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Origin, expression and possible functions of the two alternatively spliced forms of the mouse *Nedd2* mRNA

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Abstract

The *Nedd2* gene, a member of the *ICE/ced-3* family, transcribes two mRNA species by differential splicing. One of these encodes a shorter truncated version of the protein termed Nedd2s/ICH-1s. While Nedd2/ICH-1 induces apoptosis, ICH-1s was proposed to be a negative regulator of cell death. In this communication we have analyzed various aspects of the Nedd2s/ICH-1s. The coding region of mouse *Nedd2* consists of 10 exons and alternative splicing of the 7th exon results in the generation of two transcripts. Although Nedd2s expression conferred partial resistance to serum withdrawal-induced apoptosis in NIH-3T3 cells, it failed to prevent cell death induced by the overexpression of Nedd2 and was unable to suppress apoptosis in two other cell lines (N18 and FDC-P1) under factor deprived conditions. Our results indicate that Nedd2s/ICH-1s is not a general modulator of cell death.

Keywords: ICE; CED-3; ICH-1; caspases; apoptosis; protease activation

Abbreviations: ICE, interleukin 1β-converting enzyme; PCR, polymerase chain reaction; PBS, phosphate buffered saline; DMEM, Dulbecco's modified Eagle's medium

Introduction

Mammalian homologues of the product of *Caenorhabditis elegans* cell death gene, *ced-3*, play essential effector roles in apoptosis (reviewed in Kumar, 1995a; Martin and Green, 1995; Kumar and Lavin, 1996). The first known member of this growing family was interleukin-1β converting enzyme (ICE), a cysteine protease required for the processing of the interleukin-1β precursor (Thornberry *et al*, 1992; Yuan *et al*, 1993). Subsequently, several ICE-like proteins have been identified in mammalian cells and caspase has been proposed as a name for the human members of this family (Alnemri *et al*, 1996). Of the 11 known members of this family only some such as ICE and CPP32 have been characterised in detail and it is not clear whether all play a role in apoptosis. Mice deficient in ICE develop normally although show some defect in Fas-mediated apoptosis (Li *et al*, 1995; Kuida *et al*, 1995). CPP32 deficient mice show a more severe phenotype as characterised by decreased apoptosis in the brain resulting in profound hyperplasia and post-natal lethality (Kuida *et al*, 1996). Interestingly, CPP32 ($-/-$) mice only show a defect in neuronal apoptosis during development suggesting that other members of the caspase family may be involved in apoptosis in other tissues and cell lineages.

Caspases are synthesised as precursor molecules and require cleavage into two subunits prior to activation (reviewed in Kumar, 1995a). *In vitro*, caspases can be cleaved at specific aspartate residues by other members of the family (Fernandes-Alnemri *et al*, 1996; Xue *et al*, 1996; Harvey *et al*, 1996). Some of the caspase family members (eg. CPP32/Yama/apopain, Mch2 and Mch3/ICE-LAP3) have been shown to be activated during apoptosis (Martin *et al*, 1995; Chinnaiyan *et al*, 1996; Duan *et al*, 1996; Orth *et al*, 1996a,b). Once activated caspases such as CPP32/Yama/apopain and Mch3/ICE-LAP3 cleave several cellular proteins such as poly (ADP-ribose) polymerase (Nicholson *et al*, 1995; Fernandes-Alnemri *et al*, 1995; Tewari *et al*, 1995), catalytic subunit of the DNA dependent protein kinase (Casciola-Rosen *et al*, 1996; Song *et al*, 1996a,b), U1-70 kDa ribonucleoprotein (Casciola-Rosen *et al*, 1996), heteronuclear ribonucleoproteins C1 and C2 (Waterhouse *et al*, 1996); while Mch2 specifically cleaves lamin A (Orth *et al*, 1996a; Takahashi *et al*, 1996).

Mouse *Nedd2* (*N*eural precursor cell-*e*xpressed *d*evelopmentally *d*own-regulated gene 2), a member of the caspase family, was first identified as a developmentally regulated gene in the mouse central nervous system (Kumar *et al*, 1992). Subsequently, *Nedd2* was shown to encode a protein similar to ICE and CED-3 and to induce apoptosis when ectopically expressed in NIH-3T3 and N18 cells (Kumar *et al*, 1994a). Wang *et al*. (1994) identified the human counterpart of *Nedd2* and named it *ICH-1*. We also cloned the human homologue of mouse *Nedd2* and according to the recommendations of the genome nomenclature committee, called it *NEDD2* (Kumar *et al*, 1995). The new proposed name for the human ICH-1/NEDD2 is caspase-2 (Alnemri *et al*, 1996). Although, currently there is no direct evidence to firmly establish a central role for Nedd2 protease in apoptosis, several studies have provided support that Nedd2 function is required in the cell death pathway. For example, expression of antisense *Nedd2* delays apoptosis of a factor-dependent cell line under factor deprived conditions (Kumar, 1995b). Furthermore, upregulation of *Nedd2* mRNA occurs during ischaemia induced neuronal death

in Mongolian gerbil (Kinoshita *et al*, 1997), while a downregulation of *Nedd2* expression has been demonstrated upon gonadotropin-promoted follicular survival (Flaws *et al*, 1995). Proteolytic activation of Nedd2 occurs early during apoptosis induced by various stimuli including factor withdrawal, γ-irradiation and etoposide treatment in several haematopoietic cell lines (N. Harvey, A. Butt and S. Kumar, manuscript in preparation). *In vitro*, the activation of Nedd2 can be mediated by caspases such as CPP32 and Mch3 (Harvey *et al*, 1996; Xue *et al*, 1996) which are known to be activated during apoptosis (Chinnaiyan *et al*, 1996; Duan *et al*, 1996; Orth *et al*, 1996b; Martin *et al*, 1995).

Further evidence for the involvement of Nedd2 in apoptosis was provided by Wang *et al*. (1994) who identified two differentially spliced forms of *ICH-1* (*ICH-1*$_L$ and *ICH-1*s) with apparently opposing effects on apoptosis. Consistent with the Nedd2 data, ICH-1$_L$ was found to induce apoptosis in various cell types while an alternatively spliced form encoding a truncated version of the protein (ICH-1s) was shown to protect against serum deprivation-induced death of Rat-1 cells (Wang *et al*, 1994). From these studies it was unclear whether ICH-1s protein acts as a general inhibitor of apoptosis in all cell types and whether it exerts its effect by directly competing with ICH-1$_L$ or acts through another mechanism. In the present study, we have further analyzed the role of the two forms of Nedd2/ICH-1 in apoptosis. We have cloned the mouse and human *Nedd2* encoding the short forms of the protein (Nedd2s/NEDD2s) and demonstrate that both were unable to inhibit apoptosis induced by ectopic expression of Nedd2. We show that while *Nedd2s* expressing fibroblasts exhibited some protection against serum withdrawal-induced cell death, expression of *Nedd2s* in a neuroblastoma cell line and a myeloid progenitor cell line failed to show such effects. We have also determined the genomic structure of *Nedd2* to show the origin of the two *Nedd2* transcripts and analysed the expression of each form in various tissues.

