full-length clones were isolated (Fig. 2A). The viaf open reading frame exhibited no obvious homology to caspases, IAPs or other proteins currently known to be involved in the regulation or execution of the apoptotic pathway. However, VIAF was found to possess limited homology (27% over 165 residues) to phosducin, a cytosolic protein that interacts with the β subunits of G proteins and thereby regulates transmembrane signaling (67), and significant homology (57% over 239 residues) to a recently identified phosducin homolog in mouse termed mGCPHLP, also known as PhLP2 (68). Indeed, phylogenetic analysis of VIAF along with members of the phosducin family (phosducin, PhLP) from several higher order eukaryotes suggests that mGCPHLP more closely resembles VIAF than phosducin (Fig. 2B). Finally, a phosducin-like protein from Saccharomyces cerevisiae, PLP2 (69), was also found to have limited homology to VIAF (34% over 239 residues), suggesting an extensive conservation for VIAF among eukaryotic genomes.

Cellular Localization of VIAF—The cellular localization of VIAF was assessed by transfection of HEK293 cells with a plasmid encoding VIAF in fusion with the fluorescent protein dsRED in the C terminus. Following transfection, cells were examined by confocal microscopy. As shown in Fig. 3A, VIAF-dsRED was present diffusely throughout the cytoplasm, displaying no punctuate foci characteristic of mitochondria or lysosomes, and was completely excluded from the nucleus. This result was confirmed for endogenous VIAF following biochemical fractionation of HEK293 cells by differential centrifugation. Immunoblot analysis of cytoplasmic, mitochondrial, nuclear, and light membrane fractions (Fig. 3B) confirmed the results of Fig. 3A and demonstrated that VIAF is a cytosolic protein, with significant partitioning to the light membrane component. Furthermore, indirect immunofluorescence analysis of HEK293 cells expressing Op-IAP and FLAG-epitope tagged VIAF confirmed not only the cytoplasmic localization of VIAF, but also showed that Op-IAP and VIAF co-localize to the cytoplasm, consistent with their physical association (Fig. 3C).

Effect of VIAF on Cell Death—We next examined the ability of VIAF to regulate cell survival in response to several apoptotic stimuli. Since VIAF was initially isolated as an Op-IAP-interacting protein, the ability of VIAF to regulate the antiapoptotic properties of Op-IAP was tested. Op-IAP has been shown to prevent cell death in mammalian cells following several apoptotic stimuli (49, 51, 70). Specifically, transient overexpression of Op-IAP in HEK293 cells prevents cell death following co-transfection of either the pro-apoptotic Bel-2 family member Bax or the death receptor family member Fas/CD95, and this system was used to investigate the cell death regulatory properties of VIAF. Expression of VIAF alone had no effect on apoptosis in HEK293 cells following either Bax (Fig. 4A) or...
Fas transfection (data not shown), and co-transfection of VIAF along with Op-IAP resulted in no significant effect on the ability of Op-IAP to prevent apoptosis following either stimulus (Fig. 4A and data not shown). Furthermore, GST-VIAF failed to inhibit caspase activity in vitro (data not shown). These data suggest that VIAF is not a direct caspase inhibitor, and unlike other IAP-interacting proteins such as Smac/DIABLO and Omi/HtrA2, VIAF does not function as an IAP antagonist during cell death.

The above experiments investigated the ability of either overexpressed or recombinant VIAF to regulate cell death. To investigate the possibility that endogenous VIAF may function...
in the regulation of apoptosis, HEK293 cells were transfected with siRNA targeting VIAF prior to transfection with Bax. As controls, siRNA targeting GFP and the IAP homolog Survivin were also included. As shown in Fig. 4B, siRNA transfection resulted in significant reduction in both VIAF and survivin protein levels. Following Bax transfection of cells lacking VIAF or survivin, caspase activation was assessed by fluorometric analysis (Fig. 4C), as well as by immunoblot analysis for the presence of processed caspase-3 (Fig. 4D). As shown in Fig. 4, C and D, transfection of cells with either GFP- or survivin-specific siRNA had no effect on caspase activation, whereas reduction of VIAF protein by siRNA significantly reduced the levels of both caspase activity and caspase-3 processing. These data suggest that VIAF expression allows normal caspase activation to occur following Bax transfection.

