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Neuronal Apoptosis-inhibitory Protein Does Not Interact with Smac and Requires ATP to Bind Caspase-9*

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The neuronal apoptosis-inhibitory protein (NAIP) is the founding member of the mammalian family of inhibitor of apoptosis (IAP) proteins (also known as BIRC proteins) and has been shown to be antiapoptotic both *in vivo* and *in vitro*. The 160-kDa NAIP contains three distinct regions: an amino-terminal cluster of three baculoviral inhibitory repeat (BIR) domains, a central nucleotide binding oligomerization domain (NOD), and a carboxyl-terminal leucine-rich repeat (LRR) domain. The presence of the NOD and LRR domains renders NAIP unique among the IAPs and suggests that NAIP activity is regulated in a manner distinct from that of other members of the family. In this report, we examined the interaction of various regions of NAIP with caspase-9 and Smac. Recombinant NAIPs with truncations of the carboxyl-terminal LRR or NOD-LRR regions bound to caspase-9. In contrast, the full-length protein did not, suggesting some form of structural autoregulation. However, the association of the wild type full-length protein with caspase-9 was observed when interaction analysis was performed in the presence of ATP. Furthermore, mutation of the NAIP ATP binding pocket allowed full-length protein to interact with caspase-9. Thus, we conclude that NAIP binds to caspase-9 with a structural requirement for ATP and that in the absence of ATP the LRR domain negatively regulates the caspase-9-inhibiting activity of the BIR domains. Interestingly, and in contrast to the X-chromosome-linked inhibitor of apoptosis protein (XIAP), NAIP-mediated inhibition of caspase-9 was not countered by a peptide containing an amino-terminal IAP binding motif (IBM). Consistent with this observation was the failure of Smac protein to interact with the NAIP BIR domains. These results demonstrate that NAIP is distinct from the other IAPs, both in demonstrating a ligand-dependent caspase-9 interaction and in demonstrating a distinct mechanism of inhibition.

Apoptosis, or programmed cell death, is a controlled process of cellular disassembly that occurs in response to internal or external apoptotic signals. Caspases, a class of cysteine proteases, are activated in a cascade in most apoptotic pathways. Caspases proteolytically digest specific substrates and are responsible for most of the characteristic morphological changes of an apoptotic cell. The activity of the caspases is restrained through a family of proteins termed inhibitors of apoptosis (IAPs)[1] (for a recent review, see Ref. 1). IAPs are characterized by the presence of one or more baculoviral IAP repeat (BIR) domains (2), which are capable of inhibiting caspases (3–5). Seven mammalian IAPs have been identified to date, including the neuronal apoptosis-inhibitory protein (NAIP), XIAP, c-IAP1, c-IAP2, Ts-IAP, livin, and survivin (6–12). NAIP contains three BIR domains at its amino terminus, a centrally located nucleotide binding oligomerization domain (NOD), and a carboxyl-terminal leucine-rich repeat (LRR, Fig. 1). The *naip* gene was initially identified during the analysis of chromosomal deletions responsible for spinal muscular atrophy (7) and was the first mammalian IAP homologue to be described. NAIP and the other IAPs have been shown to inhibit the effector caspases caspase-3 and -7 (5, 11, 13, 14). In addition, XIAP, c-IAP1, and c-IAP2 have also been shown to inhibit caspase-9 (14–16), the initiator caspase recruited by Apaf-1 in the presence of cytochrome *c* and ATP. This complex, termed the apoptosome, initiates cell death through the intrinsic mitochondrial pathway. Several proteins have been identified recently in the mitochondria (Smac/DIABLO (17–19) and Omi/HtrA2 (20, 21)) or in association with the endoplasmic reticulum (GSPT1; Ref. 22) that interfere with caspase-9-IAP binding, thereby displacing IAPs and allowing unrestrained caspase activity. These proteins all require proteolytic processing to expose an amino-terminal tetrapeptide sequence termed an IAP binding motif (IBM). IBM-containing proteins bind to a site on BIR3, inhibiting that site from interacting with the amino terminus of the small subunit of partially processed caspase-9. Although the antiapoptotic and caspase-3/7-inhibitory activities of NAIP have been documented previously (4, 23), NAIP interaction with caspase-9 and the effects of Smac on this interaction have not been investigated.

Although the BIR domains endow NAIP with structural and functional similarities to other members of the IAP family of proteins, NAIP is unique in that it possesses a NOD. NOD-containing proteins are a diverse group with functions ranging from involvement in apoptosis (*i.e.* Apaf-1) to pathogen recognition (*i.e.* plant R genes) (24, 25). As the name implies, the NOD is proposed to mediate oligomerization in a nucleotide-dependant manner. Many of the NOD-containing proteins consist of an amino-terminal "effector" domain (such as the CARD of Apaf-1), the central NOD, and a carboxyl-terminal "sensor"

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[1] The abbreviations used are: IAP, inhibitor of apoptosis; BIR, baculoviral IAP repeat; NAIP, neuronal apoptosis-inhibitory protein; XIAP, X-chromosome-linked inhibitor of apoptosis protein; NOD, nucleotide binding oligomerization domain; LRR, leucine-rich repeat; IBM, IAP binding motif; CARD, caspase recruitment domain; GST, glutathione *S*-transferase; HA, hemagglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; Ipaf, interleukin-1β-converting enzyme protease-activating factor.