Results

cDNA cloning of the human and mouse *Nedd2s*

During a screen for the full length cDNA for *Nedd2*, we isolated several clones from a lung cDNA library which contained an extra 61 bp sequence compared to the majority of cDNA clones. As the insertion of 61 bp resulted in the creation of an inframe termination codon thereby resulting in the truncation of full length Nedd2 protein (Kumar *et al*, 1994a,b), we call the mRNA *Nedd2s*. A human cDNA for the shorter protein termed NEDD2s was isolated from a human brain cDNA library (Kumar *et al*, 1995). The human NEDD2s

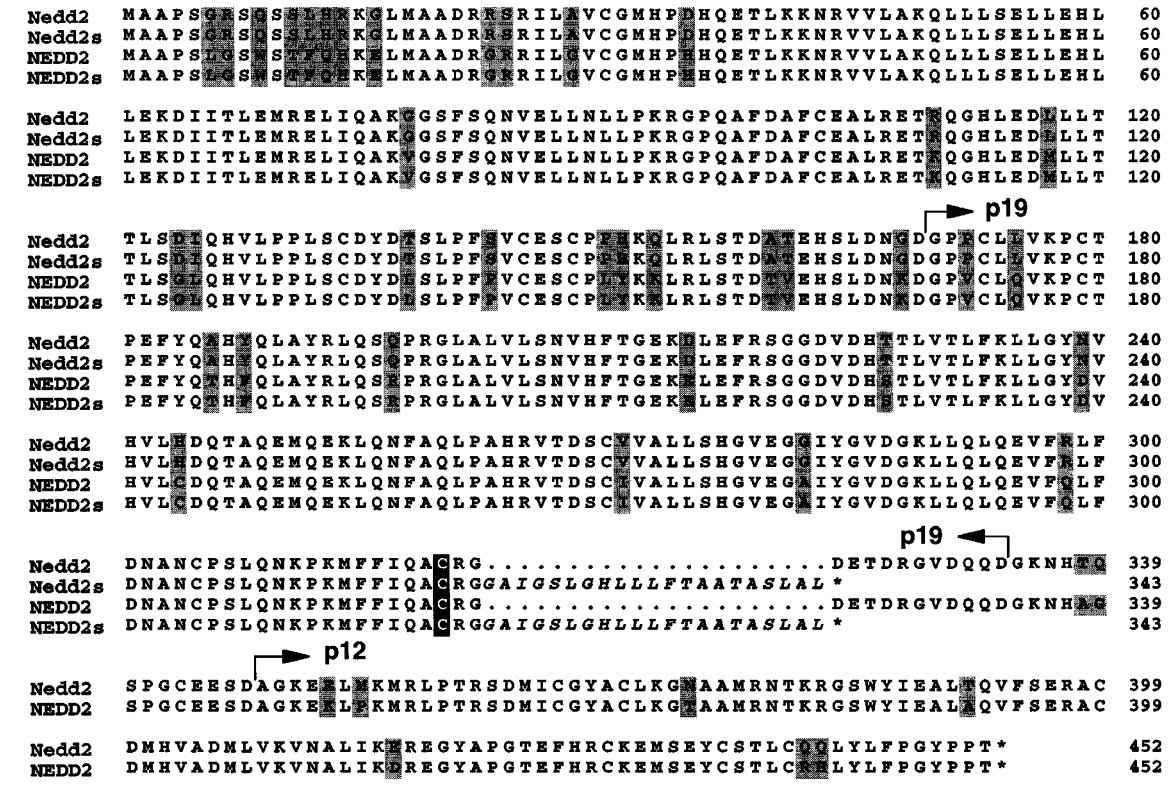

Figure 1 An alignment of the mouse and the human Nedd2 protein sequences. Mouse and human sequences are shown as Nedd2/Nedd2s and NEDD2/NEDD2s, respectively. The numbering in our human sequences (NEDD2 and NEDD2s) differs from those reported for ICH-1$_L$ and ICH-1s as the sequences reported by Wang *et al* (1994) appear to lack the amino terminal regions of these proteins. The catalytic cysteine (C) residues are shown in the black boxes and the cleavage sites in Nedd2/NEDD2 sequence which generate p19 and p12 subunits are indicated. The residues not conserved between the mouse and the human proteins are shown in shaded boxes. The sequence unique to the short form of proteins is shown in italics.

sequence was identical to that reported for ICH-1s (Wang *et al*, 1994) except that it contained 31 extra amino acid residues at the amino terminus (Figure 1). To mark this difference, we have used NEDD2s and ICH-1s respectively for our human cDNA and that of Wang *et al.* Also due to this difference at the amino terminus, our numbering of amino acid residues is different from that reported for ICH-1. The long and short forms of Nedd2/NEDD2 protein consist of 452 and 343 amino acid residues, respectively (Figure 1). The mouse sequences obtained from several lung cDNA clones contained one extra leucine residue (CTC sequence) at position 88 than reported earlier by us (Kumar *et al*, 1994a). This is consistent with the human NEDD2 sequence and with the recently reported mouse Nedd2 sequence (Allet *et al*, 1996).

The Nedd2/ICH-1_L precursor (proNedd2) is cleaved into p19 and p12 subunits by the action of other ICE-like proteases (Harvey *et al*, 1996, Xue *et al*, 1996). The cleavage of pro-Nedd2 occurs after the Asp residues as shown in Figure 1. Thus the two forms of Nedd2 protein differ from each other in two respects: (1) Nedd2s/NEDD2s lacks the region corresponding the p12 subunit and (2) it lacks 11 residues present at the carboxyl terminus of the p19 subunit, replacing them with 21 unique residues which are identical between the mouse and human proteins (Figure 1). *In vitro* translation of mRNA derived from *Nedd2* and *Nedd2s*/*NEDD2s* clones produced protein products of approximately 51 and 38 kDa, respectively (data not shown) which is consistent with the predicted sizes from their conceptual translation (Figure 1).

