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Integrity of ATP binding site is essential for effective inhibition of the intrinsic apoptosis pathway by NAIP

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ABSTRACT

The importance of the ATP binding site of human Neuronal Apoptosis Inhibitory Protein (NAIP) on its ability in prevention of intrinsic apoptotic pathway was investigated. Thus, ATP binding lysine 476 of NAIP, which is located at the Nucleotide Binding Oligomerization Domain (NOD) was mutated to threonine and the effect of this mutation on autoproteolysis of procaspase-9 and the cleavage of procaspase-3 by apoptosome was investigated. Formation of apoptosome was induced by the addition of cytochrome *c* and dATP to lysates of HeLa cells transfected with pcDNA-NAIP or pcDNA-NAIP (K476T). Full length wild type NAIP prevented the cleavage of both procaspase-9 to caspase-9 and procaspase-3 to caspase-3. However, K476T variant of NAIP did not block autocleavage of procaspase-9 efficiently. Furthermore, cleavage pattern of procaspase-9 was altered in the presence of mutant NAIP. Interestingly no effect on the procaspase-3 cleavage by apoptosome was observed. The presence of NOD domain by itself had no effect on autocleavage of procaspase-9 yet slightly reduced the cleavage of procaspase-3 by apoptosome. Pull down experiment showed direct interaction of the NOD domain of NAIP with the CARD-NOD domain of Apoptotic Protease Activating Factor 1 (APAF-1). The physical association of these domains was confirmed by pull-down assays. These observations taken with previous findings indicate that the integrity of the NOD domain is essential for effective inhibition of procaspase-9 and procaspase-3 cleavage by the NAIP protein.

1. Introduction

Neuronal Apoptosis Inhibitory Protein (NAIP), as the first member of the Inhibitor of Apoptosis Proteins (IAPs) family, was shown to be deleted in patients with severe forms of Spinal Muscular Atrophy (SMA) [1]. The deletion of the NAIP gene was originally thought to be responsible for the onset of spinal muscular atrophy, which was later proved to be inaccurate. Indeed numerous reports have shown that deletions of the NAIP contribute to the severity of the symptoms [2,3]. Further studies revealed that this protein renders cells resistant to apoptosis under both *in vivo* and *in vitro* conditions [4–7]. NAIP with the molecular weight of 160-kDa protein has three regions of Baculoviral Inhibitor of Apoptosis Repeat (BIR) domains, Nucleotide Binding Oligomerization Domain (NOD), and Leucine Rich Repeat (LRR). The apoptosis inhibitory activity of the protein was attributed to the BIR domains supposedly through the inhibition of caspases-3, -7 and -9 activities [8–10]. The ability of IAPs in caspase inhibition was later disputed emphasizing that only XIAP is a true caspase inhibitor [11]. To justify these observations, anti apoptotic activity of cIAP1, cIAP2, and livin was attributed to their ability of binding and ubiquitinating some proteins involved in apoptosis [12–15]. In the case of NAIP, however, no ubiquitin ligase domain could be found indicating that NAIP employs a different strategy to prevent apoptosis. A recent report by our group that the full length NAIP can inhibit autocleavage of procaspase-9 and cleavage of procaspase-3 by caspase-9 [10] conclusively showed that the NAIP protein is not only a true caspase inhibitor, but also showed that unlike XIAP it is unique in inhibiting the cleavage of procaspase-9. A recent study of the proteins containing the NOD domain showed heterotypic interaction of this domain and proposed that through this interaction, NOD domain may regulate the formation and activation of inflammasomes [16]. This raised the possibility that the NOD domain of NAIP may interact with NOD containing molecules involved in apoptosis. APAF-1 protein, which is absolutely essential for the initiation of the intrinsic apoptosis pathway, is classified as a member of the NOD protein family due to possession of a central Nucleotide Binding Oligomerization Domain. The NOD domain of

Abbreviations: NOD, Nucleotide Binding Oligomerization Domain; BIR, Baculoviral IAP Repeat; NAIP, Neuronal Apoptosis Inhibitory Protein; SMAC, Second Mitochondrial derived Activator of Caspases; XIAP, X-linked Inhibitor of Apoptosis Protein.

