Dimerization and Autoprocessing of the Nedd2 (Caspase-2) Precursor Requires both the Prodomain and the Carboxyl-terminal Regions*

(Received for publication, September 15, 1997, and in revised form, December 8, 1997)

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Nedd2 (caspase-2) is a cysteine protease of the caspase family that has been demonstrated to play a role in the apoptotic pathway. The 51-kDa precursor of Nedd2 undergoes cleavage into two subunits following various apoptotic stimuli. In this study, we have investigated the dimerization of the Nedd2 precursor (pro-Nedd2) in Saccharomyces cerevisiae and its self-processing activity in vivo. We demonstrate that the expression of pro-Nedd2 in yeast cells results in processing of the precursor. A catalytically inactive pro-Nedd2 mutant dimerized in yeast, and the dimerization required both the prodomain and the carboxyl-terminal residues. Aspartate mutants that block the removal of the p14/p12 subunits, but not the wild-type Nedd2, were shown to dimerize in yeast cells, suggesting that dimerization occurs prior to processing. In vitro processing of pro-Nedd2 by recombinant active Nedd2 defined the aspartate residues that are crucial for processing to occur. Both the in vivo and in vitro processing of pro-Nedd2 directly correlated with its ability to induce cell death in transient overexpression experiments.

Caspases are a family of mammalian cysteine proteases that share structural and, in some cases, functional similarity with the Caenorhabditis elegans cell death protease CED-3 (1, 2). Caspases are produced aszymogens and require cleavage into two subunits, which constitute the active enzyme (reviewed in Refs. 3–6). The small subunit of a caspase is derived from the carboxyl terminus of the precursor, while the large subunit originates from the region immediately upstream of the small subunit, leaving an amino-terminal prodomain of varying lengths in different caspases. On the basis of prodomain size, caspases can be divided into two groups, those with relatively long amino-terminal prodomains and those without. It is suggested that in caspases with long prodomains, the amino-terminal region is required for their recruitment to death receptor complexes (7, 8). The long prodomain is also likely to serve a function in dimerization and autoprocessing as suggested by a recent study with interleukin-1β-converting enzyme (caspase-1) (9). Nedd2 was initially identified as a developmentally regulated gene in the mouse central nervous system (10) and later shown to encode a cysteine protease similar to interleukin-1β-converting enzyme and CED-3 (11, 12). Several lines of evidence have implicated Nedd2 (ICH-1/caspase-2) directly or indirectly in apoptosis. Overexpression of Nedd2 induces apoptosis in various cell types (11, 12), while expression of antisense Nedd2 in FDC-P1 factor-dependent cells delays the onset of apoptosis induced by factor withdrawal (13). Abrogation of Nedd2 expression by antisense oligonucleotides rescues PC12 cells from apoptosis induced by nerve growth factor deprivation but not by superoxide dismutase down-regulation (14). In addition, an alternatively spliced form of caspase-2, which encodes a truncated protein, has been shown to protect against cell death induced by serum withdrawal in Rat-1 and NIH-3T3 cells, but not in all cell types (12, 15). Up-regulation of Nedd2 mRNA has been observed in response to ischemia-induced cell death (16, 17), while down-regulation of Nedd2 has been observed during gonadotropin-promoted follicular survival (18). Caspase-2 activation occurs early during apoptosis mediated by factor withdrawal, γ-irradiation, etoposide treatment (20, 21), tumor necrosis factor, and Fas ligation. Caspase-2 has been shown to associate via its prodomain with RAIDD, a death adaptor molecule that is thought to be involved in apoptosis mediated through death receptors via association with the death domain proteins RIP and TRADD (19).

