THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 20, Issue of May 16, pp. 13134–13139, 1997

Printed in U.S.A.

Functional Activation of Nedd2/ICH-1 (Caspase-2) Is an Early Process in Apoptosis*

(Received for publication, February 20, 1997)

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The ICE/CED-3 family of proteases (caspases) play a central role in the execution phase of apoptosis. These proteases are synthesised as precursor molecules that require processing at specific aspartate residues to produce the two subunits that comprise the active enzyme. The activation of some of these proteases has been shown to occur during apoptosis. Here we show that Nedd2/ICH-1 (caspase-2) is activated during apoptosis induced by a variety of apoptotic stimuli. This activation occurs very early upon treatment of cells with apoptotic agents and appears to precede the activation of CPP32 (caspase-3). The activation of Nedd2 was not seen in cells that are resistant to apoptosis. These observations suggest that Nedd2 is an early effector in the pathway leading to cell death. Our observations also lend weight to the hypothesis that a group of caspases containing long prodomains are the first to be activated in response to apoptotic signals and that they lie upstream of a second class of caspases such as CPP32 containing short or no prodomains.

Nedd2 was initially identified as a developmentally down-regulated gene in the mouse central nervous system (1), and its product was subsequently shown to be homologous to the *Caenorhabditis elegans* CED-3 protein and the mammalian interleukin-1β-converting enzyme (ICE)[1] (2). These enzymes are the prototypes of a growing family of aspartate-specific cysteine proteases termed caspases (3) that play a central role in the execution of apoptosis (4–6). Studies with ICE suggest that caspases are synthesized as proenzymes that are cleaved at specific aspartate residues to release two subunits of approximately 10 and 20 kDa, which heterodimerize (7) and possibly associate in a tetramer to form the active enzyme (8, 9). The substrate specificity of caspases requires an aspartate residue at the P_1 cleavage position, a property shared only by the cytotoxic T-lymphocyte serine protease granzyme B (10).

The human caspases can be subdivided into three families based on sequence homology. The ICE-like protease family includes ICE (caspase-1) (7), TX/ICH-2/ICE_{rel}-II (caspase-4) (11–13), and TY/ICE_{rel}-III (caspase-5) (13, 14). The CED-3-like family includes CPP32/YAMA/apopain (caspase-3) (15–17), Mch2 (caspase-6) (18), Mch3/ICE-lap3/CMH-1/(caspase-7) (19–21), Mch4 (caspase-10) (22), MACH/FLICE/Mch5 (caspase-8) (22–24), and ICE-lap6/Mch6 (caspase-9) (25, 26). CPP32, ICE-lap3 and Mch2 have been shown to be activated *in vivo* in response to apoptotic stimuli (20, 27–29). Nedd2/ICH-1 (caspase-2) stands alone in the third caspase subfamily, and to date no systematic study has been carried out to evaluate its activation during apoptosis.

Once activated, the caspases cleave a range of cellular substrates. The DNA repair enzyme poly(ADP-ribose)-polymerase (PARP) was one of the first identified cellular substrates cleaved during apoptosis (30). CPP32 was subsequently shown to cleave PARP with high efficiency (16, 17). Additional substrates that are specifically cleaved during apoptosis by CPP32-like proteases include proteins such as DNA-dependent protein kinase (31–33), U1–70 kDa ribonucleoprotein (31), heteronuclear riboproteins C1 and C2 (34), α-fodrin (35), and nuclear lamins (29, 36). ICE has a substrate specificity of Tyr-Val-His-Asp (YVHD), first identified as the site in pro-interleukin-1β that is cleaved by ICE to release the mature cytokine (7).

Several lines of evidence suggest a role for Nedd2 in the apoptotic pathway. Overexpression of *Nedd2* induces apoptosis in various cell types (2, 37, 38), whereas expression of antisense *Nedd2* in FDC-P1 factor-dependent cells delays the onset of apoptosis induced by factor withdrawal (39). In a similar manner, an alternatively spliced form of *Nedd2* that encodes a truncated protein has been shown to protect against cell death induced by serum withdrawal in Rat-1 and NIH-3T3 cells (37, 40). Up-regulation of *Nedd2* mRNA has been observed in response to ischaemia-induced cell death in the Mongolian gerbil (41), whereas down-regulation of *Nedd2* has been observed during gonadotropin-promoted follicular survival (42). Recently Nedd2 has been shown to associate via its pro-domain with RAIDD, a death adaptor molecule that is thought to be involved in death signaling through the TNF-R1 complex via association with the death domain proteins RIP and TRADD (43). An analogous association occurs between the pro-domain of MACH/FLICE/Mch5 and the death adaptor molecule MORT1/FADD, which recruits MACH/FLICE/Mch5 to the Fas/Apo1 signaling complex in response to Fas-induced apoptosis (23, 24). This suggests that MACH/FLICE/Mch5 activation is the most upstream enzymatic event in the Fas/Apo1 signaling pathway and that MACH/FLICE could subsequently activate other downstream caspases, resulting in apoptotic death. In a similar manner, Nedd2 activation may be an early, upstream event in TNF-R1-induced apoptosis.

