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ARTICLES

Proteolytic Cleavage and Activation of PAK2 During UV Irradiation-Induced Apoptosis in A431 Cells

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Abstract Exposure of mammalian cells to ultraviolet (UV) light elicits a cellular response and can also lead to apoptotic cell death. In this report, we show that a 36-kDa myelin basic protein (MBP) kinase detected by an in-gel kinase assay can be dramatically activated during the early stages of UV irradiation-triggered apoptosis of A431 cells. Immunoblot analysis revealed that this 36-kDa MBP kinase could be recognized by an antibody against the C-terminal regions of a family of $p21^{Cdc42/Rac}$-activated kinases (PAKs). By using this antibody and a PAK2-specific antibody against the N-terminal region of PAK2 as studying tools, we further demonstrated that UV irradiation caused cleavage of PAK2 to generate a 36-kDa C-terminal catalytic fragment and a 30-kDa N-terminal fragment in A431 cells. The appearance of the 36-kDa C-terminal catalytic fragment of PAK2 matched exactly with the activation of the 36-kDa MBP kinase in A431 cells upon UV irradiation. In addition, UV irradiation also led to activation of CPP32/caspase-3, but not ICH-1L/caspase-2 and ICE/caspase-1, in A431 cells and the kinetics of activation of CPP32/caspase-3 appeared to correlate well with that of DNA fragmentation and of cleavage/activation of PAK2, respectively. Moreover, blockage of activation of CPP32/caspase-3 by pretreating the cells with two specific tetrapeptidic inhibitors for caspases (Ac-DEVD-cho and Ac-YVAD-cmk) could significantly attenuate the extent of cleavage/activation of PAK2 induced by UV irradiation. Collectively, the results demonstrate that cleavage and activation of PAK2 can be induced during the early stages of UV irradiation-triggered apoptosis and indicate the involvement of CPP32/caspase-3 in this process. J. Cell. Biochem. 70:442-454, 1998.

Key words: UV irradiation; PAK2; apoptosis; CPP32/caspase-3; A431 cells

Apoptosis, or programmed cell death is a physiological process of cell deletion that plays a crucial role in the development and homeostasis of multicellular organisms. Disregulation of the process is associated with several human diseases including neurodegenerative disorders and cancer [Ellis et al., 1991; Thompson, 1995]. Apoptotic cell death is morphologically characterized by chromatin condensation, membrane blebbing and cell fragmentation [Kerr et al., 1972], and chromosomal DNA fragmentation is the most prominent biochemical event detected early in apoptotic cells [Wyllie, 1980]. Although the precise molecular mechanisms for apoptotic cell death have not yet been defined, a growing body of evidence has pointed out that a family of cysteine proteases, termed caspases, play important roles in apoptotic execution [for reviews see Martins and Earnshaw, 1997; Nicholson and Thornberry, 1997]. They are related to mammalian interleukin-1 β converting enzyme (ICE) [Cerretti et al., 1992; Thornberry et al., 1992] and to CED-3, a gene product that is required for developmental apoptosis in the nematode *C. Elegans* [Yuan et al., 1993]. Numerous proteins and enzymes in-

Abbreviations: ICE, interleukin-lβ-converting enzyme; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; PAK, $p21^{Cdc42/Rac}$-activated kinase; TNF-α, tumor necrosis factor-α.

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volved in diverse cell functions have been shown to be targeted for proteolytic cleavage by caspases at the onset of apoptosis [for review see Nicholson and Thornberry, 1997]. All caspases are synthesized as proenzymes that are processed to mature enzyme containing two subunits at molecular weight ~20 kDa and 10 kDa. They have a stringent requirement for Asp residue at the cleavage site and small tetrapeptidic inhibitors containg similar sequences to endogenous substrates for caspases have been developed to block caspase activity in vitro and in vivo [Thornberry et al., 1992; Nicholson et al., 1995; Milligan et al., 1995].