Previous studies have suggested that expression of pro-Nedd22 in E. coli and mammalian cells can result in self-processing (22–24). pro-Nedd2 expressed in E. coli shows polypeptides of 18, 13, and 12 kDa derived by cleavage at DNKD169, DQD333, and EED347, as determined by microsequencing (23). Since Nedd2 contains a long prodomain, it is possible that the Nedd2 precursor (pro-Nedd2) dimerizes prior to autoprocessing, in a manner similar to that suggested for interleukin-1β-converting enzyme (9). In the present paper, we have analyzed the dimerization of pro-Nedd2 using the yeast two-hybrid assay. By mutation analysis, we have also studied the mechanism of activation of Nedd2 in vitro and in vivo and its functional implications in autocatalysis and cell killing.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Experiments—The components of the yeast two-hybrid system were purchased from CLONTECH. Nedd2 constructs in Gal4 activation domain (AD) vector and Gal4 DNA-binding domain (BD) vector were co-transformed into Saccharomyces cerevisiae strain Y190, and colonies containing both vectors were selected on SD medium lacking leucine and tryptophan. Putative interacting Nedd2 fusion proteins were selected by screening for β-galactosidase activity in a colony lift filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Quantitative data on positive interactions were obtained with a liquid culture assay using o-nitrophenyl-β-D-galactopyranoside as a substrate.

* This work was supported by a Wellcome Trust Senior Research Fellowship and National Health and Medical Research Council of Australia Grant 960532 (to S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 N. L. Harvey, A. J. Butt, and S. Kumar, unpublished data.

2 The abbreviations used are: pro-Nedd2, the 51-kDa precursor of Nedd2; AD, activation domain; DB, DNA-binding domain; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MPD, Nedd2 minus prodomain; PCR, polymerase chain reaction.
Fig. 1. Various Nedd2 constructs and mutants used in the two-hybrid experiments. In A, the numbering of residues is according to Refs. 15 and 24, and the p18 and p12 subunit boundaries are derived from Ref. 23. Positions of Asp residues mutated to Ala (D135, D326, D330, and D333) or Gly (D169 and D347) and the catalytic Cys320 (C320) mutated to Gly are indicated. Gal4BD and Gal4ADWT/Cys320 constructs consist of the prodomain and p18 and p12 subunits with internal linker sequences (B). Gal4BD/ADPRO consists of only the prodomain region (C), while Gal4BD/ADMPD, with or without the Cys320 mutation, lack the prodomain (D). In all cases, Nedd2 cDNA was fused in frame to the Gal4BD and Gal4AD.

Construction of Two-hybrid Vectors—Wild-type Nedd2 plasmid pMSN2.4 carrying the entire coding region of Nedd2 and a Cys320→Gly Nedd2 mutant have been described previously (11). The coding region of Nedd2 cDNA was amplified from pMSN2.4 by 25-cycle PCR using Pfu polymerase (Stratagene) and the following oligonucleotide primers with BamHI restriction sites: primer A, 5′-CCCGGATCCACAGGAGGACAGGATTTGCAGGATTTC-3′; primer B, 5′-CCCGGATCCAACTGGTGGTGGTGGGCC-3′ (regions complementary to the Nedd2 sequence are underlined). The amplified product was cloned in frame to both the Gal4BD in the yeast expression vector pAS2.1 (Gal4BDWT) and the Gal4AD in pACT2 (Gal4ADWT). A Nedd2 Cys320→Gly mutant was amplified similarly using primers A and B and cloned in the two yeast vectors to generate Gal4BD/C320 and Gal4/AD/C320. To generate Nedd2 minus prodomain (MPD) constructs, wild-type and Cys320 mutant plasmids were used as a template for PCR using primers B and C (primer C, 5′-CCCGGATCCATAATGGTGATGGTCCT-3′) due to a lack of autoprocessing activity (11, 28). Nedd2 constructs lacking the prodomain were unable to interact with the Gal4BD/ADMPD vectors. Gal4BD/ADPRO, consisting of the prodomain region alone, were constructed by ligating the PCR-generated prodomain fragment in frame to the Gal4BD/AD. The prodomain was amplified using primers A and D (primer D, 5′-CCCGGATCAAGACCATCACCATTATCTAA-3′). Asp326→Ala, Asp326→Ala, Asp326→Ala, and Asp326→Ala mutants were a generous gift of Dr. B. Allet (24). Asp326→Gly and Asp347→Gly mutants were generated by site-directed mutagenesis (25) using pMSN2.4 as a template. Mutated cDNAs were amplified by PCR using primers A and B and cloned in frame to the Gal4BD or Gal4/AD in the yeast expression vectors.