In this report we show that proNedd2 is rapidly cleaved to generate active enzyme subunits in response to various apo-

* This work was supported by a Wellcome Trust Senior Research Fellowship in Medical Science and Grant 960532 from the National Health and Medical Research Council of Australia (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] The abbreviations used are: ICE, interleukin-1β-converting enzyme; TNF, tumor necrosis factor; IL, interleukin; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Gy, gray; PARP, poly(ADP-ribose)-polymerase.

ptotic stimuli and that Nedd2 activation precedes the activation of CPP32. These observations demonstrate that Nedd2/ICH1 is a caspase that is activated early in the apoptotic cascade.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—The human megakaryoblastic cell line Mo7e was maintained in suspension culture at 37 °C, 5% CO_2 in α-minimal essential medium supplemented with 10% heat-inactivated fetal calf serum and recombinant human interleukin-3 (IL-3) at a final concentration of 2 ng/ml (Amgen). The Burkitt's lymphoma cell lines BL30A and BL30K were maintained in suspension culture in RPMI 1640 medium containing either 20% (BL30A) or 10% (BL30K) fetal calf serum. Cells were induced to undergo apoptosis by withdrawal of IL-3 and/or serum or by exposure to either etoposide (40 μM) or 30 Gy of γ-irradiation from a ^{137}Cs source.

Preparation of Cytoplasmic Extracts—Cytoplasmic extracts were prepared essentially as described (44) with some modifications. Cells were harvested and washed with ice-cold phosphate-buffered saline and then resuspended (100 μl/10^7 cells) in ice-cold cell extraction buffer (50 mM PIPES, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Cells were allowed to swell on ice for 20 min and then lysed by freeze/thawing twice. Cell lysis was confirmed by trypan blue uptake. Lysates were centrifuged at 200 × *g* for 5 min at 4 °C, and supernatants were then removed and centrifuged at 9000 × *g* for 15 min at 4 °C. The clear cytosol was removed and either used immediately or stored at −70 °C.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—Cytoplasmic extracts (50 μg of total protein) were boiled in protein loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 20% glycerol, 4% SDS, 0.2% bromphenol blue) for 5 min and then centrifuged at 9000 × *g* for 5 min. The denatured proteins were electrophoresed on a 15% SDS-polyacrylamide gel and transferred to polyvinylidine difluoride membrane (DuPont NEN). Membranes were blocked in 5% skim milk in phosphate-buffered saline containing 0.05% Tween 20 at 4 °C overnight. Blots were probed with either an anti-ICH-1_L polyclonal antibody (Santa Cruz Biotech., Inc.) at a 1:500 dilution for 4 h, anti-Mch3 polyclonal antiserum (kindly donated by Dr. G. Cohen) at a dilution of 1:2000 for 2 h, an anti-PARP polyclonal antiserum (Boehringer Mannheim) at a dilution of 1:2000 for 1 h, or an anti-CPP32 monoclonal antibody (Transduction Labs.) at a dilution of 1:1000 for 4 h at room temperature. This was followed by incubation with anti-rabbit (ICH-1_L, Mch3, and PARP) or anti-mouse (CPP32) IgG conjugated with horseradish peroxidase (Amersham Corp.) for 1 h. Signals were detected using the ECL system (Amersham Corp.).

Cleavage of Fluorogenic Caspase Substrates—Cytoplasmic extracts (10 μg of total protein) were incubated with 100 μM DEVD-7-amino-4-trifluoromethyl coumarin or YVAD-7-amino-4-trifluoromethyl coumarin (both from Enzyme Systems Inc.) at 37 °C for 30 min in a final volume of 20 μl in cleavage buffer (25 mM HEPES, pH 7.4, 10% sucrose, 5 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS). The tetrapeptide inhibitors DEVD-CHO and YVAD-CHO (Bachem) used at a final concentration of 1 μM were preincubated for 30 min at 37 °C with cytoplasmic extracts in a 10-μl volume prior to the addition of fluorogenic substrate. Fluorescence was quantified using an Aminco Bowman Luminescence Spectrophotometer (excitation, 400 nm; emission, 505 nm).