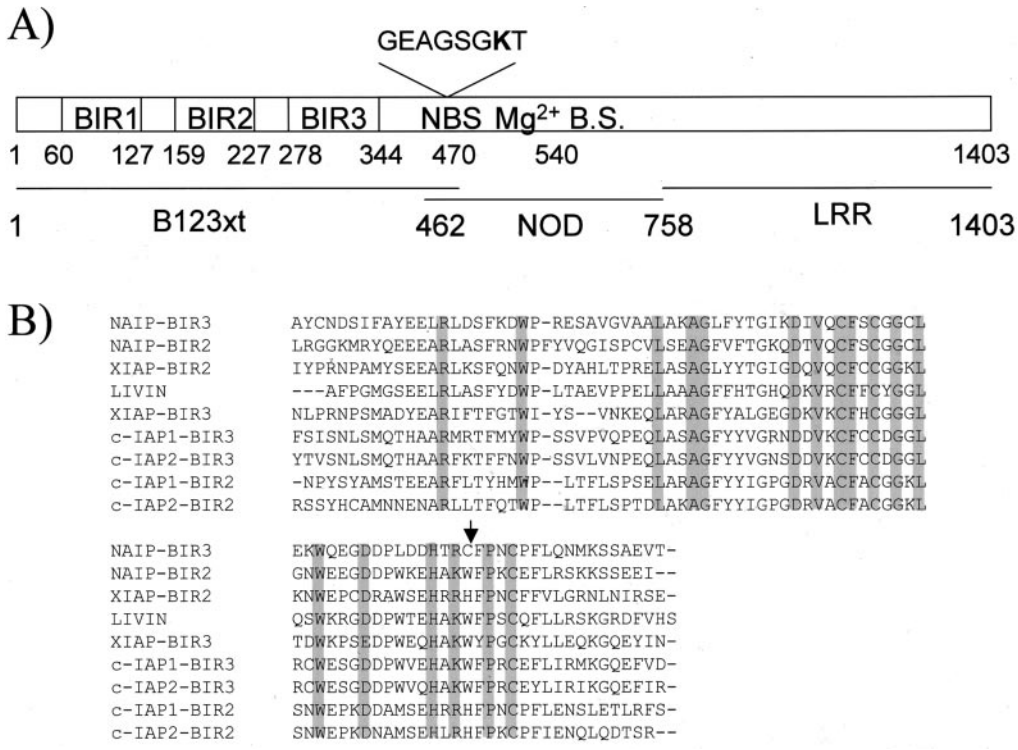

FIG. 1. *A*, organization of domains and subdomains of neuronal apoptosis-inhibitory protein. The regions of NAIP encoded by their respective constructs are *underlined*. The mutated lysine in the nucleotide binding site (*NBS*) is shown in *bold*. *B*, alignment of the BIR3 domains of IAPs.

domain (such as the WD-40 repeats of Apaf-1 that bind cytochrome *c*). Given the organizational similarity between NAIP and the NOD proteins, we investigated the effects of the NOD and LRR domains of NAIP with respect to the activity of the amino-terminal BIR domains.

In this report we show that the BIR3 domain of NAIP is capable of interaction with caspase-9 but not with Smac. However, in the context of the full-length protein, this interaction depends on the presence of ATP. Surprisingly, ATP binding does not appear to alter the quarternary structure of NAIP. Our results suggest that ATP binding to the NOD triggers a conformational change that activates BIR3-mediated caspase-9 inhibition that is not modulated by Smac.

MATERIALS AND METHODS

Expression and Purification of XIAP and NAIP BIR3 Proteins—Isolation of NAIP cDNAs and the construction of pGEX-NAIP-BIR3 and pGEX-XIAP-BIR3 have been described in detail elsewhere (4). NAIP and XIAP glutathione *S*-transferase (GST)-tagged BIR3 proteins were overexpressed in *Escherichia coli* strain BL21-TrxB-DE3 (Novagen Inc.). Overnight bacterial cultures were diluted 1 in 10 in LB medium and incubated at room temperature. When the absorbance at 600 nm reached 1.2 units, cells were induced for the production of the recombinant proteins with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested after a 2-h induction. Cell paste was resuspended in buffer A (50 mM Tris-HCl, 0.1 M NaCl, 10 mM β-mercaptoethanol, 0.5% Triton X-100, pH 8.0) and mixed at 4 °C for 15 min. After a gentle sonication, the mixture was centrifuged at 20,000 × *g* for 15 min. The supernatant was incubated with glutathione-Sephadex 4B beads (Amersham Biosciences) and equilibrated with buffer A for 1 h. The resin was washed with buffer A followed by buffer B (buffer A supplemented to 0.2 M NaCl and 10% glycerol). Recombinant NAIP and XIAP BIR3 proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, 10% glycerol, 125 mM NaCl, 5 mM β-mercaptoethanol. Protein concentrations were measured by Bio-Rad protein assay according to the manufacturer's protocol. The purity of the proteins was determined by SDS-PAGE and was generally greater than 95%.

Construction of pcDNA3–6myc-NAIP Expression Plasmids—The coding region for full-length NAIP, B123-NOD, and the LRR regions of NAIP were amplified using the following primers: 5′-dGGATCCATGGCCACCCAGCAGAAAGCCT and 5′-dCTCGAGTACATCCTCAATATGCCCCA; 5′-dGGATCCATGGCCACCCAGCAGAAAGCCT and 5′-dCTCGAGTCCAGGAGTTCAATCAGCCTCAT; and 5′-dGGATCCGATTCAGATAGGCAGGAACAT and 5′-dCTCGAGTACATCCTCAATATGCCCCA, respectively. The amplified products were digested with BamHI and XhoI and subcloned into the corresponding sites of pcDNA3–6myc. The integrity of the constructs was determined by sequencing the entire coding region. pcDNA3–6myc is a modified version of pcDNA3 (Invitrogen) that contains six repeats of the myc epitope recognized by the 9E10 monoclonal antibody, followed by BamHI and XhoI sites. The construction of HA-tagged ubiquitin Smac is described elsewhere (26).

Transfection of HeLa Cells—HeLa cells (ATCC) were transfected when ~60% confluent using LipofectAMINE Plus reagent according to the manufacturer's directions (Invitrogen). Cells were harvested 24 h post-transfection.