Structure of the coding region of the mouse *Nedd2 gene*

Using the *Nedd2* cDNA as a probe, genomic DNA fragments of the mouse *Nedd2* were isolated from a phage library. Three independent overlapping clones (λ6, λ9 and λ10) with the strongest hybridisation signals were further characterised. The restriction map and exon-intron structure for the coding region of *Nedd2* are shown in Figure 2. Nedd2 and Nedd2s are encoded by a common set of exons clustered along an approximately 15 kb region of genomic DNA. *Nedd2s* open reading frame is composed of ten exons and the presence of seventh exon (61 bp) corresponds to the unique sequence to

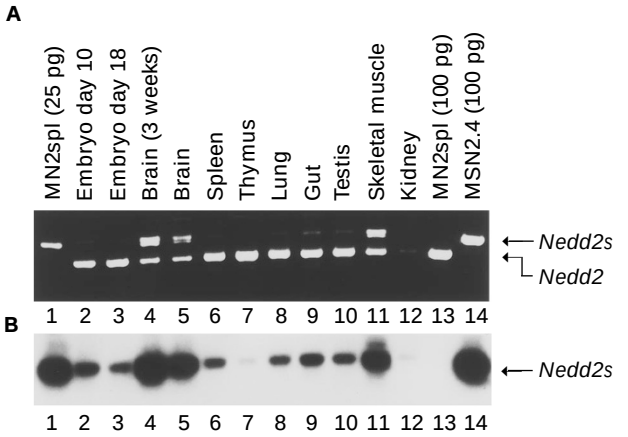

Figure 3 Relative expression of *Nedd2* and *Nedd2s* mRNA in various mouse tissues. (A) RT – PCR analysis of expression was carried out as described in the methods. The Figure shows a representative example of such an analysis in which PCR was performed for 35 cycles. Note that some of the weaker bands (eg lanes 6 and 7) for *Nedd2s* are not apparent in the picture due to a loss during photographic reproduction. In (B) the gel shown in (A) was blotted to transfer membrane and hybridised to a 40 base oligonucleotide probe specific for the *Nedd2s* form. In (A) additional bands visible in brain and skeletal muscle (lanes 4, 5 and 11) just below the *Nedd2s* band, are probably nonspecific PCR products as these were not detected in all experiments and did not hybridise to *Nedd2* probes. Weaker signals in lane 12 (kidney) are probably due to smaller amount of initial RNA sample used for cDNA synthesis. Further experiments confirmed that the levels of *Nedd2* mRNA in kidney were similar to those seen in lung and testis (lanes 8 and 10 respectively) and as previously reported (Kumar *et al*, 1994a). In minus RNA and minus cDNA controls (not shown), no PCR products were visible.

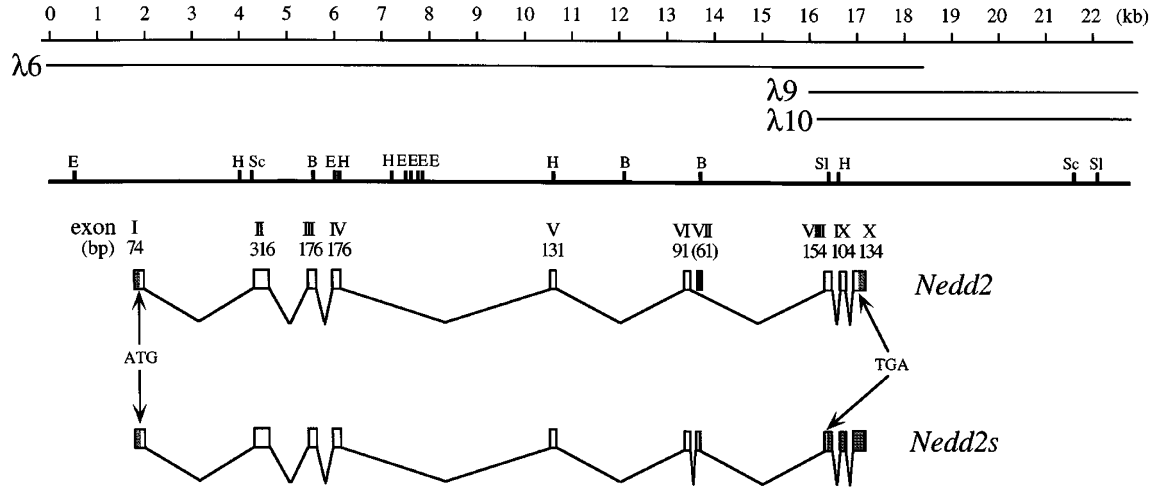

Figure 2 Structure of mouse *Nedd2* gene and origin of two alternately spliced mRNA species. Three phage clones, λ6, λ9 and λ10, are shown with the restriction map. The exon containing the first methionine codon is numbered as exon I. The length of each exon is indicated. Open and shaded segments of the boxes designate translated and untranslated regions of the exons, respectively. A black box designates the seventh exon (exon VII, 61bp), which is alternatively spliced between *Nedd2* and *Nedd2s*. Abbreviations: E; EcoRI, H; HindIII, Sc; SacI, Sl; SalI, ATG; the first methionine codon, TGA; termination codon.

Table 1 Relative levels of expression of the two alternatively spliced *Nedd2* transcripts in various mouse tissues and cell lines as determined by RT-PCR

Tissue or cell line	Approximate *Nedd2/Nedd2s ratio*
Total embryo day 10	5
Total embryo day18	5
Brain (3 week old pups)	1
Brain	1
Spleen	10
Thymus	15
Lung	8
Gut	5
Testis	5
Skeletal muscle	1
Kidney	5
Neural precursor cells	3
PCC4 (embryonal carcinoma)	2
N18 (neuroblastoma)	4
NIH-3T3 (fibroblast)	5
FDC-P1 (myeloid progenitor)	5
ES (embryonic stem cells)	2

These data are representative of several independent experiments.

Table 2 Co-expression of the two forms of *Nedd2* fails to prevent apoptosis in N18 and NIH-3T3 cells

Expression constructs	β-galactosidase-positive apoptotic cells	
	N18	NIH-3T3
Vector	1.8 ± 1.3	1.5 ± 1.4
Nedd2	96.0 ± 2.9	90.5 ± 4.2
Nedd2-Gly320	1.6 ± 1.3	1.8 ± 1.2
Nedd2-Gly320+Nedd2	93.0 ± 3.5	89.7 ± 4.8
mN2s	1.5 ± 1.2	1.7 ± 1.8
mN2s+Nedd2	97.3 ± 1.2	91.2 ± 4.0
hN2s	2.7 ± 1.1	2.9 ± 2.2
hN2s+Nedd2	98.0 ± 1.2	87.8 ± 5.7

Various *Nedd2* expression constructs were co-transfected with a β-galactosidase expression plasmid (pEF-βgal) using LIPOFECTAMINE. Cells were fixed 18–24 h post-transfection, stained with X-gal for 2–6 h , and numbers of blue cells with either normal flat, or small round and apoptotic morphology were scored. The data (mean $\pm$ SEM) are shown as percentage of morphologically apoptotic cells among all the β-galactosidase positive blue cells. The data in the Table were derived from observations on at least 600 blue cells for each cell type transfected with various constructs, and were collected from a set of experiments carried out in triplicate. Similar results were obtained in three independent experiments. mN2s and hN2s represent the mouse and human *Nedd2s/NEDD2s*, respectively.

this isoform. Therefore, the two RNA species are transcribed from a single gene and the 61-base exon is excised out to produce *Nedd2* mRNA, while alternative splicing retains this exon resulting in a frameshift to give rise to the truncated open reading frame in *Nedd2s*. A similar mechanism of alternative splicing has been reported in *ICH-1*, where the 61-base exon is spliced out for *ICH-1*$_L$ and retained for *ICH-1s* (Wang *et al*, 1994).