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APAF-1 oligomerizes in the presence of ATP/dATP along with cytochrome *c* and procaspase-9 molecules forming the apoptosome complex [17,18]. The primary role of this complex seems to juxtapose procaspase-9 molecules causing its activation through a mechanism called induced proximity dimerization model [19,20]. We have recently shown that only the full length NAIP protein but not the BIR3 domain can prevent autoproteolysis of procaspase-9 in apoptosome complex [10]. This is despite the fact that the BIR3 domain alone can prevent cleavage of procaspase-3 by the cleaved caspase-9. Therefore, a different domain of NAIP is needed to prevent autoproteolysis of caspase-9 in the apoptosome complex.

Considering that both NAIP and APAF-1 contain NOD domains, and taking into account that NOD domains can hetero-oligomerize [16], one can postulate that the presence of intact NOD domain of NAIP is essential for the inhibition of apoptosome function. Therefore, we decided to mutate lysine 476 in the NOD domain, which is the ATP binding site of the NAIP protein, to threonine in order to both block ATP binding to NAIP and maintain caspase-9 interaction [9]. Our results show that a single mutation of lysine is sufficient to sequester the inhibition of procaspase-9 cleavage by the NAIP protein. We further show a direct interaction of the NOD domain of NAIP and APAF-1 which explains altered apoptosome function of the K476T variant of the NAIP protein.

2. Materials and methods

2.1. Protein expression and purification

The cDNA of the NOD domain was amplified using forward primer 5′-GGATCCGATATCTCTTCCGATCTGGC-3′ and reverse primer 5′-GTCGACTCGAGTCCAGGAGTTCAATCAGCCTCAT-3′. The DNA fragment was subcloned into pCR2.1 vector using Topo-TA cloning kit (Invitrogen). The resulting construct was subcloned into pcDNA-myc and pET32a plasmids using BamHI and XhoI enzymes. The pET32a plasmid possessing the NOD domain of NAIP was used to express the protein in *BL21-DE3* cells. The bacteria were induced with 0.5 mM IPTG for 2 h at 30 °C. Following the lysis and centrifugation, the pellet was used for the purification of protein from inclusion bodies using Urea buffer containing 50 mM Tris, pH 8, 8 M Urea, and 5 mM βME. The protein was bound to Ni–NTA resin, washed by 50 mM Tris, 8 M Urea, 350 mM NaCl, 30 mM Imidazole, 5 mM βME, and refolded in the column using 50 mM Tris (pH 7.4), 20% glycerol, 5 mM βME. The protein was eluted by elution buffer containing 300 mM Imidazole. Finally, thioredoxin tag was removed with thrombin as described previously [21]. The CARD-NOD domain of APAF-1 coding residues 1 through 591 was subcloned into pET28a and expressed as outlined above at 26 °C [22]. The protein was purified under native conditions. Attempts to produce just the NOD domain of APAF-1 was failed due to formation of inclusion bodies that could not be refolded. Production and purification of NAIP-BIR3 was performed as described previously [9,10].

2.1.1. Site-directed mutagenesis

Primers 5′-dGAAGCTGGAAGTGGAACGACGGTCCTCCTGAAG-3′ 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.

2.2. Circular dichroism studies

The CD spectra of NAIP-NOD and APAF1-CARD-NOD domains were obtained using AVIV 215 spectropolarimeter as described previously [23]. Spectra in the far UV region of 200–260 nm were measured in a 1 mm pathlength quartz cell with a protein concentration of about 0.2 mg/mL in 20 mM Tris buffer (pH 7.4) containing 100 mM NaCl, 0.1 mM βME and 10% glycerol. The normalization of spectra was performed using the software provided by the instrument manufacturer. The secondary structure of the NAIP-NOD was predicted using CDNN 2.1 software with 33 reference spectra.