In Vitro Cleavage of PARP—The cDNA construct pBS hPARP encompassing a truncated region of human PARP cDNA from nucleotide 93 to nucleotide 1156 (amino acids 1–339 containing the DEVD cleavage site at amino acid residues 211–214) was generated by polymerase chain reaction. The upstream primer 5′-CGGAATTCTAGGTCGTGCGTCGG-3′ was designed to contain an *Eco*RI site (underlined), and the downstream primer used was 5′-GGAATATACGGTCCTGCT-3′. The pBS hPARP construct was used as a template for the production of [^{35}S]methionine (ICN)-labeled PARP protein using the Promega TNT T7 Coupled Reticulocyte Lysate System. 5 μl of labeled product was incubated in proteolysis assays at 37 °C for 1 h with cytoplasmic extracts from apoptotic and nonapoptotic cells (10 μg of total protein) in cleavage buffer in a total volume of 20 μl. Cleavage products were resolved by 15% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidine difluoride membrane (DuPont NEN) using a semi-dry apparatus (Hoeffer), and visualized by autoradiography.

RESULTS

Induction of CPP32-like Activity in Mo7e Following Apoptotic Stimuli—To investigate the magnitude and specificity of protease activity induced by various apoptotic stimuli, the tetrapeptide fluorogenic substrates DEVD-AFC and YVAD-AFC were utilized. The fluorogenic substrate DEVD-AFC mimics the cleavage site at which CPP32 and CPP32-like caspases cleave PARP, whereas the YVAD-AFC substrate is specific for caspases closely related to ICE. Cleavage of these substrates by cellular cytoplasmic extracts is thus indicative of *in vivo* proteolytic activation of these caspases in response to apoptotic signals. We initially studied caspase activation in the factor-dependent hemopoietic cell line Mo7e, which undergoes apoptosis in response to the removal of IL-3 and serum from its growth medium. Following withdrawal of IL-3 and serum from Mo7e, there was a significant, time-dependent increase in the level of DEVD-AFC cleavage, indicating the progressive activation of CPP32 and/or its close relatives in these cells. This activation reflected the progressive increase in the number of apoptotic cells as measured by trypan blue exclusion and nuclear staining (not shown), although an increase in DEVD-AFC cleavage activity was obvious prior to any significant cell death (Fig. 1*A*). Cleavage of DEVD-AFC by cytoplasmic extracts from factor-deprived Mo7e cells was completely inhibited by preincubation of the cell extracts with the tetrapeptide aldehyde inhibitor of CED-3-like caspases, DEVD-CHO. No cleavage of YVAD-AFC was observed at any time after IL-3 and serum withdrawal, indicating that ICE and its closest relatives are not activated in response to this stimulus in these cells (data not shown).

Exposure of Mo7e cells to 40 μM etoposide or 30-Gy γ-irradiation also resulted in a progressive increase in CPP32-like activity that correlated with the increase in the number of apoptotic cells as measured by trypan blue exclusion and nuclear staining (Fig. 1, *B* and *C*). As previously observed, significant substrate cleavage activity was evident before any morphological appearance of apoptosis. DEVD-AFC cleavage activity of cell extracts was inhibited by preincubation of the extracts with 1 μM DEVD-CHO. Once again, there was no increase in caspase activity on the YVAD-AFC substrate (data not shown).

Induction of CPP32-like Caspase Activity Is Restricted to Cells That Are Sensitive to Apoptotic Stimuli—In addition to the Mo7e cell line, we studied the activation of caspases in two isogenic Burkitt's lymphoma cell lines that are sensitive (BL30A) and resistant (BL30K) to the induction of apoptosis by genotoxic agents (45). As for Mo7e cells, the treatment of BL30A cells with 40 μM etoposide induced a progressive increase in the magnitude of apoptotic cells and CPP32-like activity. In contrast, BL30K cells that remain viable following etoposide treatment did not show any increase in CPP32-like activity, confirming that the activation of CPP32-like caspases occurs during cell death (Fig. 2*A*). In a similar manner, BL30A cells treated with 30-Gy γ-irradiation exhibited a time-dependent increase in DEVD-AFC cleavage activity, whereas BL30K cells were resistant to apoptosis and cytoplasmic extracts prepared from these cells did not display any increase in CPP32-like caspase activity (Fig. 2*B*).

We consistently observed a low level of DEVD-AFC cleavage in untreated Mo7e and BL30A cells. This may be a consequence of the low percentage of apoptotic cells in the normal cell population (approximately 3%).