Immunoprecipitation of myc-tagged IAPs and Their Interaction with Caspase-9—HeLa cells transfected with pcDNA3–6myc-IAPs or pcDNA3–6myc-LRR were lysed in buffer A containing leupeptin, aprotinin, and pepstatin A followed by a 30-min incubation at 4 °C. Following centrifugation at 10,000 × *g* for 10 min, 1 μg of anti-myc monoclonal antibody (Sigma) was added to the supernatant and incubated for 2 h at 4 °C. Twenty μl of 50% protein A-agarose was added and incubated for another 2 h. Immunoprecipitates were washed extensively and were immediately used for caspase-9 interaction studies as follows. Fifty units of active truncated caspase-9 (20 + 12-kDa subunits, Biomol Research Laboratories) were incubated with immunoprecipitated samples for 3 h at 4 °C and washed. The samples were analyzed by Western blot using anticaspase-9 antibody (Biomol Research Laboratories).

Site-directed Mutagenesis—Primers 5′-dGAAGCTGGAAGTGGAACGACGGTCCTCCTGAAG and 5′-dCTTCAGGAGGACCGTCGTTCCACTTCCAGCTTC-3′ were employed to mutate lysine 476 to threonine using a QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The mutant construct was sequenced using both strands as templates.

Size Exclusion Chromatography—pcDNA3–6myc-NAIP-transfected HeLa cells were resuspended in 50 mM Hepes, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, and 0.2% CHAPS buffer, lysed by two freeze-thaw cycles, and centrifuged at 20,000 × *g* for 10 min. The supernatant was loaded onto a Superose 6 HR 10/30 column equilibrated with the same buffer. To study the effect of dATP or ATP on the size of NAIP, the supernatant was incubated with 1 mM dATP or ATP for 10 min on ice and subsequently loaded onto the column. Chromatography was carried out at a flow rate of 0.4 ml/min at 4 °C using an ÄKTA Explorer 900 system

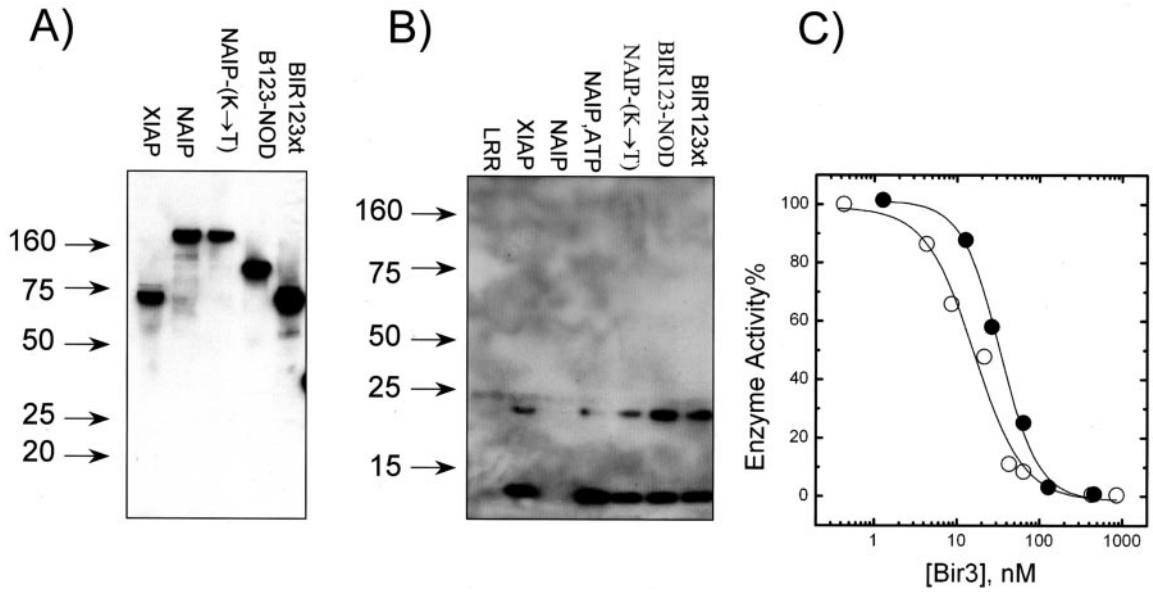

FIG. 2. **Interaction of caspase-9 with XIAP and various NAIP constructs.** *A*, Western blot analysis of HeLa cells transfected with myc-XIAP and various myc-NAIP constructs using anti-myc monoclonal antibody. *B*, the myc-LRR region of NAIP (negative control), myc-XIAP (positive control), and myc-NAIP variants were immunoprecipitated using anti-myc monoclonal antibody. They then were incubated with recombinant caspase-9 and washed. The interaction was assessed by Western blot using anticaspase-9 antibody. *C*, recombinant caspase-9 inhibition by GST-XIAP BIR3 (*open circles*) and GST-NAIP BIR3 (*filled circles*).

(Amersham Biosciences). Fractions (0.5 ml) were collected and analyzed by Western blot using anti-myc 9E10 antibody (Sigma).

Caspase-9 Inhibition Assay—Caspase-9 assays were performed in 100 mM MES, pH 6.5, 10% polyethylene glycol, 0.1% CHAPS, and 10 mM dithiothreitol using 100 units of caspase-9 and 2 mM Ac-LEHD-pNA as a substrate. The hydrolysis of the substrate was monitored at 405 nm at 30 °C for 60–90 min. The inhibitory constants were obtained using a range of inhibitor concentrations while maintaining constant levels of both the substrate and enzyme. To study the effect of AVPFVASLPN peptide (AVPF-peptide) on reversing the inhibition of caspase-9 by the BIR3 domains of NAIP and XIAP, BIR3 proteins were incubated with the final concentration of 30 μM AVPF-peptide prior to incubation with caspase-9.