2.3. Pull-down assays

For pull-down assays, S-tagged human NAIP-NOD was bound to S-protein Agarose (Novagen, the resin contains one subunit of ribonuclease S which binds to the other subunit, S-tag, fused to the NOD domain) and washed. The resin containing bovine serum albumin was used as a negative control. The resins with or without NAIP-NOD were supplemented with the CARD-NOD domain of APAF-1 in the presence and absence of 1 mM concentration of $MgCl_2$ and dATP. In order to prevent non-specific interactions, BSA with the final concentration of 0.2 mg/mL was included in all incubations. The association of the proteins was investigated by presence of CARD-NOD protein on the SDS–PAGE.

2.4. Caspase cleavage assays

HeLa cells transfected with pcDNA3myc (negative control), pcDNA3myc-NOD, -NAIP, -NAIP (K476T) were lysed using 20 mM Pipes buffer (pH 7.4) contain 10 mM KCl, 2 mM $MgCl_2$, 4 mM DTT under hypotonic conditions. Following centrifugation, the supernatant was incubated with 1 μg/mL of cytochrome *c* and 1 mM dATP at 37 °C for the indicated time. To examine the ability of the NAIP-NOD domain in prevention of caspase cleavage, supernatant of the untransfected HeLa cells was used to form apoptosome in the presence or absence of 50 nM NOD added prior to addition of cytochrome *c* and dATP. The cleavage of procaspase-3, -9 and protein expression of the transfected cells were assessed by the Western Blotting technique. Transfected cells were lysed and subjected to SDS–PAGE (10%) and blotted onto PVDF membrane. The blot was incubated with anti-myc monoclonal (Sigma), caspase-3 and caspase-9 (Cell Signaling) antibodies and detected using chemiluminescence on Biomax film (Kodak, USA).

3. Results and discussion

3.1. Inhibition of apoptosome function by the full length wild type NAIP but not by its mutant

Full length human NAIP is shown to block apoptosis both under *in vitro* and *in vivo* conditions. Anti apoptotic function of NAIP is attributed to its BIR3 domain that inhibits caspase-9 and BIR2 domain that inhibits caspase-3 [8,9]. In addition, we recently reported that the full length NAIP but not its BIR3 domain can prevent the autocatalytic processing of procaspae-9 in apoptosome complex [10]. Activation of procaspase-9 in apoptosome is essential for its auto proteolysis and subsequent cleavage and activation of procaspase-3. The question arises is why the full length protein can inhibit autoproteolysis of procaspase-9 but not the BIR3 domain which is responsible for caspase-9 inhibition. In addition to the BIR domains, NAIP possesses a Nucleotide Binding Oligomerization Domain which is highly similar to the NOD domain of APAF1. Considering that the NOD domain of different proteins interact with each other, we postulated that the presence of intact NOD domain is needed for the interaction with APAF1 and prevention of apoptosome function. To test this hypothesis, lysine 476 of human NAIP, which constitutes the ATP binding site of its