Expression of the mouse *Nedd2s*

Previous Northern blot analysis and *in situ* hybridisation analysis has shown that *Nedd2* gene is ubiquitously expressed in all tissues examined (Kumar *et al*, 1994a,b; Wang *et al*, 1994). As the two *Nedd2* transcripts only differ in size by 61 nucleotide residues and thus not discernible by Northern analysis, we analyzed the expression of *Nedd2* in various mouse tissues and cell lines by reverse transcriptase-coupled polymerase chain reaction (RT–PCR). Consistent with previous results (Wang *et al*, 1994), both transcripts were widely expressed however there was a clear difference in the relative levels of the two transcripts in various tissues and cell lines (Figure 3). Since Nedd2s has been implicated as an inhibitor of Nedd2 function, we determined approximate ratios of expression of the two forms of mRNA by densitometric analysis of signals. As shown in Table 1, *Nedd2* is the dominant form expressed in most tissues, with the exception of brain and skeletal muscle, which expressed both transcripts in low but approximately equal amounts (Figure 3). Similar results have been reported for adult Mongolian gerbil brain where expression of both forms is roughly equal and both forms are upregulated 2-fold 3–12 h post-ischaemia (Kinoshita *et al*, 1997). The lowest expression of *Nedd2s* was seen in thymus, followed by spleen and lung (Figure 3 and Table 1).

Nedd2s fails to prevent cell death induced by Nedd2

Ectopic expression of the complete coding region of *Nedd2*/*ICH-1* induces apoptosis in a number of mammalian cell lines (Kumar *et al*, 1994a; Wang *et al*, 1994). We assessed whether this cell death can be inhibited by co-transfecting the two forms in two mouse cell lines, NIH-3T3 (fibroblast) and N18 (neuroblastoma). As shown in Table 2, both mouse and human *Nedd2s*/*NEDD2s* were unable to induce apoptosis in both cell types. In addition, both also failed to prevent apoptosis induced by Nedd2. A mutant form of Nedd2 in which the catalytic cysteine residue has been replaced with a glycine residue (Nedd2-Gly320) thereby inactivating the protease activity, is unable to induce apoptosis (Table 2; Kumar *et al*, 1994a,b). Co-transfection of Nedd2-Gly320 also failed to inhibit *Nedd2*-induced apoptosis. These results suggested that both Nedd2s/NEDD2s and the catalytically inactive mutant do not act as dominant modulators of apoptosis induced by the ectopic expression of Nedd2 protease.

To further analyse the effect of the short form of Nedd2 protein, we generated NIH-3T3 and N18 cells stably transfected with *Nedd2s*/*NEDD2s*. When analyzed by Northern blotting, these cells showed detectable production of mRNA generated from the transfected cDNAs (Figure 4). When these cells were transiently transfected with the full length *Nedd2*, no significant effect was apparent on apoptosis (Table 3). Under similar experimental conditions, NIH-3T3 and N18 cells stably transfected with *Bcl-2* showed marked protection of apoptosis induced by the overexpression of *Nedd2*. Interestingly, N18 cells expressing a part of *Nedd2* cDNA in antisense orientation, showed a small but significant delay of apoptosis induced by *Nedd2* (Table 3).

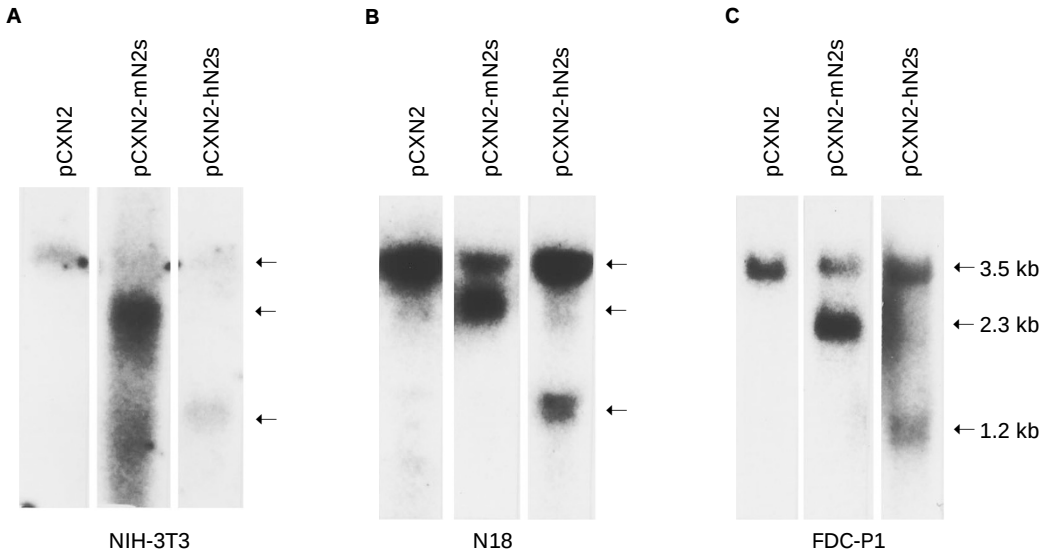

Figure 4 Expression of *Nedd2s* in the tranfected mouse cell lines. Poly A^+ RNA samples isolated from the pooled G418-selected transfected cells were analysed by Northern blotting using a mouse *Nedd2* cDNA probe. Similar results were obtained when a human probe derived from HbN2.1 was used (not shown). The blots were exposed for varying lengths of time ranging from 1–4 days. The 3.5 kb transcript in all lanes represents the endogenous mouse *Nedd2* and serves as an internal control. The ~2.3 kb and ~1.2 kb transcripts are derived from transfected mouse and human *Nedd2s*/*NEDD2s* respectively. The mouse *Nedd2s* expression construct (pCXN2-mN2s) contains an additional 1.1 kb of 3′ noncoding cDNA not present in the human expression plasmid (pCXN2-hN2s). Approximate locations of the three transcripts are shown by arrows.

