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 inhibitors 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 carboxy-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 amino-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 carboxy-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 (GSP1; 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-containing sequence that is distinct from those found in other IAPs. 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-dependent 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 carboxy-terminal “sensor”

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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 quaternary 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 Isolation of NAIP cDNAs and the construction of pGEX-NAIP-BIR3 and LRR domains of NAIP with respect to the activity of the NOD proteins, we investigated the effects of the NOD 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 quaternary 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.

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inhibit caspase-3 and -7. Functional domain-mapping experiments have established that the BIR domains of NAIP can bind 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. 2B) 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. 2A). As seen in Fig. 2B, 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. 2B, 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. 2B, 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. 2B, sixth lane), as does the deletion of both the NOD and the LRR domain (Fig. 2B, 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.

**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 have established that NAIP BIR2 potently inhibits 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.

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**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. 2C), 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 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**—Caspase-9 has an ATP-dependent structural requirement which is satisfied by the interaction of NAIP with caspase-9. This interaction results in a conformational change which is required for the inhibition of caspase-9. 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. 2B, 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.

**NAIP Does Not Interact with Smac and Requires ATP to Bind 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. 2C), 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 in E. coli or in eukaryotic cells using the recombinant adeno-NAIP virus.

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NAIP with [35S]methionine-labeled cell extract did not reveal 160-kDa NAIP monomer. Immunoprecipitation analysis of/H11011 680 kDa (Fig. 3), roughly equivalent to four subunits of the or absence of ATP, we performed size exclusion chromatogra-
changes in the higher order structure of NAIP in the presence 
combines with the CARD (29). To investigate potential 
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 
bind Smac (Fig. 5A). In contrast, no evidence of 
which triggers NAIP-caspase-9 interaction, did not induce 
activates XIAP BIR3. As expected, a 30-micromolar excess of the AVPF-
activity of isolated BIR domains has been inves-
activation mechanism and its failure to be inhibited by IBM-
NAIP is based entirely on the conservation of the BIR domains, whereas the overall organization of the protein suggests mem-
proteins. IBM-containing proteins are believed to mediate their 
which XIAP is proteolytically processed favors our contention 
among all ATP-binding proteins, abolished this requirement.

To substantiate these findings, we assessed whether an 
were investigated by affinity pull-down of 
full-length NAIP. 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 

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changes in the higher order structure of NAIP in the presence 
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 
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 carboxy-
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 mem-

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.
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 IC₅₀ 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 IC₅₀ 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 P₃ position of the IBM peptide contacts with the indole ring of Trp⁹⁰ of XIAP (18, 19). Trp⁹⁰ 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 trypto-
phan 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 be-
lieved to maintain caspase-9 in a monomeric and inactive con-
formation (35, 38). Our results would suggest that NAIP BIR3
bypasses the initial IBM-mediated interaction and instead re-
elies exclusively on similar surface contacts with caspase-9. Co-
crystallization of NAIP BIR3 with caspase-9 will conclusi-
vely resolve this issue.

Several members of the NOD family of proteins have been
shown to undergo homo-oligomerization through their respect-
ive 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 mono-
mers 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 ap-
pears 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 proteo-
lytic 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 con-
firm 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, suggest-
ing that at least under the conditions that lead to the cleavage of
XIAP, NAIP is not processed.

In summary, we have documented several unique and unex-
pected aspects of NAIP function. Previous experiments have
demonstrated that NAIP is profoundly antiapoptotic in vivo,
and the structural organization of the protein raise the possi-
bility that NAIP function may be activated by pathogen-asso-
ciated molecular patterns. Activation of proinflammatory cyto-
kines 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 possi-
bility that NAIP function may be activated by pathogen-asso-
ciated 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 tar-
geted deletion of the entire NAIP gene cluster should be ex-
quisitely sensitive to proinflammatory stimuli that would nor-
mally result in interleukin secretion.
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