RESULTS

NAIP Interaction with Caspase-9 Is ATP-dependent—Previous experiments have established that the BIR domains of NAIP can inhibit caspase-3 and -7. Functional domain-mapping experiments established that NAIP BIR2 potently inhibited caspase-3 to a degree equivalent to full-length XIAP (K_i = 10 ± 6 nM and K_i = 16 ± 6 nM, respectively; Ref. 4). Intriguingly, NAIP BIR3 displayed significantly poorer inhibition (K_i = 185 ± 15 nM) yet provided significantly better cytoprotection in cell survival assays, suggesting additional, as yet uncharacterized activities for BIR3. Furthermore, these experiments utilized recombinant proteins consisting of isolated BIR domains and thus did not assess BIR domain function in the context of the full-length protein. Given the known interaction of XIAP BIR3 with caspase-9 (14), we sought to determine whether NAIP also binds caspase-9. We used epitope-tagged recombinant proteins expressed and immunoprecipitated from tissue culture cells as a source of NAIP or XIAP and examined their ability to interact with purified active caspase-9. This approach was also used to determine what effect, if any, the NAIP NODs and LRR domains had on this interaction.

Caspase-9 interaction with NAIP was examined by Western blot analysis of affinity-purified 6myc-NAIP using anti-caspase-9 antibody (Fig. 2*B*) with 6myc-XIAP used as a positive control. Transfection efficiency was monitored by Western blot of whole cell lysate using anti-myc monoclonal antibody (Fig. 2*A*). As seen in Fig. 2*B*, *second* and *third lanes*, NAIP did not interact with caspase-9 under conditions that allowed XIAP to bind. However, the addition of ATP to the reaction mixture resulted in a NAIP-caspase-9 interaction (Fig. 2*B*, *fourth lane*), suggesting that NAIP undergoes an ATP-dependent conformational change necessary for the interaction to occur. Previous studies have shown that lysine to threonine mutations within the ATP binding site render NOD proteins constitutively active. Thus to further investigate the ATP dependence of NAIP, lysine 476 in the putative nucleotide binding site was mutated to threonine. Interestingly, NAIP caspase-9 interaction was then observed in the absence of ATP (Fig. 2*B*, *fifth lane*). We therefore conclude that the caspase-9-NAIP interaction has a structural requirement for ATP, rather than an energetic one requiring ATP hydrolysis. Finally, deletion of the carboxyl-terminal LRR domain in the BIR123-NOD construct eliminates the requirement for ATP (Fig. 2*B*, *sixth lane*), as does the deletion of both the NOD and the LRR domain (Fig. 2*B*, *BIR123xt* (*seventh lane*)). Taken together, these results suggest that the LRR region in some way sequesters the BIR domains of NAIP, a sequestration which is reversed by ATP binding.

NAIP BIR3 Inhibits Caspase-9—Having established that the NAIP BIR domains can bind caspase-9, we next sought to determine the functional consequences of this interaction on caspase-9 enzymatic activity. Previous reports have established that the BIR3 domains of the three BIR-containing IAPs (XIAP, c-IAP1, and c-IAP2) specifically bind and inhibit caspase-9. We therefore expressed and purified a GST-NAIP BIR3 fusion protein from *E. coli*. GST-XIAP BIR3 was expressed and purified for use as a positive control. Chromogenic assays using a caspase-9 peptide substrate revealed that both XIAP BIR3 and NAIP BIR3 proteins function as potent inhibitors of caspase-9 (Fig. 2*C*), with IC_{50} values of 17 and 33 nM, respectively. We also sought to confirm caspase-9 inhibition by full-length NAIP. However, we could not address this issue because the full-length NAIP is extensively degraded when expressed either in *E. coli* or in eukaryotic cells using the recombinant adeno-NAIP virus.

NAIP Exists as a Tetramer in Both the Presence and Absence of ATP—NOD-mediated homo-oligomerization has been reported previously (27, 28). The most highly characterized of these is Apaf-1, which in its latent state is present as a monomer. Cytochrome *c* released from the intramembrane space of

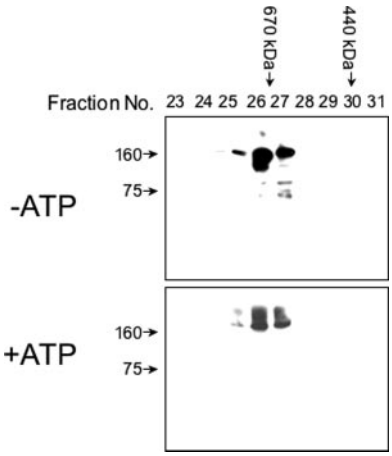

FIG. 3. **Western blot analysis of NAIP eluted from a size exclusion column in the presence and the absence of ATP.** The presence of weak bands on the blot may be partly because of nonspecific antibody binding and/or partial proteolysis of the NAIP.

the mitochondria binds to the Apaf-1 WD-40 sensor domain and allows an ATP-dependent self-oligomerization process mediated through the CARD (29). To investigate potential changes in the higher order structure of NAIP in the presence or absence of ATP, we performed size exclusion chromatography. NAIP was observed to migrate at a molecular mass of ~680 kDa (Fig. 3), roughly equivalent to four subunits of the 160-kDa NAIP monomer. Immunoprecipitation analysis of NAIP with [^{35}S]methionine-labeled cell extract did not reveal the presence of additional protein species within this complex (data not shown). We therefore concluded that NAIP exists as a tetramer. Surprisingly, a similar migration pattern was observed for the wild type protein in the presence of ATP (Fig. 3) or dATP as well as for the constitutively active K476T mutant (data not shown). We therefore propose that ATP binding facilitates the interaction of caspase-9 and NAIP through a change in the conformation of NAIP but not through a change in its quaternary structure.