VIAF Is Ubiquitinated by Op-IAP—As with many other IAP proteins, Op-IAP contains a RING domain at the C terminus that possesses E3 ubiquitin ligase activity (78). While VIAF failed to affect Op-IAP-mediated protection from Bax-induced apoptosis, the physical interaction between VIAF and Op-IAP nevertheless suggested that these two proteins may co-regulate; therefore the ability of Op-IAP to catalyze the ubiquitination of VIAF was assessed. HEK293 cells were transiently transfected with plasmids encoding HA-Op-IAP along with FLAG-VIAF, as well as a plasmid expressing His-tagged ubiquitin. As a control, a sample transfected with FLAG-VIAF along with the E3 ubiquitin ligase-deficient Op-IAP variant ΔRING was also included. Cell lysates were prepared, ubiquitinated proteins were precipitated using Ni-NTA beads, and the presence of VIAF in precipitated complexes was determined by immunoblotting for the FLAG epitope. As shown in Fig. 5, VIAF appears to be modestly ubiquitinated in the absence of co-expressed Op-IAP, but the presence of Op-IAP significantly increased the amount of ubiquitinated VIAF observed. As expected, not only did Op-IAPΔRING fail to increase VIAF ubiquitination, a decrease in VIAF ubiquitination was observed in
Op-IAP was then performed by confocal microscopy. As a nuclear marker, cells were additionally stained with Hoechst and VIAF localization was determined by confocal microscopy.

Numerous studies have characterized the ability of IAPs to function as a dominant-negative protein. However, equally compelling data exists that implicate IAPs in the regulation of cellular processes distinct from caspase inhibition. The majority of cellular IAPs that have been described were identified in searches of genomic databases through their homology with the prototype baculoviral IAPs, and only two members of the entire family, c-IAP1 and c-IAP2, have been isolated biochemically (13). The realization that c-IAP1 and c-IAP2 are central, functional components of TNF receptor superfamily signaling complexes through their interactions with TRAF1 and TRAF2, while at the same time being capable of suppressing the apoptotic signaling cascade through caspase inhibition (72), suggests that a complex and interconnected relationship exists between these two pathways. Additionally, the modes of action of the prototype baculovirus IAPs, including Op-IAP, are not entirely understood. While the baculovirus IAPs may normally suppress cell death in the context of a virus infection through the inhibition of lepidopteran caspases, Op-IAP can also suppress apoptosis in mammalian cells (49, 51), despite no apparent ability to inhibit mammalian caspases. We therefore took a two-hybrid approach by screening a human B-cell cDNA library with a Gal4-Op-IAP chimera in an attempt to identify mammalian factors that might interact with Op-IAP. From this screen we isolated a novel human factor, which we designated VIAF.

The identification of human VIAF as an Op-IAP-interacting protein raised several important questions. First, given the wide evolutionary distance between human and lepidopteran genomes, has the viaf gene been conserved throughout evolution, and secondly, if a primary function of VIAF is to function through IAPs, does human VIAF interact with human IAPs? We therefore examined the public sequence databases and found expressed transcripts in a diverse range of species, including Drosophila, zebrafish, and yeast, which display homology to human VIAF; molecular cloning and characterization of these transcripts confirmed that the sequences represent true orthologs of the human gene (Fig. 2A).

Several distinct classes of proteins have been shown to interact with various IAP family members, of which the two most widely recognized are caspases and the IAP antagonists, such as Smac/DIABLO and Omi/HtrA2 in mammals and the Drosophila IAP antagonists Hid, Reaper, Grim, and Dose. These two classes of IAP-binding proteins utilize overlapping determinants within IAPs for binding. In the case of Smac/DIABLO and Omi/HtrA2, well defined N-terminal tetrapeptide sequences (AVPI and AVPS, respectively) have been shown to be required for IAP binding, and similar IAP binding motifs are present in the Drosophila IAP antagonists (73, 74). Despite a robust interaction with Op-IAP both in vitro and in intact cells, no readily identifiable IBM is present in the VIAF primary sequence. Unlike Smac, which fails to bind XIAP when expressed in fusion with an N-terminal epitope tag, recombinant VIAF containing an N-terminal fusion retains the ability to interact with Op-IAP, which would not be the case if an IBM were present at the N terminus of VIAF. Furthermore, VIAF overexpression fails to prevent Op-IAP from protecting mammalian cells from apoptosis. Collectively, these observations reveal that VIAF is not an IAP antagonist in the classical sense, and suggest that VIAF represents the first of a new class of IAP-binding protein.