In Vitro Proteolysis Experiments—Recombinant active caspase-3 was generated as described previously (26). For mouse caspase-2 expression, PCR-amplified coding region of Nedd2 was cloned into the pET32 vector (Novagen), and recombinant enzyme was expressed according to instructions provided by Novagen. Wild-type and mutant Nedd2 cDNAs cloned into pBluescript vector (Stratagene) were used as a template for the production of [35S]methionine-labeled protein using a coupled transcription/translation kit (Promega). 2.5–5 μl of translated product was incubated with recombinant enzyme for 3 h at 37 °C, electrophoresed on SDS-polyacrylamide gels, transferred to PolyScreen polyvinylidene difluoride membrane (DuPont), and exposed to x-ray film.

Transient Transfection—For expression in mammalian cells, wild-type and mutant Nedd2 cDNAs were cloned into the pCMX vector (27). For cell death assays, NIH-3T3 cells were plated at 2.5 × 10^5/well in 6-well dishes the day before transfection. For each well, we used 2 μg of the Nedd2 expression plasmid mixed with 0.5 μg of the β-galactosidase expression plasmid (pEF-bgal). All transfections were carried out using Superfect transfection reagent (Qiagen) according to the manufacturer’s protocol. Cells were fixed and stained with X-gal 18–24 h post-transfection as described previously (11). For immunoblotting experiments, cells plated at a density of 10^6/60-mm dish were transfected with 5 μg of the Nedd2 expression constructs and harvested 12 h after transfection.

Immunoblotting—Transiently transfected NIH-3T3 cell pellets were boiled in SDS-polyacrylamide gel electrophoresis buffer prior to electrophoresis. Nedd2 constructs fused to the Gal4/AD in pACT2 were transformed into S. cerevisiae Y180, and colonies were selected in medium lacking Leu. Yeast extracts were prepared using the trichloroacetic acid protein extraction method following the CLONTECH protocol. Proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membrane. Immunoblotting was carried out using a 1:500 dilution of an anti-caspase-2 rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.), which detects the precursor and the p14 and p12 subunits of the human Nedd2 protein (20), or a Gal4AD antibody (CLONTECH) at a 1:10,000 dilution. After probing with an appropriate horseradish peroxidase-coupled antibody, signals were detected by ECL (Amersham Corp.).