A variety of extrinsic and intrinsic signals can trigger apoptosis including environmental stress such as ultraviolet (UV) irradiation [Sachs and Lotern, 1993; Buttke and Sandstrom, 1994; Thompson, 1995]. Exposure of mammalian cells to UV light causes damage to DNA and elicits a gene induction response called mammalian UV response [Herrlich et al., 1992]. The c-Jun N-terminal kinases (JNKs) (also known as stress-activated protein kinases - SAPKs), which are emerging members of the mitogen-activated protein kinase (MAPK) family, can be potently activated by UV irradiation and are key components in the signaling pathway that results in this response [Drijard et al., 1994; Kyriakis et al., 1994]. Although activation of JNK/SAPK has been shown to be required for apoptotic induction in several cell types under certain circumstances [Xia et al., 1995; Verheij et al., 1996], there are reports that activation of JNK/SAPK is associated with stimulation of proliferative and differentiated functions on other cell types [Derijard et al., 1994; Su et al., 1994]. Hence, the role of JNK/SAPK in the apoptotic signaling pathway remains to be further established. In addition to JNK/SAPK, recent studies have shown that several protein kinases can be activated during apoptosis and may also involve in the transduction of apoptotic signals. These kinases include cyclin-dependent kinases [Shi et al., 1994; Lahti et al., 1995], protein kinase C [Emoto et al., 1995], double-stranded RNA-dependent kinase [Lee and Esteban, 1994], and cAMP-dependent kinase [Vintermyr, 1993]. However, it remains undetermined whether activation of these kinases is essential for apoptotic triggering or signals specific apoptotic events.

p21-activated kinases (PAKs) were identified as a family of 62–68 kDa serine/threonine kinases that can bind to and be activated by small (21 kDa) guanosine triphosphatases (GTPases) Cdc42 and Rac that regulate actin polymerization [Manser et al., 1994; Martin et al., 1995; Bagrodia et al., 1995]. Three isoforms of PAK, namely, α-, β-, and γ-PAK (or PAK1, 3, and 2) have been found in various mammalian tissues and all have similar sequences consisting of a N-terminal regulatory region with a p21 binding site and a C-terminal kinase domain [for review see Sells and Chernoff, 1997]. Recent studies have indicated PAKs as downstream mediators of the effects of Cdc42 and Rac in regulating morphological and cytoskeletal changes in several cell types [Brzeska et al., 1997; Manser et al., 1997;Sells et al., 1997].

In the present study, apoptosis of human epidermoid carcinoma A431 cells triggered by UV irradiation in combination with an in-gel renaturable kinase assay is employed to investigate the possible kinase candidate directly involved in the apoptotic signal transduction. We show that a 36 kDa myelin basic protein (MBP) kinase can be activated during UV irradiation-induced apoptosis in A431 cells and activation of this kinase is closely associated with DNA fragmentation in these cells. We demonstrate by immunoblot analysis that this 36 kDa kinase is in fact a C-terminal catalytic fragment derived from proteolytic cleavage of PAK2 in apoptotic cells. In addition, we examine the identity of the protease(s) responsible for the cleavage/activation of PAK2 in apoptotic A431 cells and find that UV irradiation also induces activation of CPP32/caspase-3 in A431 cells. By using specific tetrapeptidic caspase inhibitors as studying tools, we further show that CPP32/caspase-3 is involved in the proteolytic cleavage and activation of PAK2 in apoptotic A431 cells induced by UV irradiation.

MATERIALS AND METHODS

[γ-^{32}P]ATP and was purchased from Amersham (Buckinghamshire, UK). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). Dulbecco's modified Eagle's medium (DMEM), Tween 40, guanidine hydrocholoride, goat antirabbit, and antimouse IgG antibodies conjugated with alkaline phosphatase were from Sigma (St. Louis, MO). Acetyl Asp-Glu-Val-Asp aldehyde (Ac-DEVD-cho) and Acetyl Tyr-Val-Ala-Asp chloromethylketone (Ac-YVAD-cmk) were from Calbiochem (La Jolla, CA). Anti-αPAK (C19) antibody and the 19-

amino acid control peptide (C19) for this antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-CPP32/caspase-3 and anti-ICH-1L/caspase-2 antibodies were from Transduction Laboratories (Lexinton, KY). Anti-ICE/caspase-1 antibody was from Upstate biotechnology (Lake Placid, NY). CDP-Star™ (a chemiluminescent substrate for alkaline phosphatase) was from Boehringer Mannheim (Mannheim, Germany). Molecular weight marker proteins (Mark12) were from Novel Experimental Technology (San Diego, CA). BCA protein assay reagent was from Pierce (Rockford, IL).