Cleavage of PARP during Apoptosis in Mo7e and BL30A Cells—To further investigate the proteolytic activity in apoptotic and nonapoptotic extracts from Mo7e, BL30A, and BL30K cells, we examined the cleavage of PARP both *in vitro* and *in vivo*. It has been previously shown that CPP32, Mch3, and Mch2 cleave PARP efficiently (16–19), whereas ICE, TX, and Nedd2 do so poorly (46). Cytoplasmic extracts from Mo7e cells following factor withdrawal or treatment with etoposide or

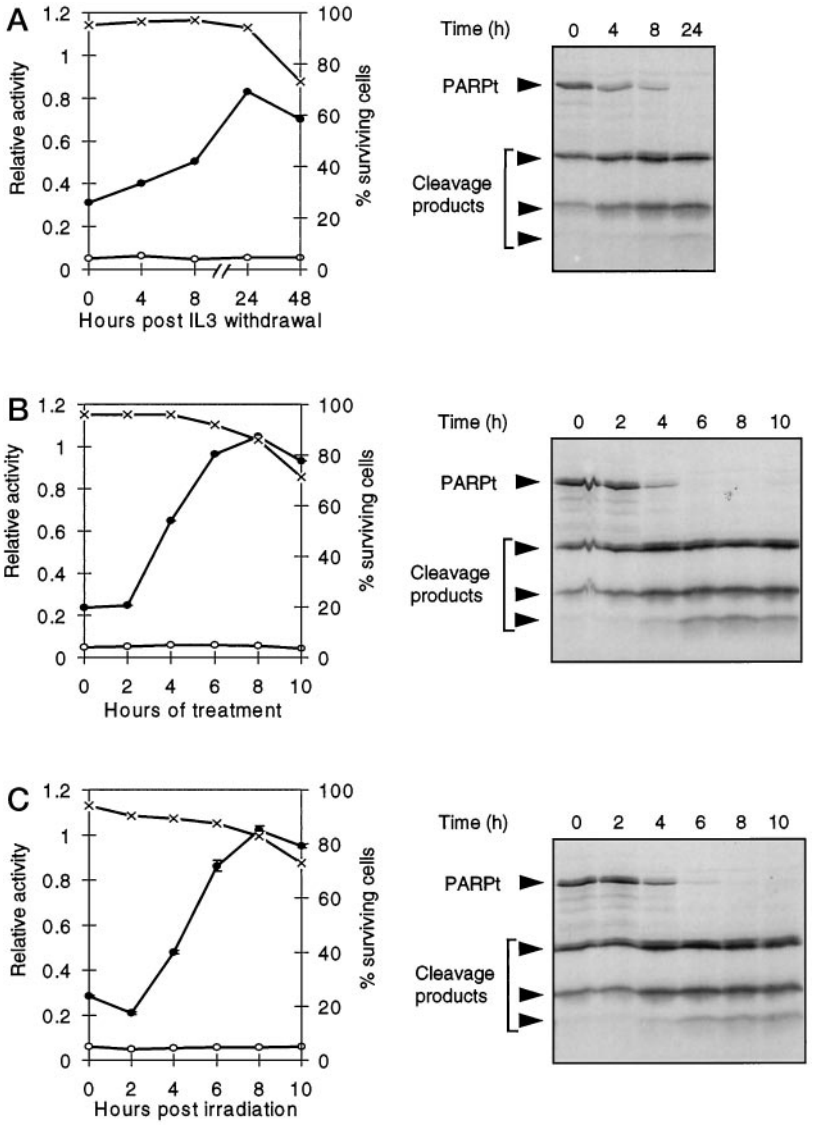

FIG. 1. **Activation of CPP32-like activity in Mo7e cells in response to apoptotic stimuli.** The *left-hand panel* displays composite graphs illustrating cell viability as measured by trypan blue exclusion (×), relative DEVD-AFC cleavage (●) and inhibition of DEVD-AFC cleavage by DEVD-CHO (○) for Mo7e cells following treatment with IL-3 and serum withdrawal (*A*), 40 μM etoposide (*B*), or 30-Gy γ-irradiation (*C*). In DEVD-AFC cleavage assays, 10 μg of cytoplasmic extract from Mo7e cells at indicated times post treament with apoptotic stimuli was incubated with the fluorogenic substrate DEVD-AFC (100 μM) in the presence or the absence of the tetrapeptide inhibitor DEVD-CHO (1 μM) for 30 min at 37 °C. Cleavage of the fluorogenic substrate was quantitated using a Luminescence Spectrophotometer (exctitation, λ 400 nm; emisssion, λ 505 nm). Mean and standard error of triplicate samples are shown. The *right-hand panel* illustrates *in vitro* cleavage of PARP. In these assays, [^{35}S]methionine-labeled truncated PARP (*PARPt*) was incubated with 10 μg cytoplasmic extract for 60 min at 37 °C. Cleavage products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