NAIP Is Not Cleaved by Caspase-9—Our results indicate that the LRR domain of NAIP functionally sequesters the BIR domains and maintains NAIP in a latent state. Caspase processing of c-IAP1 and XIAP has been reported previously (15, 30). XIAP cleavage between BIR2 and BIR3 was originally proposed to liberate the two functional halves of the protein, with BIR1-2 free to inhibit caspase-3 or -7 and the BIR3-RING fragment free to inhibit caspase-9. Because we observed that truncated NAIPs lacking the LRR domain and/or NOD were constitutively active with respect to caspase binding, we sought to determine whether NAIP undergoes caspase-dependent cleavage *in vivo*. HeLa cells were co-transfected with NAIP or XIAP expression vectors and a plasmid encoding caspase-9. Western blot analysis with anti-myc antibody demonstrated that approximately half of the expressed XIAP was cleaved to a size consistent with the reported processing between BIR2 and BIR3. This processing was caspase-9-dependent as no processing was observed in the absence of caspase-9 (Fig. 2*A*). In contrast, no evidence of NAIP processing was observed, either with the wild type protein or with the "activated" forms bearing the NOD point mutation or the truncation of the LRR domain and the NOD (Fig. 4). The absence of NAIP cleavage under conditions in which XIAP is proteolytically processed favors our contention that an ATP-dependent conformational change in NAIP, rather than its cleavage, enables NAIP to bind caspase-9.

NAIP Does Not Interact with Smac—Both NAIP and XIAP bind and inhibit caspase-9, yet there are significant amino acid differences in their respective BIR3 binding pockets (Fig. 1). This binding pocket is believed to bind the amino terminus of the small subunit of caspase-9 following autocatalytic cleavage at Asp^{315}. This processing event exposes a tetrapeptide sequence termed an IAP binding motif, which has also been identified in several *Drosophila* and mammalian proapoptotic proteins. IBM-containing proteins are believed to mediate their proapoptotic function, at least in part, by competing for interaction with the IAPs, thus displacing bound caspases, which are then free to continue amplifying the caspase cascade (for review, see Ref. 31).

To determine whether NAIP is capable of interacting with IBM-containing proteins, HeLa cells were co-transfected with NAIP or XIAP expression vectors and a plasmid that expresses mature cytosolic Smac-HA protein lacking the mitochondrial leader peptide (26). myc-tagged NAIPs and XIAPs were immunoprecipitated using anti-myc antibody and immunoblotted with anti-HA antibody for the detection of Smac (Fig. 5*A*). Although XIAP bound the Smac protein, wild type NAIP did not interact with Smac. Furthermore, the presence of ATP, which triggers NAIP-caspase-9 interaction, did not induce binding. The constitutively active forms of NAIP also failed to bind Smac (Fig. 5*A*).

To substantiate these findings, we assessed whether an AVPF-initiating peptide would reverse caspase-9 inhibition by NAIP BIR3. As expected, a 30-micromolar excess of the AVPF-peptide almost fully restored caspase-9 activity when XIAP BIR3 was employed as an inhibitor (Fig. 5*C*). In contrast, no discernible effect on NAIP BIR3-mediated inhibition of caspase-9 was observed (Fig. 5*B*). These observations support our contention that NAIP is unique among the IAPs in both its activation mechanism and its failure to be inhibited by IBM-containing proteins such as Smac.

DISCUSSION

NAIP, the founding member of the mammalian IAPs, is a 160-kDa protein with three distinct regions: an amino-terminal cluster of three BIR domains, a central NOD, and a carboxyl-terminal LRR domain. These regions consist of ~350, 300, and 640 amino acids, respectively (Fig. 1). In this respect NAIP is a much larger protein than the other known IAPs, the largest of which is less than 70 kDa. In fact, classification of NAIP as an IAP is based entirely on the conservation of the BIR domains, whereas the overall organization of the protein suggests membership in the NOD family of proteins with diverse physiological functions (24, 25). The antiapoptotic function of full-length NAIP has been documented in a number of tissue culture and animal model systems (2, 23, 32, 33), and the caspase-3- and -7-inhibiting activity of isolated BIR domains has been investigated *in vitro* (4). However, to date, no studies have examined the effect of the NOD and the LRR domain on the function of full-length NAIP.

To address this issue, the association of caspase-9 with myc-tagged IAPs was investigated by affinity pull-down of caspase-9. Although caspase-9 interacted with full-length XIAP, full-length NAIP did not show any binding, suggesting either that NAIP and caspase-9 do not interact or that the binding site for caspase-9 is somehow maintained in a latent state. The presence of a potential ATP binding pocket in the NOD of NAIP prompted us to perform the pull-down in the presence of ATP. Interestingly, the addition of ATP allowed the interaction of full-length wild type NAIP with caspase-9, and mutation of lysine 476 to threonine, which is conserved among all ATP-binding proteins, abolished this requirement. Taken together, these data suggest that ATP binding within the NOD induces a conformational change that leads to the exposure of the caspase-9 binding site within the BIR domains

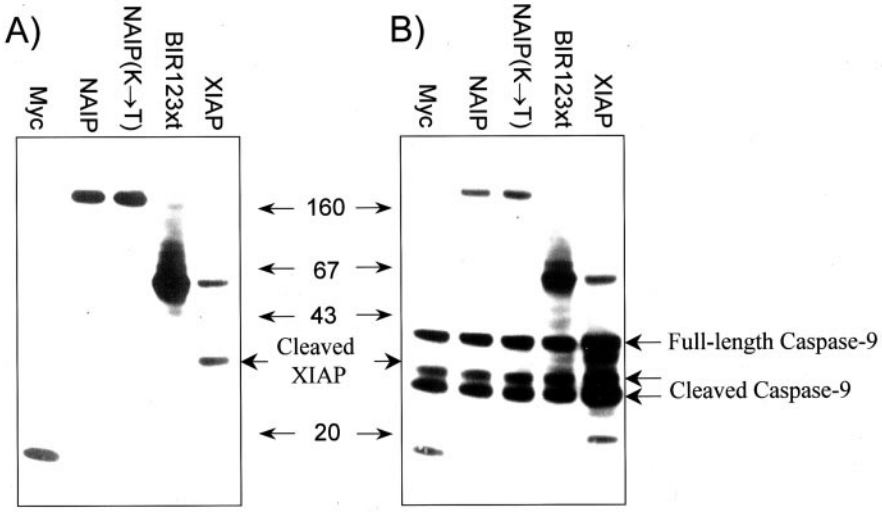

FIG. 4. **Cleavage of XIAP but not NAIP when co-transfected with caspase-9.** *A*, Western blot analysis of XIAP and NAIP variants using anti-myc monoclonal antibody. *B*, the membrane from *A* was probed with anticaspase-9 antibody without stripping anti-myc monoclonal antibody.