RESULTS

Dimerization and Processing of pro-Nedd2 in Yeast—Since pro-Nedd2 can undergo processing when expressed in E. coli and mammalian cells (22, 23), we used a functionally inactive pro-Nedd2 mutant in which the catalytic Cys320 residue was replaced by a Gly residue for our protein-protein interaction studies using the yeast two-hybrid system. This mutant is unable to induce apoptosis in mammalian cells, presumably due to a lack of autoprocessing activity (11, 28). Nedd2 constructs were cloned into both the Gal4BD and Gal4AD vectors (Fig. 1) and transformed into S. cerevisiae. Analysis of Nedd2 protein expression in yeast showed that the 51-kDa wild-type Nedd2 fused to 19-kDa Gal4/AD was present as a band of ~56 kDa, suggesting that autoprocessing occurred from the C terminus removing a ~14-kDa fragment (Fig. 2). The Nedd2 Cys320 mutant was expressed mostly as full-length precursor protein (~70 kDa), suggesting that the inability of this mutant to induce apoptosis in mammalian cells is due to a lack of autoprocessing activity. Following cotransformation, no interaction between the BD and AD wild-type pro-Nedd2 was observed, suggesting that processing occurs rapidly upon dimerization and that full-length Nedd2 precursor is required for stable dimerization to take place. Indeed, the two full-length Cys320 mutant fusion proteins were able to interact, as evident by the activation of the LacZ reporter gene and β-galactosidase activity, measured by X-gal staining of colonies (not shown) and colorimetric assays (Table I). Cotransformation of the two parent vectors or either vector carrying the Cys320 mutant did not show any activation of the reporter genes. Since the prodomain in interleukin-1β-converting enzyme has been shown to require full-length Nedd2 precursor in dimerization. When prodomain constructs in AD and BD plasmids were cotransformed into S. cerevisiae, strong activation of the reporter gene was observed, suggesting that the prodomain region can homodimerize readily. Furthermore, both wild-type Nedd2 and Cys320 constructs lacking the prodomain were unable to interact with the full-length Cys320 mutant (Table I), indicating that the prodomain region is essential for dimerization of pro-Nedd2. We also assessed whether the prodomain alone was sufficient to medi-
Aspartate Mutants Affect Dimerization and Processing of pro-Nedd2—Since wild-type pro-Nedd2 was unable to homodimerize, we further examined whether processing of this caspase affects dimerization. Since the processing of caspases occurs by cleavage of the precursor at the C terminus of Asp residues, we used Nedd2 constructs in which Asp residues at which cleavage is reported to occur (Asp169, Asp330, and Asp347) had been replaced with either Gly or Ala residues by site-directed mutagenesis. In addition, we used three other Asp mutants (Asp135, Asp326, and Asp347) since these Asp residues lie in the vicinity of the above cleavage sites and have been shown to abolish the proapoptotic activity of Nedd2 (24). When cotransformed with the Cys320 mutant, Asp135, Asp347, and Asp347 failed to show any activation of the reporter gene, while Asp326, Asp330, and Asp333 were able to dimerize with Cys320 (Table II). Except for the Asp135 mutant, in all cases, the ability to dimerize appeared to correlate with the inability to undergo autoproteolytic processing. The Asp326 mutant blocked the removal of p14, an intermediate for the p12 subunit, and was able to dimerize with Cys320, indicating that residues in p14 are required for dimerization (Fig. 2). Asp330 and Asp333 mutants were also poorly processed in yeast (data not shown) and mammalian cells (see below) and were able to dimerize with Cys320 (Table II). Asp347, cleavage at which results in the generation of p12 (23), was processed in S. cerevisiae, presumably by cleavage at Asp333, removing the p14 subunit (Fig. 2). Similarly, the Asp169 mutant showed a smaller product than expected due to cleavage at the C-terminal Asp residues. Although the Asp135 mutant was not well processed in S. cerevisiae, it failed to interact with Cys320. Asp135 was also unable to homodimerize or heterodimerize with all other Asp mutants or the prodomain (Table II).

In Vitro Processing of pro-Nedd2 by Recombinant Caspases—We and others have previously shown that pro-Nedd2 can be cleaved into two subunits in vitro by several caspases including caspase-2 and caspase-3 (22, 23). To ascertain whether the mutations in various Asp residues can affect the cleavage of the precursor, mutant proteins were subjected to cleavage by recombinant Nedd2 (Fig. 3). While the wild-type Nedd2 and Asp347 mutants were efficiently processed by recombinant Nedd2, as apparent by the disappearance of the 51-kDa pro-Nedd2 and the appearance of smaller polypeptides representing intermediates and subunits, Asp135 and Asp326 only showed partial cleavage, and most of the precursor remained intact (Fig. 3B). Mutations at Asp330 and Asp333 suppressed the release of p14. Asp169 showed significant processing, but the cleavage profile of this mutant was significantly altered from the wild-type pro-Nedd2. Since CPP32 (caspase-3) has previously been shown to mediate the cleavage of pro-Nedd2 (22, 23), we subjected various Asp mutants to proteolysis by recombinant CPP32 (Fig. 3C). Interestingly, Asp135 and Asp326 mutants, which are poorly processed by Nedd2, were efficiently cleaved by CPP32. CPP32 cleavage of Asp330 and Asp333 (P4