γ-irradiation cleaved *in vitro* translated [^{35}S]methionine-labeled PARP at the CPP32-like caspase recognition sequence, liberating 24- and 14-kDa polypeptides from the truncated 38-kDa PARP substrate (Fig. 1, *A*, *B*, and *C*). Similarly, cytoplasmic extracts prepared from BL30A cells following treatment with apoptosis inducing stimuli contained PARP cleavage activity, whereas those prepared from BL30K cells did not (Fig. 2, *A* and *B*). As previously seen in fluorogenic substrate cleavage experiments, a marginal base-line level of CPP32-like activity was observed in untreated cells. These experiments confirm that activation of CPP32-like proteases occurs in both Mo7e and BL30A cells early during apoptosis.

Western analysis indicated that in Mo7e cells, PARP was rapidly cleaved *in vivo* to 89- and 24-kDa products in response to IL-3 withdrawal, etoposide, and γ-irradiation, with the 115-kDa precursor completely cleaved within the first 4 h following IL-3 withdrawal and within the first 2 h following etoposide treatment or γ-irradiation (Fig. 3). The complete proteolysis of PARP significantly preceded the appearance of apoptotic cells, suggesting that caspase activation occurs prior to the morphological changes of apoptotic cell death.

Activation of Nedd2/ICH-1 during Apoptosis—Activation of Nedd2 requires the cleavage of the 51-kDa precursor molecule into subunits of 19 and 12 kDa (47, 48). Having established that the cytoplasmic extracts from cells treated with apoptotic stimuli contained CPP32-like caspase activity, we investigated whether proNedd2 is cleaved to generate the p19 and p12 subunits in response to apoptotic stimuli. We examined the activation of Nedd2 by Western blotting with an antiserum against the p12 subunit of human Nedd2 that recognizes both p12 and p51 proNedd2. In Mo7e cells, IL-3/serum withdrawal and etoposide or γ-irradiation treatment resulted in the cleavage of p51 and the appearance of p12 (Fig. 3, *A*, *B*, and *C*). From time course studies, the activation of Nedd2 appears to be rapid, detectable as early as 4 h after IL-3 and serum withdrawal and as early as 2 h following exposure to etoposide and γ-irradiation. This correlates well with the first significant increase in fluorogenic substrate cleavage and PARP cleavage, both *in vitro* and *in vivo* (Figs. 1–3). In BL30A cells, but not in apoptosis-resistant BL30K cells, progressive proteolysis of p51 proNedd2 over time was observed in response to treatment with both etoposide and γ-irradiation (Fig. 4, *A* and *B*).

Although the majority of activity on DEVD-AFC observed in our experiments is probably due to CPP32 and caspases closely related to it, we and others have observed the ability of Nedd2 to cleave the DEVD substrate (49).[2] Therefore, it is possible that some DEVD-AFC cleavage may be attributed to activated

[2] N. L. Harvey and S. Kumar, unpublished observations.