Table 3 Induction of apoptosis by *Nedd2* in cells stably expressing *Nedd2s*

Cells	β-galactosidase-positve apoptotic cells (%) Vector	Nedd2
NIH/vector	0.9±0.6	91.1±4.8
NIH/mN2s	0.9±0.4	85.1±3.1
NIH/hN2s	0.8±0.7	83.6±2.5
NIH/Bcl-2	0.8±0.2	30.2±6.2
N18/vector	1.3±0.8	95.0±3.3
N18/mN2s	2.3±1.3	90.4±5.9
N18/hN2s	1.7±1.5	89.7±1.9
N18/Bcl-2	2.4±1.1	31.2±5.9
N18/Nedd2AS	2.5±1.5	60.7±2.3

Pooled NIH-3T3 or N18 cells stably transfected with the indicated expression constructs were transiently transfected using LIPOFECTAMINE with either pCXN2 (Vector) or pCXN2-N2 (Nedd2), and pEF-βgal. Cells were fixed 18–24 h post-transfection, stained with X-gal for 2–6 h and scored as in Table 2. Although the data (mean±SEM) shown in the table were derived from a single experiment done in triplicate, similar results were obtained in three separate experiments. mN2s and hN2s represent the mouse and human *Nedd2s*/*NEDD2s*, respectively. Nedd2AS contains part of the mouse *Nedd2* cDNA in antisense orientation.

Inhibition of apoptosis in Nedd2s expressing cells under serum- or factor-deprived condition

Since ICH-1s has been shown to inhibit apoptosis in Rat-1 cells under serum-free conditions, in further experiments we analyzed whether NIH-3T3 and N18 cells expressing Nedd2s show any suppression or delay of cell death under factor-deprived conditions. As shown in Figure 5A, more than 90% of the NIH-3T3 transfected with pCXN2 vector alone died within 7 days, while more than 50% of the cells expressing Bcl-2 remained viable during this period. NIH-3T3 cells transfected with either the mouse or the human *Nedd2s*/*NEDD2s* showed small but significant inhibition of death induced by serum deprivation and at 7 days post-serum withdrawal, >20% of the cells were still viable (Figure 5A). While N18 cells transfected with *Bcl-2* showed significant resistance to death induced by serum-depletion, in these cells, both human and mouse *Nedd2s*/*NEDD2s* were unable to inhibit or delay apoptosis (Figure 5B). The levels of mRNA for *Nedd2s*/*NEDD2s* were similar in two transfected cell lines (Figure 4). Therefore it appears that the differences observed in these experiments are due to cell type. This was further evident when a factor dependent myeloid progenitor cell line FDC-P1 was used in similar experiments. In the absence of cytokine (GM-CSF or IL-3), FDC-P1 cells die rapidly by apoptosis. Although FDC-P1 cells transfected with *Bcl-2* were significantly resistant to apoptosis induced by GM-CSF withdrawal, no significant inhibitory effect was seen with either human or mouse *Nedd2s*/*NEDD2s* expression (Figure 5C).

Co-expression of the p19 and p12 subunits of Nedd2 fails to induce cell death

The main difference between the p19 subunit of Nedd2/ICH-1_L and Nedd2s/ICH-1s is that in Nedd2s/ICH-1s the 11 amino acid residues at the carboxyl terminus are replaced by 21 amino acid residues derived from the alternatively spliced exon 7. Importantly, Nedd2s/ICH-1s protein retains the crucial residues of p19 subunit which correspond to the ones implicated in catalysis and substrate binding in the p20 subunit of ICE (Walker *et al*, 1994; Wilson *et al*, 1994). Thus it was of interest to check whether the co-expression of Nedd2s with the p12 subunit would generate an active enzyme and

induce apoptosis. We generated expression constructs carrying only the regions of cDNA corresponding to the p19, p12 and p20 (Nedd2s) subunits. Surprisingly, co-transfection of p19 and p12 subunits of Nedd2 into NIH-3T3 cells failed to induce apoptosis (Figure 6). Indeed, cotransfection of the p20 Nedd2s with p12 was also unable to induce apoptosis in NIH-3T3 cells (Figure 6). In further experiments, we cotransfected p12 expression construct and constructs carrying either the prodomain+p19 (pCXN2-N2N) or prodomain+p20 (pCXN2-N2s) into NIH-3T3 cells. Again, these constructs failed to induce apoptosis (Figure 6). Since full length cDNA encoding the p51 pro-Nedd2 is capable of inducing apoptosis owing to its protease activity, these experiments suggest that an intact precursor is required for the correct processing and folding of the protease.

Discussion

Among the members of the caspase family, Nedd2/ICH-1 shares limited similarity with the ICE-subfamily and the CPP32-subfamily (reviewed in Kumar and Lavin, 1996). Nedd2 protease has not been extensively characterised and its substrate specificity and downstream targets remain unknown. Some of our recent work has shown that Nedd2 fails to cleave proteins that are cleaved by CPP32 and Mch3 (Waterhouse *et al*, 1996; our unpublished data), despite the fact it shows some activity on DEVD-AFC, a fluorogenic tetrapeptide substrate of CPP32 (Harvey *et al*, 1996; Xue *et al*, 1996). In addition, Nedd2 is only partially inhibited by the baculovirus p35 and not inhibited by the cowpox virus protein CrmA (Wang *et al*, 1994a,b; Xue and Horvitz, 1995; L. Dorstyn