![Fig. 2. Processing of various Nedd2 fusion proteins in S. cerevisiae.](http://www.jbc.org/) Extracts from S. cerevisiae Y190 cells transformed with various Gal4AD constructs were analyzed by immunoblotting with an antibody that detects the Gal4 activation domain (≈19 kDa). Full-length pro-Nedd2 fused to AD (Gal4ADWT) is approximately 70 kDa. Removal of p14 from the C terminus in wild-type pro-Nedd2 (Gal4ADWT) and the Asp169 (Gal4ADD169) and Asp347 (Gal4ADD347) mutants generates a ~56-kDa fragment, detected by the AD antibody. Cys320 (Gal4ADC320), Asp135 (Gal4ADD135), and Asp326 (Gal4ADD326) mutants are not processed efficiently in S. cerevisiae, since most of the protein was present in precursor form. AD-fused proteins containing the Nedd2 prodomain alone (Gal4ADPRO) or the prodomain-minus region (Gal4ADMPD and Gal4ADMPDC320) are detected as ~37- and ~53-kDa bands, respectively. Note that the Nedd2 protein lacking the prodomain does not seem to undergo autocatalytic cleavage in S. cerevisiae.

**Table I**

| Cotransformed plasmids | Gal4AD | Gal4ADWT | Gal4ADDC320 | Gal4ADMPD | Gal4ADMPC320 | Gal4ADPRO | Gal4ADSV40 largeT |
|------------------------|--------|----------|-------------|------------|--------------|------------|-----------------|
| Gal4BD                 | +      | +        | -           | -          | -            | -          |                 |
| Gal4BDWT               | -      | -        | -           | -          | -            | -          |                 |
| Gal4BDC320             | -      | -        | +           | -          | -            | -          |                 |
| Gal4BDMPD              | -      | -        | -           | +          | -            | -          |                 |
| Gal4BMPDPC320          | -      | -        | -           | -          | +            | -          |                 |
| Gal4BDPRO              | -      | -        | -           | -          | -            | +          |                 |
| Gal4BDp53              | -      | -        | -           | -          | -            | -          | ++              |

**Table II**

| Cotransformed plasmids | Gal4ADD135 | Gal4ADD169 | Gal4ADD326 | Gal4ADD330 | Gal4ADD333 | Gal4ADD347 | Gal4ADPRO |
|------------------------|------------|------------|------------|------------|------------|------------|-----------|
| Gal4BDC320             | -          | -          | +          | +          | +          | -          |           |
| Gal4BDD135             | -          | -          | +          | +          | +          | -          |           |
and P1 positions in the D330QQD333 sequence in pro-Nedd2 mutants generated a fragment larger than 14 kDa, suggesting that CPP32 cleaves pro-Nedd2 at Asp333. These results suggest that the mechanisms of processing of pro-Nedd2 by caspase-2 and caspase-3 are different and that the two caspases may have different preferences for various cleavage sites in the precursor molecule.

In Vivo Processing and Apoptotic Activity of Nedd2 Mutants—The first biological assays to show a role for Nedd2 in apoptosis utilized pro-Nedd2 overexpression in transiently transfected cells (11, 12). These experiments clearly showed that overexpression of pro-Nedd2 induced apoptosis, but it was not clear how the cell death was mediated. We and others have suggested that the overexpression of caspases can result in autoprocessing (11, 12, 24, 28), perhaps in a concentration-dependent manner, but it has not been firmly established in the transient transfection system, despite the fact that these assays are now widely used. We analyzed the death-inducing activities and processing of the various Nedd2 mutants in transiently transfected NIH-3T3 fibroblasts (Figs. 4 and 5). Consistent with the yeast experiments and in vitro Nedd2 processing data above, mutations at Asp169 and Asp347 retained their apoptotic activity, while substitutions at Cys320, Asp135, Asp326, and Asp333 completely abolished the death-inducing activity of pro-Nedd2. Asp330 retained some apoptotic activity (45%). We also analyzed the in vivo processing of various mutants using an antibody against the small subunit of caspase-2. NIH-3T3 cells were transfected with various constructs and harvested 12 h later. Immunoblotting of extracts from these cells showed that wild-type pro-Nedd2 and the Asp347 mutant were efficiently processed in vivo; however, in the Asp347 mutant, p14 but not p12 was released, indicating that cleavage at Asp347 generates the mature p12 subunit. Asp330 and Asp333 showed partial processing, with release of the prodomain generating a p18 intermediate that is further processed to some extent in the case of Asp330 to generate p14 and p12 subunits. Asp135 and Asp326 mutants were not processed (Fig. 5), which is consistent with their inability to induce apoptosis. Some processing of Cys320 mutant was also evident. Since this mutant is catalytically inert, we believe that partial processing of Cys320 in transfected cells may be mediated by other endogenous proteases/caspases rather than autocatalysis.