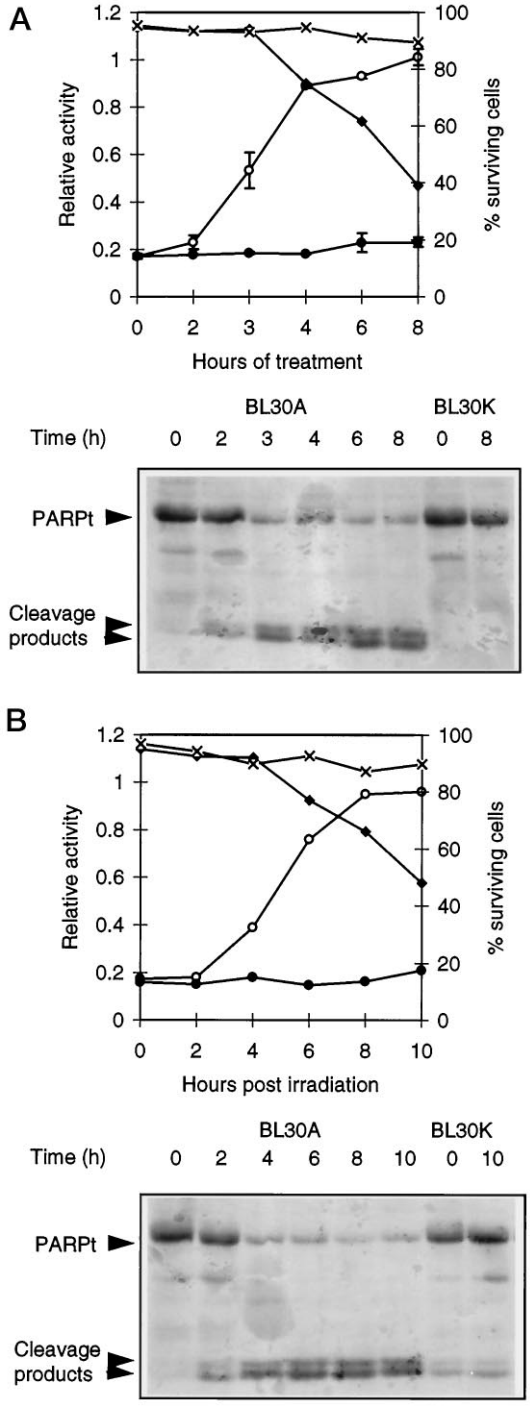

FIG. 2. **Induction of CPP32-like activity in response to apoptotic stimuli in BL30A cells that are sensitive to apoptosis but not in BL30K cells that are resisitant to apoptosis.** Cell viability as measured by trypan blue exclusion and relative DEVD-AFC cleavage activity by cytoplasmic extracts from cells post treatment with 40 μM etoposide (*A*) or 30-Gy γ-irradiation (*B*) are illustrated in the *upper panels*. In the *lower panels*, cleavage of PARP is shown. Experimental details are described in the legend to Fig. 1. ♦ represents BL30A cell viability; ○ represents cleavage of DEVD-AFC by cytoplasmic extracts from BL30A cells; × illustrates BL30K viability; ● shows corresponding DEVD-AFC cleavage by cytoplasmic extracts from BL30K cells. PARPt, truncated PARP.

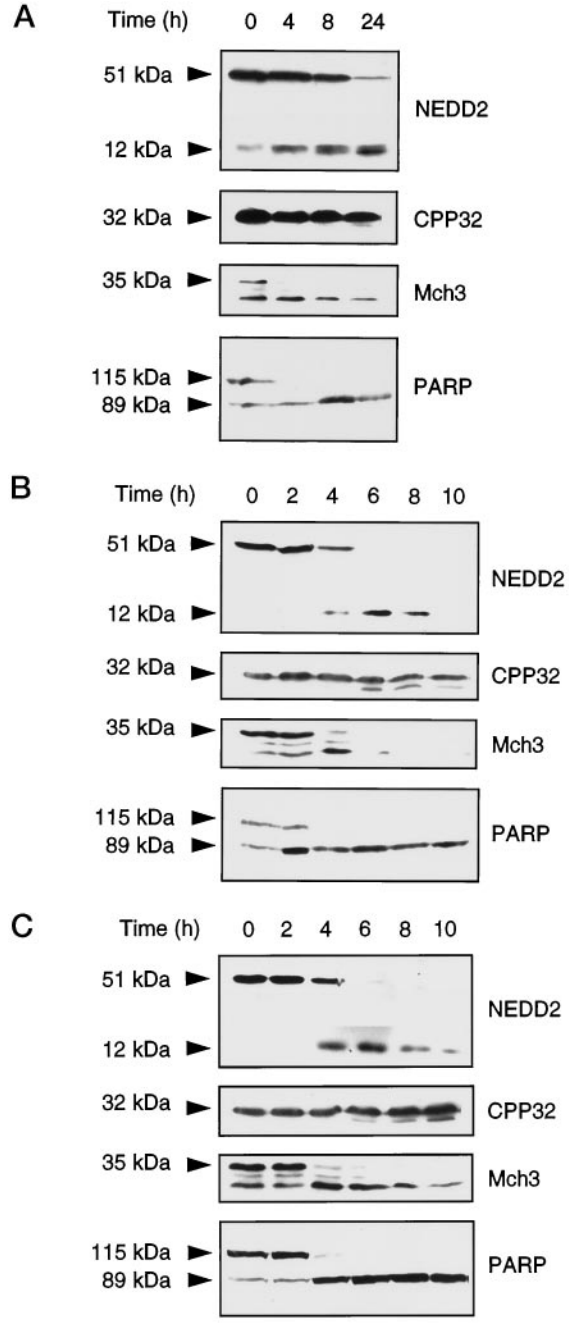

FIG. 3. **Time-dependent activation of caspases and cleavage of the caspase substrate PARP in Mo7e cells following treament with various apoptosis inducing stimuli.** Western blot analyses of the activation of Nedd2, CPP32, and Mch3 and *in vivo* cleavage of PARP in Mo7e cells in response to IL-3 and serum withdrawal (*A*), 40 μM etoposide (*B*), or 30-Gy γ-irradiation (*C*) at various times following exposure to each apoptotic stimulus. Conditions used for each Western blot are described under "Materials and Methods." Caspase activation is evident from the disappearance of the precursor and/or the appearance of p20-like and p10-like subunits that comprise the active enzyme.