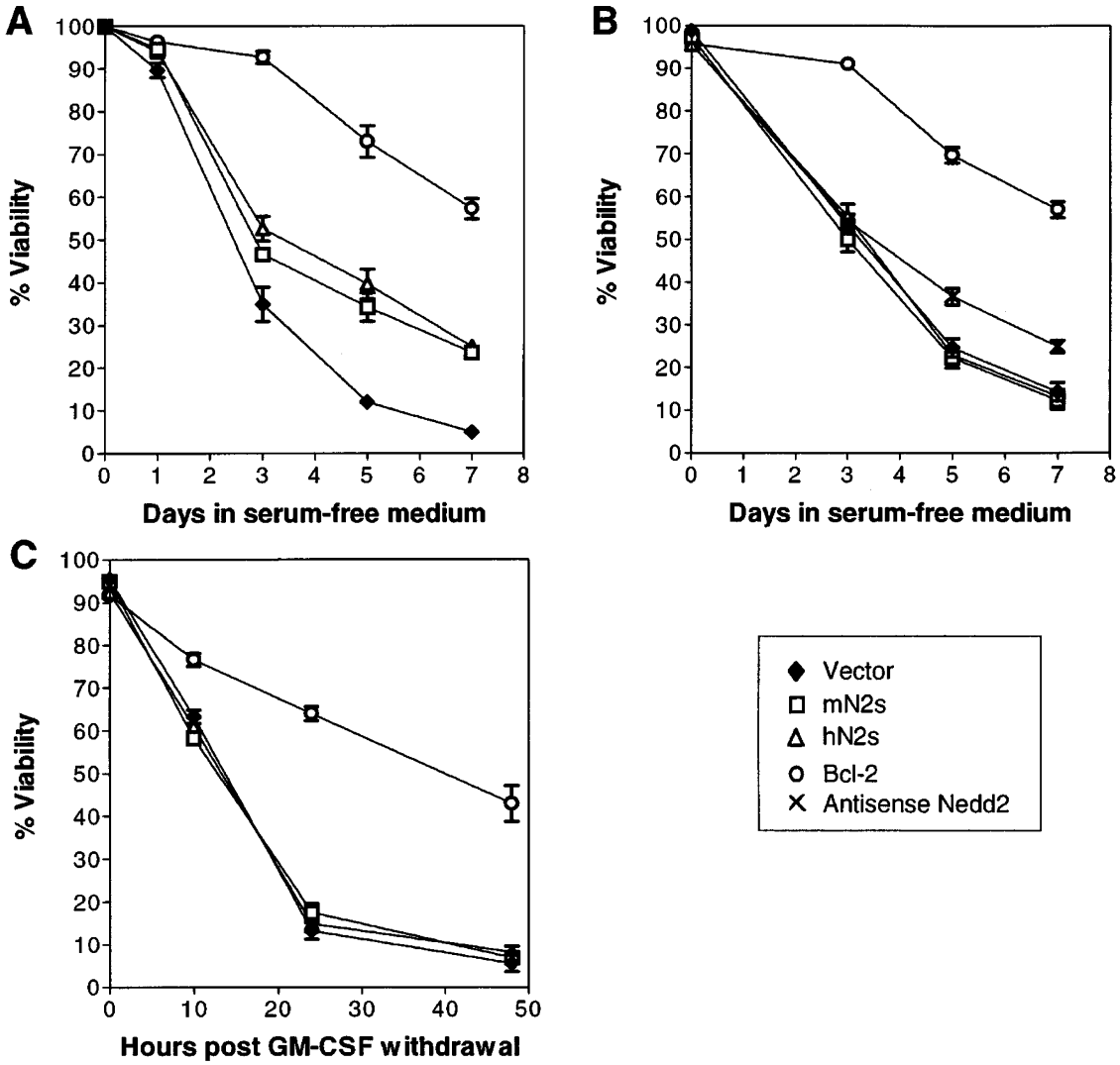

Figure 5 Effect of serum/factor withdrawal on apoptosis in cells expressing the smaller form of *Nedd2*. (A) NIH-3T3; (B) N18 and (C) FDC-P1. Pooled populations of transfectants were plated in complete medium. Following day, cells were washed and medium replaced with serum-free (NIH-3T3 and N18) or GM-CSF-free medium (FDC-P1). At indicated times, cells were analyzed for viable population by trypan blue exclusion. Data shown were derived from a single experiment done in triplicate. Similar results were obtained in at least two experiments.

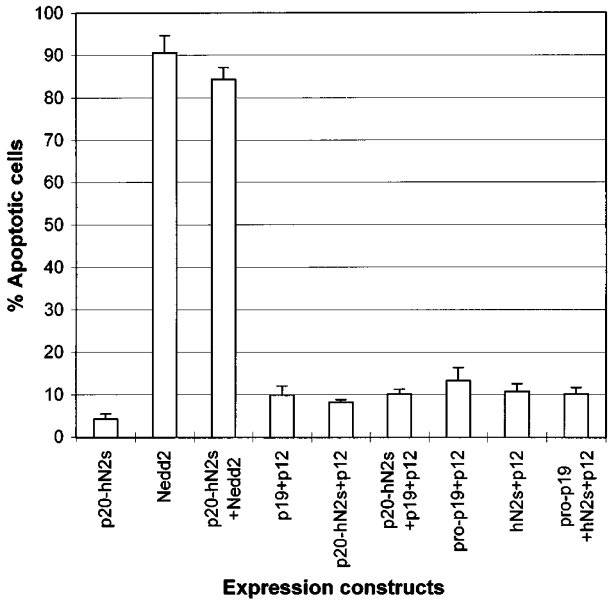

Figure 6 Co-transfection of the cDNA encoding two subunits of Nedd2 fails to induce apoptosis. Various expression constructs were co-transfected with pEF-βgal into NIH-3T3 cells by lipofection using DOSPER. At 18 h post-transfection cells were fixed, stained with X-gal and blue cells observed for apoptosis. Bars represent apoptotic cells as % of total β-galactosidase +ve cells $\pm$SEM. At least 600 blue cells were scored for each dish. The data shown were derived from an experiment done in triplicate. Similar results were obtained in three independent experiments. pCXN2-p20s (p20-hN2s) lacks the NEDD2s prodomain. pCXN2-p19 (p19) and pCXN2-p12 (p12) contain only the regions of cDNA corresponding to the two subunits of Nedd2. pCXN2-N2N (pro-p19) contains the region of cDNA encoding the amino terminal 328 amino acid residues.

and S. Kumar, unpublished data). Although, as mentioned earlier, there are several lines of evidence to suggest a role for Nedd2 in apoptosis, a direct demonstration for this is lacking. Perhaps the most convincing evidence was the demonstration by Wang *et al*. (1994) that the short form of ICH-1 generated by alternative splicing can inhibit serum deprivation induced apoptosis in Rat-1 fibroblasts.

In most tissues, the expression of *Nedd2* is low (Kumar *et al*, 1994). Except for brain and skeletal muscle, where the levels of both transcripts are low but both are expressed in equal ratio, *Nedd2s* is a minor transcript. One can argue that lower Nedd2 : Nedd2s ratio in tissues with low cell turnover such as brain and skeletal mucle, and higher ratio in tissues where substantial cell death occurs such as thymus and spleen, might suggest an apoptosis inhibitory function for the shorter protein. Whether this is relevant in a physiological context remains to be determined. Co-transfection of the two forms, which is likely to generate equal levels of mRNA for the two proteins, kills cells as efficiently as the long form of Nedd2. This suggests that the level of the long form of Nedd2 and not that of Nedd2s, might dictate the fate of the cell. This is further supported by our observation that in Mongolian gerbils, both forms of *Nedd2* are upregulated to similar extent upon transient forebrain ischaemia (Kinoshita *et al*, 1997).