Protein Purification

Myelin basic protein (MBP) was purified from porcine brain following the procedure as described in a previous report [Yu and Yang, 1994a].

Production of Anti-PAK2 (N17) Antibody

The anti-PAK2 (N17) antibody was produced in rabbits by using the peptide, MSDNGELEDKPPAPPVR, corresponding to the NH_2-terminal region from amino acids 1–17 of the sequence of human and rabbit PAK2 [Martin et al., 1995; Jakobi et al., 1996] as the antigen. This peptide was synthesized using an automated Applied Biosystems peptide synthesizer 430A and purified on a preparative C-18 reverse-phase high performance liquid chromatography column. A cysteine residue was added to the C-terminus to facilitate coupling the peptide to Keyhole Limpets hemocyanin (KLH) according the procedure described by Reichlin [1980] using glutaraldehyde as the crosslinker. The methods used for production and affinity-purification of the antipeptide antibody were detailed in previous reports [Yu and Yang, 1994b,c]. In this report, the antibody can potently and specifically immunoblot the 62 kDa PAK2 from the A431 cell extracts on blotted membrane after separation by SDS-PAGE.

Culture of A431 Cells and UV Irradiation

The human epidermoid carcinoma A431 cells were cultured as previously described [Yu and Yang, 1994b,c]. One day before experiments, cells (~5-6 X 10^6) were plated on 100 mm culture dishes. UV irradiation (254 μm, UVC) was carried out with a UV gene linker equipped with an energy output control (Stratagene, model 1800). Prior to irradiation, the medium was removed from the culture dishes and the dishes, with the lids off, were exposed to UV light at various doses as indicated in a UV gene linker. The medium removed before UV exposure was added again immediately into the dishes after UV irradiation and the dishes were incubated at 37°C in a CO_2 incubator. After incubation for 2 h, if the time points not specified, the cells were washed twice with ice-cold PBS and lysed in 600 μl of solution A (20 mM Tris-HCl at pH 7.0, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 20 mM sodium pyrophosphate, and 1 mM sodium orthovanadate) on ice for 10 min. The cell lysates were collected and then sonicated on ice by Sonicator (model W-380, Heat Systems-Ultrasonics) for 3 x 10 s at 50% power output followed by centrifugation at 15,000 x g for 20 min at 4°C. The resulting supernatants were used as the cell extracts.

Immunoblots

Immunoblotting was carried out essentially as described in previous reports [Yu and Yang, 1994b,c]. Affinity-purified anti-PAK2 (N17) antibody (1 μg/ml), commercial α-PAK (C19) antibody (0.2 μg/ml), anti-CPP32/caspase-3 monoclonal antibody (0.25 μg/ml), anti-ICH-1L/caspase-2 monoclonal antibody (0.25 μg/ml), or anti-ICE/caspase-1 antibody (0.5 μg/ml) was used to immunoblot proteins transferred from SDS-gel to PVDF membrane. The interested proteins were detected by using goat antirabbit or antimouse IgG antibody conjugated with alkaline phosphatase and CDP-StarTM (a chemiluminescent substrate for alkaline phosphatase) according to the procedure provided by the manufacturer.

For reprobing the same membrane by another kind of antibody, the blotted membrane was first incubated with 200 ml of stripping buffer (62.5 mM Tris-HCl at pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS) at 50°C for 30 min with occasional agitation to strip off the bound antibodies. After washing three times in TTBS buffer (20 mM Tris-HCl at pH 7.4, 0.5 M NaCl, and 0.05% Tween 20), the stripped membrane was re-subjected to immunoblotting with another kind of antibody.