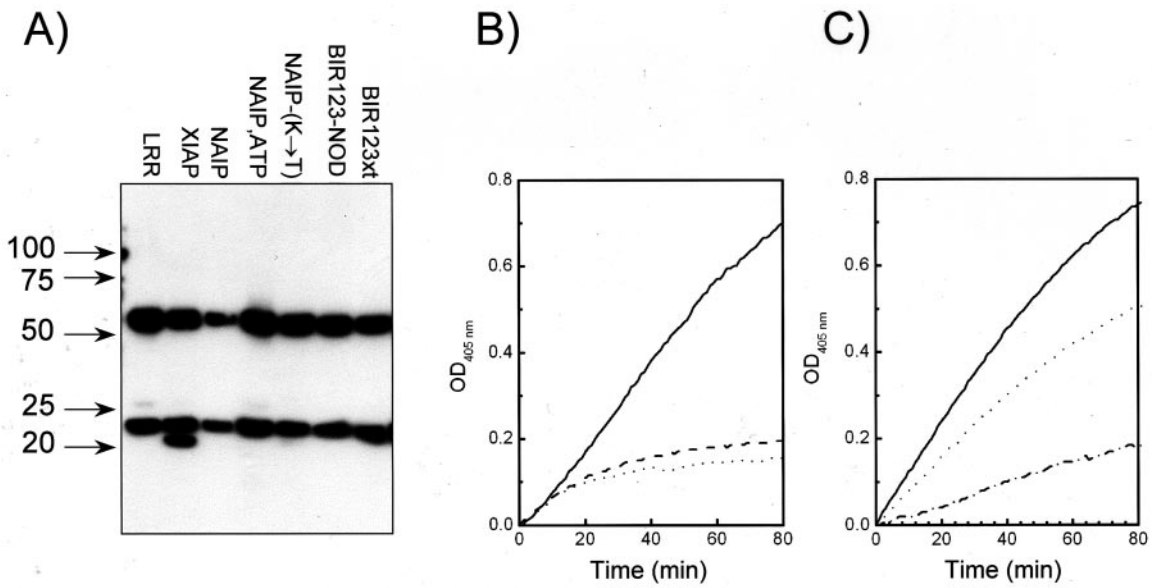

FIG. 5. **Interaction of Smac with XIAP and NAIP variants.** *A*, the Smac-HA plasmid was co-transfected with either XIAP or NAIP plasmids. IAPs were immunoprecipitated using anti-myc monoclonal antibody and probed for the presence of Smac with anti-HA antibody. Caspase-9 activity in the absence (*solid lines*) or presence (*dashed lines*) of 50 nM NAIP-BIR3 (*B*) or 30 nM XIAP-BIR3 (*C*) was measured. The caspase-9-inhibitory activity of both NAIP-BIR3 and XIAP-BIR3 (*dotted lines*) in the presence of 30 μM AVPF-initiating peptide was assessed.

of NAIP. We thus propose that the LRR region of the NAIP functions as a negative regulator of the BIR domains, hindering the interaction with caspase-9. This concept is based on the conformational changes that occur in Apaf-1, which in the latent state is folded in such a way that the carboxyl-terminal WD-40 repeat domain occludes the amino-terminal CARD. Apaf-1 interaction with cytochrome *c* and ATP is required for the CARD interaction with other proteins. Consistent with this model, truncation of the NAIP carboxyl-terminal LRR region alleviated the ATP requirement for NAIP-caspase-9 interaction. Intriguingly, interleukin-1β-converting enzyme protease-activating factor (Ipaf, also known as CARD12) is also a NOD family member and exhibits the highest overall amino acid similarity to NAIP. Ipaf contains a CARD-NOD-LRR organization and binds caspase-1 via CARD-CARD interactions (34). As is the case with NAIP, truncation of the LRR domain of Ipaf renders the protein constitutively active. The effect of nucleotide triphosphate binding on Ipaf-1 activity has not yet been studied.

Having established a direct interaction between caspase-9 and NAIP, we next established whether this interaction is accompanied by inhibition of caspase-9. Previous work has demonstrated that the third BIR domains of XIAP, c-IAP1, and c-IAP2 are responsible for caspase-9 inhibition (16, 35). We therefore expressed and purified the BIR3 domains of both XIAP and NAIP as GST fusions in *E. coli*. Both BIR3 proteins were found to inhibit caspase-9 with very low IC_{50} values, the averages of which were 17 and 33 nM for XIAP and NAIP, respectively. Although XIAP is frequently cited in the literature as the most potent of the IAPs, the IC_{50} values for the individual BIR domains of NAIP and XIAP are comparable for caspase-3, -7, and -9 interaction (this work and Ref. 4).

We then decided to see whether IBM-containing proteins, such as Smac, could reverse NAIP-mediated caspase-9 inhibition. In contrast to XIAP, the NAIP BIR3 domain does not appear to interact with Smac. Furthermore, an AVPF-containing peptide reversed XIAP-mediated but not NAIP-mediated caspase-9 inhibition. The crystal structure of XIAP BIR3-Smac complexes revealed that the proline in the P_3 position of the IBM peptide contacts with the indole ring of Trp^{323} of XIAP (18, 19). Trp^{323} is conserved among all IAP BIR3 domains except Ts-IAP and NAIP, which contains a cysteine residue in this

position (see Fig. 1). This change of amino acids from tryptophan to cysteine might explain, at least in part, why NAIP does not bind to Smac.