NOD domain, was mutated to threonine. In order to assess the functional consequence of this mutation, the wild type full length NAIP, K476T variant of NAIP, and the NOD domain were introduced into HeLa cells using pcDNA3myc plasmid (results not shown). Transfected cells were lysed and the supernatants were used to investigate their effect on procaspase-9 and procaspse-3 cleavage upon formation of apoptosome by the addition of cytochrome *c* and dATP. As expected full length wild type NAIP prevented autoproteolysis of procaspase-9 and cleavage of procaspase-3 by caspase-9 (Fig. 1). Furthermore, the BIR3 domain of NAIP prevented caspase-9 mediated cleavage of procaspase-3 but not the cleavage of procaspase-9 to caspase-9. On the other hand, the NOD domain of NAIP failed to inhibit procaspase-9 cleavage (Fig. 1A), neither did it prevent cleavage of procaspase-3 by procaspase-9 (Fig. 1B). Similar results were obtained with refolded NOD protein from inclusion bodies (data not shown). Therefore, it can be concluded that the presence of both the BIR domains and the NOD domain is essential for effective inhibition of procaspase-9 cleavage. Interestingly, mutant NAIP while preventing the proper cleavage of procaspase-9, did not inhibit the cleavage of procaspase-3 by procaspase-9. This observation suggests that the integrity of the ATP binding domain is essential for proper function of the full length NAIP in inhibition of procaspase-3 cleavage by caspase-9. It is surprising that a mutation in the NOD domain can interfere with function of the BIR3 domain which is responsible for the inhibition of the cleaved caspase-9. The reason behind this observation is not clear but it can be postulated that the mutation in the NOD domain alters the global structure of the protein and prevents proper alignment of the BIR3 domain with the active site of caspae-9 protein. Another interesting observation was that not only limited procaspase-9 cleavage occurred in the presence of the mutant NAIP, but also the cleavage sites of both procaspase-9 and procaspase-3 were altered. These observations suggest that the mutant NAIP protein affected the three dimensional structure of procaspase-9 such that its specificity for cleavage sites was altered. Activation of procaspase-9 by participation in apoptosome complex leads to auto catalytic cleavage of the enzyme at aspartates 315 and 330 producing the 35 kDa large N-terminal and the 12 kDa small C-terminal subunits [24]. It seems that the mutant NAIP prevented the cleavage at aspartate 315 producing a band around 37 kDa. In fact this 37-kDa fragment is similar in size to a very faint band of the caspase-9 observed in the presence of wild type NAIP protein. To determine if longer incubation of the sample at 37 degrees would result in further cleavage of the 37-kDa to the regular 35-kDa fragment, the lysates containing the mutant NAIP or no NAIP were incubated for a longer time (Fig. 2). Ninety minute incubation resulted in further cleavage of the procaspase-9 to the 37-kDa fragment without the appearance of the 35-kDa band. Therefore, it appears that the mutant NAIP is capable of inhibiting procaspase-9 autoproteolysis at Asp315 and decreasing the rate of auto proteolysis at Asp 330.

3.2. NOD domain of NAIP interacts with the CARD-NOD domain of APAF-1

NOD-NOD heterotypic interaction of NOD containing proteins has been observed previously. It was demonstrated that the association of NOD domain of CLAN with Nod1 and Nod2 proteins inhibits NF-κB activation [16]. Although, association of the NAIP-NOD with CLAN was also observed, the interaction was very weak suggesting non-specific nature of the interaction. These observations taken with the alterations of autoproteolysis pattern of procaspase-9 by mutant NAIP (lysine 476 is located at the ATP binding site of the NOD domain) prompted us to hypothesize the heterotypic interaction of NAIP-NOD with the CARD-NOD domain of APAF-1. To examine this possibility, the NOD domain of NAIP and the CARD-NOD domain of APAF-1 were produced in *Escherichia coli*. The NOD domain of NAIP protein was produced mostly in the form of inclusion bodies. The protein was solubilized by guanidine hydrochloride. However, refolding was proved to be problematic both through dialysis against 0.5 M concentration of arginine or inside the column prior to elution. Nonetheless, a portion of the protein could be solubilized by Urea or sarkosyl. In spite of numerous efforts under various conditions for refolding, the protein could only be refolded in a soluble form inside the Ni–NTA column, perhaps due to restrictions imposed on protein refolding by the resin. Even then, the protein was prone to aggregation upon storage. Unlike the NOD domain of NAIP, the CARD-NOD domain of APAF-1 was produced in a soluble form when expressed at 26 °C.