In general and despite their physical interaction, VIAF does not appear to regulate the function of Op-IAP, at least with respect to the ability of Op-IAP to prevent apoptosis in mammalian cells. However, this does not rule out the possibility that Op-IAP and potentially other cellular IAPs regulate the function of VIAF, possibly through ubiquitination. It has recently been shown that Op-IAP fails to directly inhibit caspases but prevents Bax-mediated apoptosis in mammalian cells.
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VIAF displays significant homology to a recently identified phosducin homolog in mouse termed mGCPHLP. It should be noted that while highly similar, mGCPHLP is localized to mouse chromosome 5 (68), and is therefore distinct from mouse VIAF (Fig. 2A), which is localized to mouse chromosome 2 (data not shown). Whereas little is known about the function of mGCPHLP, this gene has been shown to functionally complement for deletion of the yeast gene PLP2, the likely homolog of VIAF. Expression of mGCPHLP is restricted to male and female germ cells, in contrast to VIAF, which was present in all tissues tested including testis and ovary. VIAF did not affect the protective properties of Op-IAP, yet a role for VIAF in modulating full caspase activation was nevertheless revealed. In light of the similarities between VIAF and mGCPHLP, a role for VIAF in biological processes distinct from apoptosis remains likely, and it is tempting to speculate that VIAF and mGCPHLP may perform overlapping yet distinct cellular functions, which likely exhibit tissue specificity.

In summary, we describe here the cloning and molecular characterization of VIAF, a novel IAP associated factor. While not an IAP antagonist, VIAF nevertheless was found to play a regula-

Fig. 4. VIAF does not affect Op-IAP protection but regulates Bax-mediated caspase activation. A, HEK293 cells were transiently transfected with GFP along with control, Op-IAP, VIAF, or Op-IAP and VIAF expression plasmids in the absence and presence of Bax. Sixteen hours after transfection, viability was determined by morphological examination of GFP-positive cells. B–D, HEK293 cells were transfected with siRNA oligonucleotides targeting GFP, VIAF, or survivin. 48 h later, cells were transfected with Bax. B, suppression of VIAF and survivin expression was confirmed by immunoblot analysis. Note that siGFP had no effect on either VIAF or survivin levels, and siRNA to VIAF and survivin were highly specific to each gene. C, lysates from transfected HEK293 cells were subjected to a fluorogenic caspase activity assay. D, caspase-3 processing in lysates from HEK293 cells was determined by immunoblot analysis using an antibody that only recognizes the cleaved forms of caspase-3.

A.

B.

C.

D.
Op-IAP ubiquitates VIAF. HEK293 cells were transiently transfected with His-tagged ubiquitin and either control (pEBB) or FLAG-tagged VIAF expression plasmids along with control, HA-Op-IAP or FLAG-tagged VIAF expression plasmids. The ubiquitin proteins (VIAF) in precipitated complexes was detected by immunoblot analysis. Equivalent protein expression was confirmed by immunoblot analysis of input samples using anti-FLAG and anti-HA, respectively.