These results raise the seemingly contradictory issue of how NAIP is able to bind caspase-9 but not Smac, given that both of these interactions have been proposed to involve IBM-peptide interactions with the BIR3 binding pocket (36). Autocatalytic processing of caspase-9 at the sequence ^{315}DATPF generates the amino-terminal IBM of the small subunit of caspase-9 that binds XIAP BIR3. However, removal of the IBM by caspase-3 processing of caspase-9 at ^{330}DAISS does not prevent XIAP-mediated inhibition, which is maintained by extensive contacts between BIR3 and caspase-9 (37). These interactions are believed to maintain caspase-9 in a monomeric and inactive conformation (35, 38). Our results would suggest that NAIP BIR3 bypasses the initial IBM-mediated interaction and instead relies exclusively on similar surface contacts with caspase-9. Co-crystallization of NAIP BIR3 with caspase-9 will conclusively resolve this issue.

Several members of the NOD family of proteins have been shown to undergo homo-oligomerization through their respective NODs (24, 27, 39, 40). We therefore decided to assess the quaternary structure of NAIP in the presence and absence of ATP. NAIP was found to migrate at a molecular mass of ~700 kDa, suggesting that NAIP exists as a complex of four monomers or is associated with other cellular components. NAIP immunoprecipitation analysis indicated that no other proteins were associated with the complex (data not shown), supporting the proposed NAIP tetrameric structure. Furthermore, the presence of ATP or dATP did not alter the elution profile of NAIP in the size exclusion column experiments. Thus, it appears that the ATP-induced conformational change in NAIP that leads to interaction with caspase-9 is not accompanied by a change in the oligomeric structure of the protein. This finding does not, however, rule out the possibility that an unknown ligand(s) might interact with the LRR domain and alter the quaternary structure of NAIP.

XIAP has been shown to undergo caspase-mediated proteolytic processing during apoptosis. The cleavage of XIAP into BIR1-BIR2 and BIR3-RING fragments allows the domains to act independently with respect to caspase-3/7 and caspase-9 interactions (15). Consequently, we determined whether NAIP sustains similar proteolysis when co-transfected with a caspase-9 expression plasmid. Western blot analysis was used to confirm the production of p37 as well as p35 fragments of caspase-9, consistent with the proteolytic activation of this enzyme. In these experiments, XIAP was cleaved and generated a fragment with an approximate molecular mass of 45 kDa, consistent with the sum of the molecular masses of the amino-terminal 6myc epitope tag and the amino-terminal BIR1 and BIR2 domains. Unlike XIAP, neither NAIP nor any of carboxyl-terminal truncation mutants were cleaved, suggesting that at least under conditions that lead to the cleavage of XIAP, NAIP is not processed.

In summary, we have documented several unique and unexpected aspects of NAIP function. Previous experiments have demonstrated that NAIP is profoundly antiapoptotic *in vivo*, yet the mechanism of action remained poorly defined. Here we show that NAIP is unique among the IAPs, requiring an activation event, following which the BIR domains are roughly equivalent to XIAP in terms of potency. NAIP thus behaves much like other members of the NOD family of proteins, over 20 of which contain carboxyl-terminal LRR domains, including Ipaf, Nod1, Nod2, and cryopyrin (for review, see Ref. 41). In several cases, NOD protein LRR domains have been shown to be responsive to bacterial lipopolysaccharide or other pathogen-associated molecular patterns. Activation of proinflammatory cytokines such as interleukin-1β requires the activity of the group I caspases, which, when overexpressed, can activate the caspase cascade. The expression of NAIP in macrophages and the structural organization of the protein raise the possibility that NAIP function may be activated by pathogen-associated molecular patterns concomitant with proinflammatory caspases. We propose that a key role of NAIP may therefore be to allow caspase-1 activation while suppressing unintentional activation of caspase-3/7 and -9. If true, then mice with targeted deletion of the entire NAIP gene cluster should be exquisitely sensitive to proinflammatory stimuli that would normally result in interleukin production.