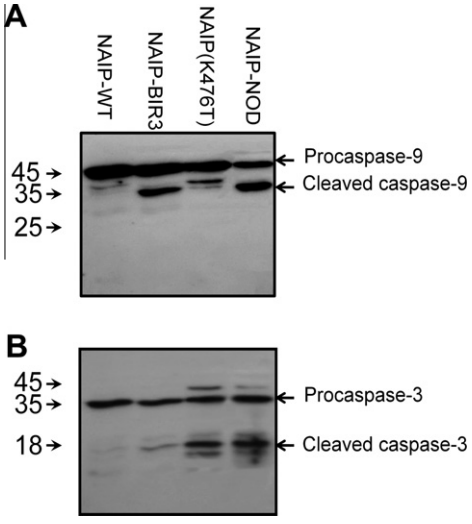

Fig. 1. Cleavage of procaspase-9 (A) and procaspase-3 (B) in the presence of various NAIP domains. HeLa cells transfected with the pcDNA-NAIP constructs including wild type NAIP, NAIP-WT, BIR3 domain of NAIP, NAIP-BIR3, lysine 476 threonine mutant of NAIP, NAIP (K476T), and the NOD domain of NAIP, NAIP-NOD, were lysed and the supernatants were used for the cleavage assays. The Apoptosome was formed by the addition of cytochrome *c* and dATP and incubated at 37 °C for 30 min. The cleavage of caspases was assessed by Western Blot as described in Section 2.

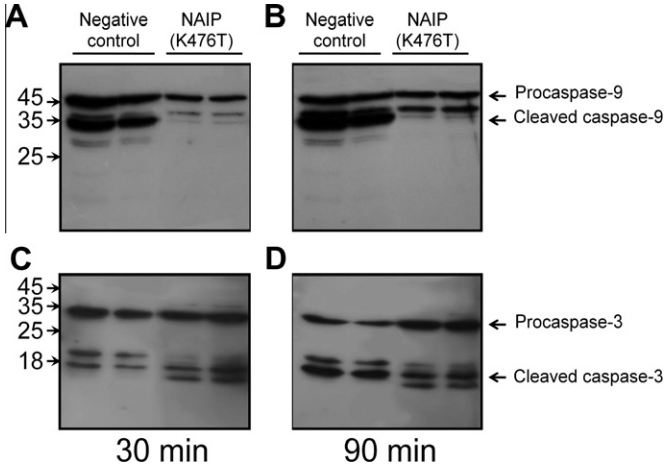

Fig. 2. Time course of the cleavage of procaspase-9 (A and B) and procaspase-3 (C and D) upon induction of intrinsic pathway. The supernatants form pcDNA-myc and pcDNA-myc-NAIP(K476T) transfected HeLa cells were supplemented with cytochrome *c* and dATP and incubated at 37 °C for 30 min (A and C) and 90 min (B and D). The cleavage of the caspases was assessed by Western Blot using anti caspase antibodies.

In order to assess the direct interaction of the two proteins, the NAIP-NOD domain bound to S-resin was used to affinity isolate the CARD-NOD domain of APAF-1 (Fig. 3). Indeed, the interaction of the two was observed with the stoichiometry of 1:1, when dATP and $MgCl_2$ were present, while no protein was bound to S-protein Agarose. Bovine serum albumin was present during the pull-down assays to prevent non-specific interactions. In the presence of EDTA, on the other hand, CARD-NOD interacted nonspecifically with the resin precluding us from making any conclusion with respect to dependence of this interaction upon dATP and $MgCl_2$.