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REFERENCES
1. Deveraux, Q. L., and Reed, J. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10170–10175
2. Hawkins, C. J., Ekert, P. G., Uren, A. G., Holmgreen, S. P., and Vaux, D. L. (1996) Cell 85, 1319–1328
3. Manji, G. A., Hozak, R. R., LaCount, D. J., and Friesen, P. D. (1997) J. Biol. Chem. 272, 28824–28830
4. Jordan, B. W., Dinev, D., Lemellay, V., Troppmair, J., Gotz, R., Wixler, L., Sendtner, M., Ludwig, S., and Rapp, U. (2001) J. Biol. Chem. 276, 4708–4714
5. Riedl, S. J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C., Fesik, S. W., and Reed, J. C. (2000) Genes & Dev. 14, 2206–2208
6. Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, S. H., Herrmann, J. M., and Fesik, S. W. (1999) J. Clin. Invest. 104, 10170–10175
7. Yamaguchi, K., Nagai, S., Ninomiya-Tsuji, J., Nishita, M., Tamai, K., Irie, K., Ueno, N., Nishida, E., Shihbuya, H., and Matsumoto, K. (1999) EMBO J. 18, 179–187
8. Shiozaki, E. N., Chai, J., Rigotti, D. J., Riedl, S. J., Li, P., Srinivasula, S. M., Alnemri, E. S., Fairman, R., and Shi, Y. (2003) Mol. Cell 11, 519–527
9. Huang, Q., Deveraux, Q. L., Maeda, S., Salvesen, G. S., Stennicke, H. R., Herrmann, J. M., and Fesik, S. W. (1999) Nature 401, 818–822
10. Vaux, D. L., and Silke, J. (2003) Nature 421, 112–116
11. Liston, P., Ackermann, E. J., Bennett, C. F., Rothermel, A. L., Plescia, J., Tognin, S., Villa, A., Marchisio, P. C., and Altieri, D. C. (1999) Mol. Cell. Biol. 19, 3300–3309
12. Rees, D. C., and Reed, J. C. (1998) Trends Cell Biol. 8, 323–328
13. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) J. Cell Biol. 138, 38699–38706
14. Joazeiro, C. A. P., Bonfoco, E., Kamada, S., Leverson, J. D., and Fesik, S. W. (2000) Cell 100, 26542–26549
15. Chai, J., Shiozaki, E., Srinivasula, S. M., Wu, Q., Dataa, P., Alnemri, E. S., and Herrmann, J. M., and Fesik, S. W. (1999) Nature 401, 818–822
16. Li, X., Yang, Y., and Ashwell, J. D. (2002) J. Biol. Chem. 277, 7003–7014
17. Yamaguchi, K., Nagai, S., Ninomiya-Tsuji, J., Nishita, M., Tamai, K., Irie, K., Ueno, N., Nishida, E., Shihbuya, H., and Matsumoto, K. (1999) EMBO J. 18, 179–187
18. Levkau, B., Garton, K. J., Ferri, N., Kloke, K., Nofer, J. R., Baha, H. A., Raines, E. W., and Breitbard, G. (2001) Circ. Res. 89, 282–290
19. Hofer-Warbinek, R., Schmid, J. A., Stehlik, C., Binder, B. R., Lipp, J., and de Vlaming, M. (2001) J. Biol. Chem. 276, 22064–22068
20. Matsumoto, K., Hofmann, B. D., and Reed, J. C. (2000) J. Biol. Chem. 275, 1746–1750
21. Deveraux, Q. L., and Reed, J. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6707–6711
22. Deveraux, Q. L., and Ulevitch, R. J. (1999) Mol. Cell. Biol. 19, 2168–2174
23. Birnbaum, M. J., Clem, R. J., and Miller, L. K. (1994) J. Biol. Chem. 269, 2921–2927
24. Sendtner, M., Ludwig, S., and Rapp, U. R. (2001) J. Cell Biol. 154, 1283–1287
25. Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, S. H., Herrmann, J. M., and Fesik, S. W. (1999) J. Clin. Invest. 104, 10170–10175
26. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) J. Cell Biol. 138, 38699–38706
27. Manji, G. A., Hozak, R. R., LaCount, D. J., and Friesen, P. D. (1997) J. Biol. Chem. 272, 1680–1683
28. Deveraux, Q. L., Schmaier, A. H., Schmieder, B. A., and Rapp, U. R. (1991) J. Biol. Chem. 266, 8877–8880
29. Herrmann, J. M., and Fesik, S. W. (2000) J. Biol. Chem. 275, 28824–28830
30. Shiozaki, E. N., Chai, J., Rigotti, D. J., Riedl, S. J., Li, P., Srinivasula, S. M., Alnemri, E. S., Fairman, R., and Shi, Y. (2003) Mol. Cell 11, 519–527
31. Deveraux, Q. L., and Reed, J. C. (1999) Trends Cell Biol. 9, 223–228
32. Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, S. H., Herrmann, J. M., and Fesik, S. W. (1999) Nature 401, 818–822
33. Herrmann, J. M., and Fesik, S. W. (1999) Nature 401, 818–822
34. Yamaguchi, K., Nagai, S., Ninomiya-Tsuji, J., Nishita, M., Tamai, K., Irie, K., Ueno, N., Nishida, E., Shihbuya, H., and Matsumoto, K. (1999) EMBO J. 18, 179–187
35. Chai, J., Shiozaki, E., Srinivasula, S. M., Wu, Q., Dataa, P., Alnemri, E. S., and Shi, Y. (2001) Cell 104, 769–780
36. Shiozaki, E. N., Chai, J., Rigotti, D. J., Riedl, S. J., Li, P., Srinivasula, S. M., Alnemri, E. S., Fairman, R., and Shi, Y. (2003) Mol. Cell 11, 519–527
37. Sun, C., Cai, M., Gunasekera, A. H., Meadows, D., Wu, H., Chen, J., Zhang, H., Wu, W., Xu, N., Ng, S. C., and Fesik, S. W. (1999) Nature 401, 818–822
38. Riedl, S. J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C., Fesik, S. W., Liddington, R. C., and Salvesen, G. S. (2001) Cell 101, 791–800
39. Liston, P., Fong, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. (2000) Science 287, 884–877
40. Duckett, C. S., Nava, V. E., Gedrich, R. W., Clem, R. J., Van Dongen, J. L., and Fesik, S. W. (1999) J. Biol. Chem. 274, 22064–22068
41. Joazeiro, C. A. P., Bonfoco, E., Kamada, S., Leeverson, J. D., and Hunter, T. (2000) J. Biol. Chem. 275, 26661–26664
VIAB Binds Op-IAP and Regulates Caspase Activation