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REFERENCES

1. Martin, S. J. (2002) *Cell* **109,** 793–796
2. Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J. E., MacKenzie, A., and Korneluk, R. G. (1996) *Nature* **379,** 349–353
3. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) *Nature* **388,** 300–304
4. Maier, J. K., Lahoua, Z., Gendron, N. H., Fetni, R., Johnston, A., Davoodi, J., Rasper, D., Roy, S., Slack, R. S., Nicholson, D. W., and MacKenzie, A. E. (2002) *J. Neurosci.* **22,** 2035–2043
5. Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) *EMBO J.* **16,** 6914–6925
6. Rajcan-Separovic, E., Liston, P., Lefebvre, C., and Korneluk, R. G. (1996) *Genomics* **37,** 404–406
7. Roy, N., Mahadevan, M. S., McLean, M., Shutler, G., Yaraghi, Z., Farahani, R., Baird, S., Besner-Johnston, A., Lefebvre, C., Kang, X., Salih, M., Aubry, H., Tamai, K., Guan, X., Loannou, P., Crawford, T. O., de Jong, P. J., Surh, L., Ikeda, J., Korneluk, R. G., and MacKenzie, A. (1995) *Cell* **80,** 167–178
8. Farahani, R., Fong, W. G., Korneluk, R. G., and MacKenzie, A. E. (1997) *Genomics* **42,** 514–518
9. Kasof, G. M., and Gomes, B. C. (2001) *J. Biol. Chem.* **276,** 3238–3246
10. Ambrosini, G., Adida, C., and Altieri, D. C. (1997) *Nat. Med.* **3,** 917–921
11. Richter, B. W., Mir, S. S., Eiben, L. J., Lewis, J., Reffey, S. B., Frattini, A., Tian, L., Frank, S., Youle, R. J., Nelson, D. L., Notarangelo, L. D., Vezzoni, P., Fearnhead, H. O., and Duckett, C. S. (2001) *Mol. Cell. Biol.* **21,** 4292–4301
12. Lagace, M., Xuan, J. Y., Young, S. S., McRoberts, C., Maier, J., Rajcan-Separovic, E., and Korneluk, R. G. (2001) *Genomics* **77,** 181–188
13. Suzuki, Y., Nakabayashi, Y., Nakata, K., Reed, J. C., and Takahashi, R. (2001) *J. Biol. Chem.* **276,** 27058–27063
14. Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G. S., and Reed, J. C. (1998) *J. Biol. Chem.* **273,** 7787–7790
15. Deveraux, Q. L., Leo, E., Stennicke, H. R., Welsh, K., Salvesen, G. S., and Reed, J. C. (1999) *EMBO J.* **18,** 5242–5251
16. Deveraux, Q. L., Roy, N., Stennicke, H. R., Van Arsdale, T., Zhou, Q., Srinivasula, S. M., Alnemri, E. S., Salvesen, G. S., and Reed, J. C. (1998) *EMBO J.* **17,** 2215–2223
17. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) *Cell* **102,** 33–42
18. Liu, Z., Sun, C., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000) *Nature* **408,** 1004–1008
19. Wu, G., Chai, J., Suber, T. L., Wu, J. W., Du, C., Wang, X., and Shi, Y. (2000) *Nature* **408,** 1008–1012
20. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) *Mol. Cell* **8,** 613–621
21. Martins, L., Iaccarino, I., Tenev, T., Gschmeissner, S., Totty, N. F., Lemoine, N. R., Savopoulos, J., Gray, C. W., Creasy, C. L., Dingwall, C., and Downward, J. (2002) *J. Biol. Chem.* **277,** 439–444
22. Hegde, R., Srinivasula, S. M., Datta, P., Madesh, M., Wassell, R., Zhang, Z., Cheong, N., Nejmeh, J., Fernandes-Alnemri, T., Hoshino, S., and Alnemri, E. S. (2003) *J. Biol. Chem.* **278,** 38699–38706
23. Hutchison, J. S., Derrane, R. E., Johnston, D. L., Gendron, N., Barnes, D., Fliss, H., King, W. J., Rasquinha, I., MacManus, J., Robertson, G. S., and MacKenzie, A. E. (2001) *J. Neurotrauma* **18,** 1333–1347
24. Inohara, N., and Nunez, G. (2001) *Oncogene* **20,** 6473–6481
25. Chamaillard, M., Girardin, S. E., Viala, J., and Philpott, D. J. (2003) *Cell. Microbiol.* **5,** 581–592
26. Hunter, A. M., Kottachchi, D., Lewis, J., Duckett, C. S., Korneluk, R. G., and Liston, P. (2003) *J. Biol. Chem.* **278,** 7494–7499
27. Yang, X., Chang, H. Y., and Baltimore, D. (1998) *Science* **281,** 1355–1357
28. Chaudhary, D., O'Rourke, K., Chinnaiyan, A. M., and Dixit, V. M. (1998) *J. Biol. Chem.* **273,** 17708–17712
29. Cain, K., Bratton, S. B., Langlais, C., Walker, G., Brown, D. G., Sun, X. M., and Cohen, G. M. (2000) *J. Biol. Chem.* **275,** 6067–6070
30. Clem, R. J., Sheu, T. T., Richter, B. W., He, W. W., Thornberry, N. A., Duckett, C. S., and Hardwick, J. M. (2001) *J. Biol. Chem.* **276,** 7602–7608
31. Liston, P., Fong, W. G., and Korneluk, R. G. (2003) *Oncogene* **22,** 8568–8580
32. Matsumoto, K., Nakayama, T., Sakai, H., Tanemura, K., Osuga, H., Sato, E., and Ikeda, J. E. (1999) *Mol. Reprod. Dev.* **54,** 103–111

33. Mercer, E. A., Korhonen, L., Skoglosa, Y., Olsson, P. A., Kukkonen, J. P., and Lindholm, D. (2000) *EMBO J.* **19,** 3597–3607
34. Poyet, J. L., Srinivasula, S. M., Tnani, M., Razmara, M., Fernandes-Alnemri, T., and Alnemri, E. S. (2001) *J. Biol. Chem.* **276,** 28309–28313
35. Shiozaki, E., Chai, J., Rigotti, D., Riedl, S., Li, P., Srinivasula, S., Alnemri, E., Fairman, R., and Shi, Y. (2003) *Mol. Cell* **11,** 519–527
36. Srinivasula, S. M., Hegde, R., Saleh, A., Datta, P., Shiozaki, E., Chai, J., Lee, R. A., Robbins, P. D., Fernandes-Alnemri, T., Shi, Y., and Alnemri, E. S. (2001) *Nature* **410,** 112–116
37. Zou, H., Yang, R., Hao, J., Wang, J., Sun, C., Fesik, S., Wu, J., Tomaselli, K., and Armstrong, R. (2003) *J. Biol. Chem.* **278,** 8091–8098
38. Renatus, M., Stennicke, H. R., Scott, F. L., Liddington, R. C., and Salvesen, G. S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 14250–14255
39. Acehan, D., Jiang, X., Morgan, D. G., Heuser, J. E., Wang, X., and Akey, C. W. (2002) *Mol. Cell* **9,** 423–432
40. Linhoff, M. W., Harton, J. A., Cressman, D. E., Martin, B. K., and Ting, J. P. (2001) *Mol. Cell. Biol.* **21,** 3001–3011
41. Inohara, N., and Nunez, G. (2003) *Nat. Rev. Immunol.* **3,** 371–382

Neuronal Apoptosis-inhibitory Protein Does Not Interact with Smac and Requires ATP to Bind Caspase-9

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