Nedd2, the optimal substrate of which is thus far unknown.

Activation of Nedd2 Occurs Prior to That of CPP32 during Apoptosis—To investigate at which stage in the apoptotic pathway Nedd2 activation occurs in relation to that of other caspases, we assessed the activation of CPP32 by Western blotting using an anti-CPP32 monoclonal antibody that detects both proCPP32 and the p17 subunit. We did not observe significant cleavage of CPP32 in Mo7e cells subjected to IL-3/serum withdrawal or treated with either etoposide or γ-irradiation at time points where proNedd2 was completely cleaved (Fig. 3, *A*, *B*, and *C*). In later time points some cleavage of proCPP32 was evident (data not shown). These results indicate that in the apoptotic pathway, Nedd2 activation occurs upstream of CPP32 activation in Mo7e cells.

Activation of CPP32 was clearly observed in BL30A cells

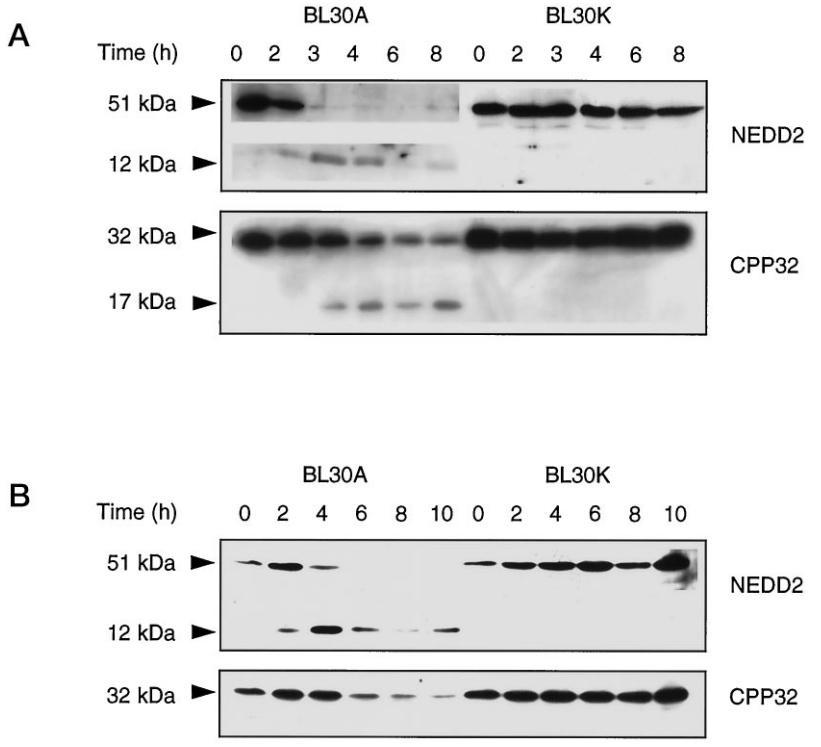

FIG. 4. **Time-dependent activation of proNedd2 and proCPP32 occurs in BL30A cells in response to apoptotic stimuli but not in BL30K cells resistant to apoptosis.** Western blot analyses of proNedd2 and proCPP32 cleavage in response to 40 μM etoposide (*A*) and 30-Gy γ-irradiation (*B*) were performed as described under "Materials and Methods."

following treatment with both etoposide and γ-irradiation (Fig. 4, *A* and *B*). The appearance of the CPP32 p17 subunit was visible approximately 3 h post treatment with etoposide, once again a later stage than the initial activation of Nedd2, visible 2 h post treatment (Fig. 4*A*). An analogous situation was observed in response to γ-irradiation (Fig. 4*B*). In contrast, no cleavage of CPP32 precursor was seen in extracts from the nonresponsive BL30K cells treated with etoposide or γ-irradiation.

Activation of Mch3 in Mo7e Cells Following Apoptotic Stimuli—Because no early activation of CPP32 was observed in Mo7e cells, we investigated the activation of Mch3, the caspase most homologous to CPP32. Western blotting with an anti-Mch3 polyclonal antiserum revealed that proMch3 is rapidly processed, as evident by the disappearance of the precursor, in response to all apoptotic stimuli (Fig. 3, *A–C*). In our experiments, the activation of Mch3 appears to occur concurrently with that of Nedd2 and correlates with the cleavage of PARP we observed *in vitro* and *in vivo*.