3.3. Circular dichroism spectroscopy of the purified proteins

Expression of the NOD domain of NAIP and the CARD-NOD domain of APAF-1 also allowed us to compare the secondary structure content of these proteins by circular dichroism spectroscopy. Due to aggregation of the protein during dialysis, G-10 size exclusion column was used to remove imidazole from the freshly purified samples. As a result, the samples were subjected to CD spectroscopy immediately following the buffer exchange. The CD spectra of both CARD-NOD domain of APAF-1 and the NOD domain of NAIP appeared very similar exhibiting two minima at 222 and 208 nm (Fig. 4A), indicating that both proteins consist of mostly α-helical structures. The secondary structure content of the NOD domain of NAIP was estimated to be 66% α-helix, 6% β-sheets, 28% random coil and other structures. These numbers are very similar to the secondary structure of the NOD domain of APAF-1, which consists of 56% α-helix and 8% β-sheets as determined by X-ray crystallography [22]. Considering the high primary structure similarity of NOD domains of the NOD family of proteins, it can be postulated that α-helices should dominate the structure of these proteins. Addition of various nucleotides including, ATP, dATP, GTP, or dGTP did not have a significant effect on the CD spectrum of the NAIP-NOD domain (data not shown) suggesting that the interaction of these molecules does not lead to a significant change in the secondary structure of the protein. However, presence of $MgCl_2$ caused an up shift in elipticity at 208 nm indicating the interaction of magnesium ions with the protein (Fig. 4B).

In summary, the pull-down assay shows a direct interaction of the NOD domains of NAIP and APAF1. Furthermore, procaspase-9 cleavage assays clearly indicate that the integrity of ATP binding site of NAIP is essential for the inhibition of procaspase-9 auto proteolysis by NAIP. While the NOD domain by itself is not capable of inhibiting procaspase-9 activity, it seems that the NOD domain functions in the alignment of the BIR domains in the apoptosome complex through the interaction with APAF1 such that the activity of the procaspase-9 is hindered. We therefore propose that the BIR3 domain of NAIP binds to procaspase-9 and the NOD domain interacts with the NOD domain of APAF1 forming a bridge between procaspase-9 and APAF1. Finally, the integrity of the NOD domain is essential for formation of such bridge and effective inhibition of procaspase-9 [24].

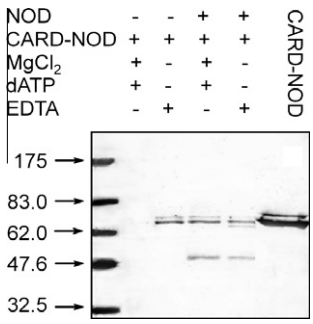

Fig. 3. Affinity pull-down of the CARD-NOD domain of APAF1 with the NOD domain of NAIP. The NOD domain of NAIP was bound to S-protein Agarose and subsequently incubated with the CARD-NOD domain of APAF1 in the presence and absence of dATP and $MgCl_2$ as described in Section 2.

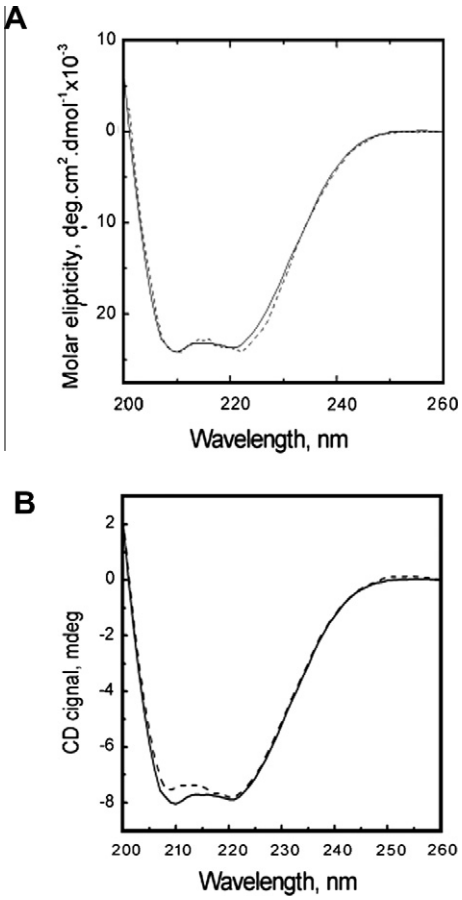

Fig. 4. (A) Circular dichroism spectra of NAIP-NOD compared to the CARD-NOD domain of APAF1. (B) The CD spectra of the NOD domain of NAIP in the absence, solid line, and presence, dashed line, of 0.5 μM concentration of $MgCl_2$.

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