DISCUSSION

In this paper, we show that pro-Nedd2 is able to dimerize in S. cerevisiae. Dimerization of pro-Nedd2 only occurred in mutants that lacked the ability to autoprocess, and removal of either the prodomain or the C terminus abolished this interaction. This is consistent with the assumption that dimerization occurs prior to the processing of pro-Nedd2. Preceding this study, only interleukin-1β-converting enzyme had been shown to be able to form dimers in vivo (9), but it is likely that all caspases containing long amino-terminal prodomains are capable of forming homodimers. This would be in keeping with the processing of many other enzymes unrelated to caspases, where the prodomain region is required for optimal folding and maturation of the active enzyme (29). Interestingly, the prodomain alone, devoid of the rest of the sequence, was able to homodimerize. However, the prodomain did not heterodimerize.
Nedd2 Dimerization and Processing

Using a Semliki Forest virus expression system, Allet et al. (24) showed that the Asp135 mutant is unable to be processed in infected Chinese hamster ovary cells. This mutant was also unable to induce cell death when microinjected into sympathetic neurons (24). Our results are consistent with these earlier studies. Since the Asp135 mutant was not well cleaved by recombinant Nedd2, even at Asp residues in the C-terminal region of pro-Nedd2, it appears that the Asp135 residue is important in maintaining the overall structure of the precursor molecule, and in the mutant this structure is disrupted in such a way that other cleavage sites are no longer accessible to caspase cleavage. This is further supported by our data showing that despite the fact that Asp135 is not processed, unlike Cys320, it is unable to homodimerize or to heterodimerize with Cys320 in S. cerevisiae. As expected, the Asp135 mutant also failed to induce apoptosis when overexpressed in mammalian cells. Mutation of Asp326 also inhibited processing and cell killing activity of Nedd2, suggesting a situation similar to Asp135.

Our results clearly suggest that pro-Nedd2 can homodimerize prior to processing. We propose that homodimerization of the precursor molecules is important for its autoprocessing and proapoptotic activity and is distinct from oligomerization of subunits that constitute the active enzyme. Our data also indicate that autoprocessing occurs in a concentration-dependent manner and that killing of cells by overexpression of pro-Nedd2 is mainly due to autoactivation. ProNedd2 is widely expressed in many cell types without having any adverse effect (11, 12, 15). There is some evidence for transcriptional activation of Nedd2 preceding cell death in various systems (16–18). However, recruitment through adapters, such as RAIDD (19), may also be important in achieving optimum precursor concentration in specific cellular compartments, promoting auto cleavage. Since pro-Nedd2 can also be cleaved into active subunits by downstream caspases, such as caspase-3, once these caspases are activated, a cascade event would ensure that all precursor is rapidly processed in cells undergoing apoptosis, thus amplifying the apoptotic response.

Acknowledgments—We are grateful to Dr. Bernard Allet for providing the Asp135, Asp262, Asp330, and Asp333 mutants and to Dr. Jun-ichi Miyazaki for the pCXN2 mammalian expression vector.

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