DISCUSSION

Previous studies have demonstrated the activation of CPP32, Mch2, and ICE-LAP3 caspases in response to various apoptotic stimuli (20, 27–29). We sought to investigate whether the Nedd2/ICH1 caspase is activated in response to various apoptosis inducing stimuli and at what stage in the protease cascade pathway this occurs. Here we show that Nedd2 is activated early in the passage of events that leads to the execution of cell death, preceding the activation of CPP32. In the megakaryoblastic cell line Mo7e, the activation of Nedd2 occurred much before the activation of CPP32; however, in the Burkitt's lymphoma cell line BL30A, CPP32 activation followed Nedd2 activation much more rapidly.

It is becoming clear that in addition to the division of the caspases into subfamilies by virtue of their relatedness, caspases can be divided into two classes based on the length of their amino-terminal pro-domains and their position in the caspase heirachy. Proteases containing a long pro-domain include CED-3, Nedd2/ICH-1, Mch4, MACH/FLICE/Mch5, ICE, TX, and ICE_{rel}-III, whereas those with very short or absent pro-domains include CPP32, Mch2, Mch3, and Mch6. The pro-domains of Nedd2 and MACH/FLICE/Mch5 have been shown to contain protein motifs that mediate their association with similar motifs present in the amino termini of RAIDD and MORT1/FADD, respectively (23, 24, 43). The carboxyl termini of the RAIDD and MORT1/FADD molecules harbor a "death domain," a motif first identified in TNF-R1 (50) and Fas/Apo1 receptors (51) that serves to mediate homo- and heterotypic protein associations necessary for cell death. A death domain also mediates the association of MORT1/FADD with TRADD, an adaptor protein involved in the TNF-R1 cell death pathway (52), suggesting that these two cell death pathways may be linked. Mch4 also contains a long pro-domain that harbors death effector domains (22). Mch4 and Mch5 have both been shown to activate all known caspases *in vitro*, although they differ with respect to their inhibition by CrmA (53), suggesting that several parallel pathways to cell death may exist that utilize the same effector machinery but are initialized by different upstream caspases in response to different stimuli.

From our data obtained in Mo7e, it appears that CPP32 may not be involved in the apoptotic pathway in these cells. Instead, the function of CPP32 may be replaced by Mch3, which we observed to be rapidly cleaved following apoptotic stimuli. Mch3 may be the caspase responsible for PARP cleavage in these cells. It is not known whether Nedd2 is responsible for Mch3 cleavage in Mo7e cells or whether another caspase member such as Mch4 or Mch5 is activated in response to these stimuli and proceeds to activate Mch3. We have previously shown that Nedd2 is unable to cleave CPP32 *in vitro* (47), which suggests that another protease that is activated early in the caspase cascade such as Mch4 or Mch5 may be responsible for the cleavage of CPP32 we observed in BL30A cells. Alternatively, Nedd2 may activate an unidentified caspase, which in turn activates CPP32. These results lend weight to the classification of caspases in a hierarchy based on their sequence of activation in response to apoptotic stimuli. It appears that caspases containing long pro-domains that mediate their direct

physical coupling to signal transduction machinery constitute the first class of proteases to be activated in response to apoptotic signals and that they in turn activate the second class of caspases, which are the cell death effectors.

Our results show that Nedd2/ICH-1 is activated in response to various apoptotic stimuli and that this activation occurs at an earlier stage in the caspase hierarchy than that of CPP32, suggesting that Nedd2 belongs to the first group of caspases activated in response to apoptotic stimuli. The interaction of Nedd2 with RAIDD suggests that Nedd2 might be activated in response to TNF or Fas-mediated apoptosis, but our results demonstrate that Nedd2 activation also occurs in response to other apoptosis inducing stimuli. Nedd2 may therefore also comprise part of the signaling complex that transduces death signals from stimuli such as factor withdrawal, drug treatment, and γ-irradiation, although the identity of the other components of this complex and their location in the cell is currently not known. In future studies it will be interesting to examine if Nedd2, once activated, can further process other members of the caspase family, thereby initiating the proteolytic cascade characteristic of the execution phase of apoptosis. It will also be interesting to see whether the Nedd2 prodomain can interact with other, as yet unidentified, adaptor molecules.

Acknowledgments—We thank Martin Lavin and Dianne Watters for providing the BL30A and BL30K cells, Gerry Cohen and Marion MacFarlane for kindly donating the Mch3 antiserum and for helpful discussions, Gayathri Parasivam for technical assistance, and Andreas Strasser and David Vaux for useful comments.

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