62. Yu, H., and Kopito, R. R. (1999) J. Biol. Chem. 274, 36852–36858
63. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
64. Johnson, B. W., Cepero, E., and Boise, L. H. (2000) J. Biol. Chem. 275, 31546–31553
65. Duckett, C. S., Gedrich, R. W., Gilfillan, M. C., and Thompson, C. B. (1997) Mol. Cell. Biol. 17, 1535–1542
66. Beltrami, R., Plescia, J., Wilkinson, J. C., Duckett, C. S., and Altieri, D. C. (2004) J. Biol. Chem. 279, 2077–2084
67. Lahue, M. J., Blum, K., Danner, S., and Krasel, C. (1996) Biochem. Soc. Trans. 24, 975–980
68. Lopez, P., Yaman, R., Lopez-Fernandez, L. A., Vidal, F., Puel, D., Clerc, P., Cuzin, F., and Rassoulzadegan, M. (2003) J. Biol. Chem. 278, 1751–1757
69. Flanary, P. L., DiBello, P. R., Estrada, P., and Dohlman, H. G. (2000) J. Biol. Chem. 275, 18462–18469
70. Miura, M., Friedlander, R. M., and Yuan, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8318–8322
71. Fesik, S. W., and Shi, Y. (2001) Science 294, 1477–1478
72. Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) EMBO J. 16, 6914–6925
73. Silke, J., Verhagen, A. M., Ekert, P. G., and Vaux, D. L. (2000) Cell Death Differ. 7, 1275
74. Wright, C. W., and Clem, R. J. (2001) J. Biol. Chem. 277, 2454–2462
75. Jacob, J., Louis, J. M., Richter, B. W., Duckett, C. S., and Torchia, D. A. (2004) J. Biol. Chem. 279, 197–198
76. Lee, R. H., Whelan, J. P., Lolley, R. N., and McGinnis, J. F. (1988) Exp. Eye. Res. 46, 829–840
77. Craft, C. M., Xu, J., Slepak, V. Z., Zhan-Poe, X., Zhu, X., Brown, B., and Lolley, R. N. (1998) Biochemistry 37, 15758–15772
78. Wilkinson, J. C., Wilkinson, A. S., Scott, F. L., Csomos, R. A., Salvesen, G. S., and Duckett, C. S. (2004) J. Biol. Chem. 279, 51082–51090