Our results with NIH-3T3 cells expressing either the mouse or the human *Nedd2s* are consistent with the Rat-1 results, however, no such inhibition of cell death was seen in two other cell lines. As suggested by Wang *et al*. (1994), in NIH-3T3 and Rat-1 cells the partial inhibition of apoptosis induced by serum withdrawal may be due either to (1) the competition between the p19/p20-Nedd2s for the p12 subunit, or (2) interaction of Nedd2s with some other apoptosis regulator. If the first possibility was true, the levels of expression of the two form would determine the extent of apoptosis. This however does not appear to be the case as in transient co-transfection experiments where two forms are likely to be expressed in roughly equal levels, no inhibition of *Nedd2* induced apoptosis was evident. Moreover, in N18 and FDC-P1 cells, where the ratio of two forms is comparable to that in NIH-3T3 cells, no protection from serum- or factor- withdrawal induced apoptosis is seen. In isolated *Nedd2s*/*NEDD2s* transfected clones expressing varying levels of the transcript, results similar to those shown in Figure 5 were obtained (data not shown). These observations suggest that Nedd2s/ICH-1s is unlikely to act by competing with Nedd2/ICH-1. In NIH-3T3 and Rat-1 cells, the effect of Nedd2s/ICH-1 may be mediated by interaction with components other than Nedd2/ICH-1. Cross species interactions between subunits derived from different ICE-like proteases has been demonstrated (eg. Gu *et al*, 1995; Fernandes-Alnemri *et al*, 1995). Whether Nedd2s also functions to inhibit apoptosis in Rat-1 and NIH-3T3 cells by interacting with the smaller subunits of other caspases, remains to be determined. The differential effect of Nedd2s seen in three cell lines used in our study may be due to the different levels of expression of various caspases or other molecules which interact with Nedd2s in these cells.

Some members of the caspase family such as Nedd2, ICE, ICE_{rel}-II, ICE_{rel}-III, Mch4 and Mch5/FLICE/MACH contain relatively long prodomains, while others including CPP32, Mch2 and Mch3, the prodomain consists of a very short stretch of amino acid residues (see Kumar and Lavin, 1996 for some examples). Based on recent findings it appears that the function of the long prodomains in some caspases is to act as an adaptor to link the proteases to a death signalling complex. For example, the prodomain of FLICE/MACH binds to FADD, a protein which is recruited to the death complex upon activation of Fas/APO-1 receptor (Boldin *et al*, 1996; Muzio *et al*, 1996). Another function of the prodomain of caspases is evident from a recent study which shows that the prodomain of ICE is required for dimerisation prior to the processing of pro-ICE (Van Criekinge *et al*, 1996). The experiments reported in this paper demonstrate that co-transfection of the p12 and p19 subunits fails to induce apoptosis. We have also noticed that *in vitro* mixing and refolding of the p19 and 12 subunits of Nedd2 expressed in *E. coli* fails to generate catalytically active Nedd2 enzyme (N. Harvey *et al*, unpublished observations). A recent study by Allet *et al* (1996) also supports our conclusions. These authors have shown that while microinjection of the full length *Nedd2* cDNA expression construct into sympathetic neurons results in apoptosis, expression constructs lacking the

prodomain or containing only the regions corresponding to the two subunits (p19 and p12) fail to trigger cell death. These data suggest that the intact prodomain of Nedd2 is essential for function and might be required for proper dimerisation of the precursor prior to processing, or interaction with other proteins. Nedd2 and Nedd2s share identical prodomains and p19 differs from Nedd2s by only a few amino acid residues at the carboxyl terminus. Thus, Nedd2 and Nedd2s may heteromerise through their prodomains. Whether this occurs *in vivo* and has an effect on the outcome of the apoptotic response of a cell, awaits further studies.

Materials and Methods

cDNA cloning of the human and mouse *Nedd2s/NEDD2s*

Using an EcoRI fragment of mouse *Nedd2* cDNA as a probe (Kumar *et al*, 1994a,b), we screened approximately 5×10^5 clones from a human foetal brain cDNA library (Stratagene) by standard protocols (Sambrook *et al*, 1989) and isolated two *NEDD2*/*ICH-1* cDNA clones (HbN2.1 and HbN2.3) (Kumar *et al*, 1995). These clones contained approximately 1.1 kb and 0.8 kb cDNA inserts, respectively, covering a part of the 5′ region of the human *NEDD2* mRNA. HbN2.1 clone contained the entire coding region of the shorter form of NEDD2/ICH-1 protein (NEDD2s/ICH-1s) (Kumar *et al*, 1995). Three partial cDNA clones containing the 61 bp sequence from the alternatively spliced exon were isolated from a mouse lung cDNA library (Stratagene). To create a complete coding cDNA for the mouse Nedd2s, the 0.29 kb HindIII/SalI fragment of the clone MSN2.4, encoding the long form of Nedd2 (Kumar *et al*, 1994a) was replaced by a 0.35 kb HindIII/SalI fragment from clone MLN2.3 containing the 61 bp exon. This plasmid was named MN2spl.

Isolation and mapping of the mouse *Nedd2* genomic DNA clones

A mouse genomic DNA library in λFIX II (Stratagene) was screened with [α^{32}P]-labelled probe generated by a random priming method from a cDNA fragment isolated from clone MN2.4, which contains the entire open reading frame of *Nedd2* (Kumar *et al*, 1994a). By screening 4×10^5 plaques we isolated 11 positive clones after three cycles of screening and harvested phage DNA from three independent clones (λ6, λ9 and λ10) with the strongest hybridisation signals. Restriction mapping (AccI, BamHI, EcoRI, EcoRV, HindIII, MluI, NotI, PstI, SalI, SmaI, SpeI, SstI, XbaI and XhoI) combined with Southern hybridisation using several oligonucleotide probes revealed that the three clones overlapped and λ6 (18.5 kb) contained all the exons for the coding regions of *Nedd2* and *Nedd2s* mRNAs (Figure 2). Several of the restriction fragments of these clones were subcloned in pBluescript SK(−) (Stratagene) and sequences around the exon-intron junctions determined by using a series of primers synthesised based on the cDNA sequence.

RNA isolation and expression analysis

Total RNA was prepared from cultured cells and mouse tissues using RNAzol B according to the instructions provided by the manufacturers (Tel-Test, Inc.). Poly A^+ RNA was isolated by oligo dT batch absorption (Sambrook *et al*, 1989). Samples were electrophoresed on 1.2% agarose/2.2 M formaldehyde gels, transferred to Biodyne A membrane (PalI) and hybridised to probes labelled with [α-^{32}P]-dCTP by random priming. Mouse *Nedd2* probe was a 2.2 kb BamHI/XhoI fragment of the clone MSN2.4 representing the entire coding region of the cDNA (Kumar *et al*, 1994a). Human *NEDD2*/*ICH-1* probe was derived as a 0.8 kb EcoRI fragment of the clone HbN2.3 (Kumar *et al*, 1995). For PCR detection of *Nedd2* and *Nedd2s* transcripts, two oligonucleotide primers 5′-CAGAATTTTGCACAGTTACCTGCAC and 5′-CAAGCATAGCCACATATCATGTCTG, representing the sequences upstream and downstream of the 61 bp alternatively spliced sequence in *Nedd2s* were used. Using these primers, *Nedd2* and *Nedd2s* transcripts generate PCR products of 343 and 404 bp respectively. cDNA was synthesized from 1 μg of polyA$^+$ RNA using Superscript (Life-Technologies). Ten percent of the cDNA or 25–100 pg of plasmid DNA containing *Nedd2*/*Nedd2s* cDNA were amplified by 35 cycles of PCR with each cycle consisting of 1 min incubation each at 92°, 65° and 72°C. PCR products were analyzed on 1.5% agarose gels and when necessary tranferred to Biodyne A (PalI) and hybridised either to a ^{32}P- labelled oligonucleotide probe specific for *Nedd2s* form (a 40 base antisense oligonucleotide from exon 7 sequence in Figure 2), or a cDNA probe as described above. Relative signal intensities for the two transcripts were estimated by densitometry as described previously (Kinoshita *et al*, 1997).

Construction of expression vectors

Construction of the *Nedd2* expression plasmids pCXN2-N2, pCXN2-N2 Gly-320, pCXN2-N2N (containing the region of cDNA encoding *Nedd2* prodomain plus the p19 subunit) and pCXN2-N2AS have been described previously (Kumar *et al*, 1994a; Kumar, 1995b). pCXN2-N2 contains the entire coding region of Nedd2 cDNA in pCXN2 mammalian expression vector (Niwa *et al*, 1991). An antisense expression vector (pCXN2-N2AS) was constructed by cloning the blunt ended 1.0 kb SalI fragment in antisense orientation with respect to β-actin promoter in pCXN2 vector. pCXN2-N2 Gly-320 contains entire ORF of *Nedd2* but carries a cysteine to glycine substitution at position 320 (Figure 1). pCXN2-mN2s was created by blunt end ligation of the T4 polymerase treated 2.2 kb ApaI/XbaI fragment from pMN2spl into the EcoRI digested/T4 polymerase treated pCXN2 vector. To generate pCXN2-hN2s, the T4 polymerase treated 1.1 kb fragment of HbN2.1 was inserted into pCXN2 as above. To construct the p19, p12 and p20 (hN2s minus the prodomain) expression vectors (pCXN2-p19, pCXN2-p12 and pCXN2-p20s, respectively), regions of the *Nedd2* cDNA corresponding to these subunits were amplified by PCR and cloned blunt ended into pCXN2. Following primers were used for PCR: 5′-GCCGCCATGGGTCCTCCCTGTCTTCTG and 5′-GAGAGATCAATCTTGCTGGTCGACACC (p19); 5′-GCCGCCATGGCTGGCAAAGAGGAGTTG and 5′-GAGAGATCACGTGGGGTGGGTAGCC (p12); 5′-GCCGCCATGGGTCCTGTCTGCCTTCAG and 5′-GAGAGATTACAGAGCAAGAGAGGC (p20s).

In all cases, an optimised Kozak sequence with ATG (underlined) was inserted in frame at the start of protein coding sequence by PCR.

Cell culture and transfection

Cells were maintained as described previously (Kumar *et al*, 1994b; Kumar, 1995). In transient transfection studies, NIH-3T3 and N18 cells were plated at a density of 2×10^5 cells/35 mm dish. The following day, 2.5 μg of pCXN2 plasmid constructs and, where indicated, 0.5 μg of a β-galactosidase expression vector (pEF-βgal) were co-transfected using 10 μl of Lipofectamine (Life Technologies) as described (Kumar *et al*, 1994a). In some experiments (eg. Figure 6), DOSPER reagent (Boeringer-Mannheim) was used for transfection. For β-galactosidase expression analysis, at 18–24 h post-transfection cells were rinsed with PBS, fixed with 2% formaldehyde, 0.2% glutaraldehyde in PBS for

5 min, washed twice with PBS, and stained for 2–6 h with 0.1% X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS. After rinsing in PBS, β-galactosidase positive cells were microscopically observed for apoptosis. All transient trasfection experiments were carried out in triplicate and were performed at least three times. In these assays, some variations were obvious between different sets of experiments employing different batches of cells, but overall results were similar. NIH-3T3 and N18 cells expressing Bcl-2 have been described elsewhere (Kumar *et al*, 1994a). Murine myeloid progenitor cell line FDC-P1 was transfected and selected using Lipofectamine as described previously (Kumar, 1995b). Briefly, 4 μg of plasmid DNA in 0.5 ml OPTIMEM (Life Technologies) was mixed with 10 μl of Lipofectamine in 0.5 ml OPTIMEM and incubated at room temperature for 30 min. FDC-P1 cells (10^6/transfection) were washed once in OPTIMEM, resuspended in DNA/Lipofectamine mix, supplemented with 40 units/ml GM-CSF, and plated into a 6-well dish. After 6 h incubation at 37°C, each well was fed with 3 ml of OPTIMEM containing 20% FCS and 40 units/ml GM-CSF. The following day, cells were pelleted, washed and plated in DMEM supplemented with 10% FCS, 40 units/ml GM-CSF and appropriate drug for selection. Transfected cells were selected for antibiotic resistance for 2–4 weeks prior to cloning.

Cell death assays

For serum withdrawal experiments NIH-3T3 or N18 cells were plated at a density of 2×10^5 cells/well in a 6-well tray. Semiconfluent cell monolayers were washed three times in PBS and refed with DMEM without foetal calf serum. At 24 h intervals, cells were harvested and assessed for viability. Detached cells in the culture supernatants were collected by centrifugation. Adherent cells were washed with PBS and trypsinised. Fractions from supernatant, washes and trypsinised cells were pooled and recovered by centrifugation. Pellets were resuspended in 0.5–1.0 ml PBS and viability and apoptosis determined by trypan blue exclusion and nuclear staining respectively. In experiments employing FDC-P1 cells, cell were washed in DMEM supplemented with FCS three times and resuspended at 2×10^6 cells/ml in the same medium. One hundred μl aliquots were incubated in 96-well tray for the desired length of time. In control experiments, cells were washed in a similar way and resuspended in medium containing 40 units/ml of GM-CSF to account for the cell death which may occur during washing procedures. Equal volumes of cell suspension were mixed with 0.8% trypan blue and dye positive and negative cells were counted using a haemocytometer. In these experiments, at time zero (soon after the washing steps), typically more than 90% cells were viable as determined by trypan blue dye exclusion. In all cell death experiments, cells were examined and scored for apoptosis-associated morphological changes.

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