DNA Fragmentation

DNA fragmentation was analyzed according to the method of Zhu and Wang [1997]. Briefly, A431 cells (~1x10^6 cells) detached from culture dishes by trypsin/EDTA were collected, washed once with ice-cold PBS, and centrifuged. After removing the supernatants, cells were dispersed in 30 µl lysis buffer (10 mM Tris-HCl at pH 7.4, 100 mM NaCl, 25 mM EDTA, and 1% sarkosyl) by gentle vortexing, and 4 µl of proteinase K (10 µg/µl) was then added and the cell lysates were incubated at 45°C for 2 h. Two microliters of RNase (10 µg/µl) was added and the cell lysates were incubated for another 1 h at room temperature. The resulting reaction mixtures (20 µl/each sample) were subjected to electrophoresis on 2% agarose gels for DNA fragmentation analysis.

In-Gel Kinase Assay

In-gel kinase assays were performed basically according to the method as described by Kameshita and Fujisawa [1989]. Briefly, 0.5 mg/ml of MBP was co-polymerized in the separating gel of 10% SDS-ploy acrylamide gels, and extracts (40–80 µg) of A431 cells were subjected to electrophoresis in these MBP-containing 10% SDS-gels. The gels were then washed with two changes of buffer B (50 mM Tris-HCl at pH 8.0 and 5 mM 2-mercaptoethanol) containing 20% 2-propanol at room temperature for 1 h followed by washing with buffer B for another 1 h. The washed gels were incubated with two changes of denaturation solution (6 M guanidine.HCl in buffer B) for 1.5 h at room temperature followed by renaturation by incubating with five changes of buffer B containing 0.04% Tween 40 at 4°C for 16–20 h. The gels were then incubated in phosphorylation buffer (50 mM Hepes at pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, 20 mM $Mg(CH_3COO)_2$, and 50 µM [γ-^{32}P]ATP (50–75 µCi)) at room temperature for 1 h and stained with Coomassie Brilliant Blue in 45% methanol/9% acetic acid. After destaining, the gels were dried and subjected to autoradiography with a Fuji RX x-ray film using Kodak X-Omatic cassette with intensifying screen
at −70°C.

Analytic Methods

Protein concentrations were determined by using the BCA protein assay reagent from Pierce (Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [1970] using 10% gels. Molecular weight markers used are as follows: myosin (200,000), β-galatosidase (116,300), phosphorylase b (97,400), bovine serum albumin (66,300), glutamate dehydrogenase (55,400), lactate dehydrogenase (36,500), carbonic anhydrase (31,000), and trypsin inhibitor (21,500).

RESULTS

Activation of a 36-kDa MBP Kinase During UV Irradiation-induced Apoptosis in A431 Cells

UV irradiation can induce apoptosis in various cell types including A431 cells. As shown in Figure 1A,B, DNA fragmentation, the most prominent biochemical event in the early stages of apoptosis, could be induced by UV irradiation at doses above 200 J/m^2 within 2 h in A431 cells and was initially detected at 60 min after the cells exposed to 200 J/m^2 UV light. To investigate the underlined mechanisms involved in the apoptotic signal transduction induced by UV irradiation, cell extracts prepared from A431 cells exposed to UV light as described above were subjected to kinase activity detection in gels containing MBP (in-gel kinase assays). It was found that a renaturable kinase activity at ~36 kDa was dramatically activated by UV irradiation in a dose- and time-dependent manner (Fig. 1C,D). The dose and time course required for activation of the 36-kDa MBP kinase appeared to correlate well with those required for induction of DNA fragmentation in A431 cells upon UV irradiation (Fig. 1), indicating a close association of this kinase activation with UV irradiation-triggered apoptosis in A431 cells. As the apparent molecular weight of this UV irradiation-activated MBP kinase (36 kDa) is distinct from those of JNK/SAPK isozymes (46 kDa for JNK1 and 54 kDa for JNK2), this 36-kDa MBP kinase can not be JNKs.

UV Irradiation-Activated 36-kDa MBP Kinase Can Be Recognized by Antibody Against PAK

Previously, we identified a 36-kDa serine/threonine kinase termed autophosphorylation-dependent protein kinase (auto-kinase) in pig brain and liver, which is a potent MBP kinase when it becomes activated by autophosphorylation [Yang et al., 1987a, b]. Recently, we further demonstrated that the 36-kDa auto-kinase from

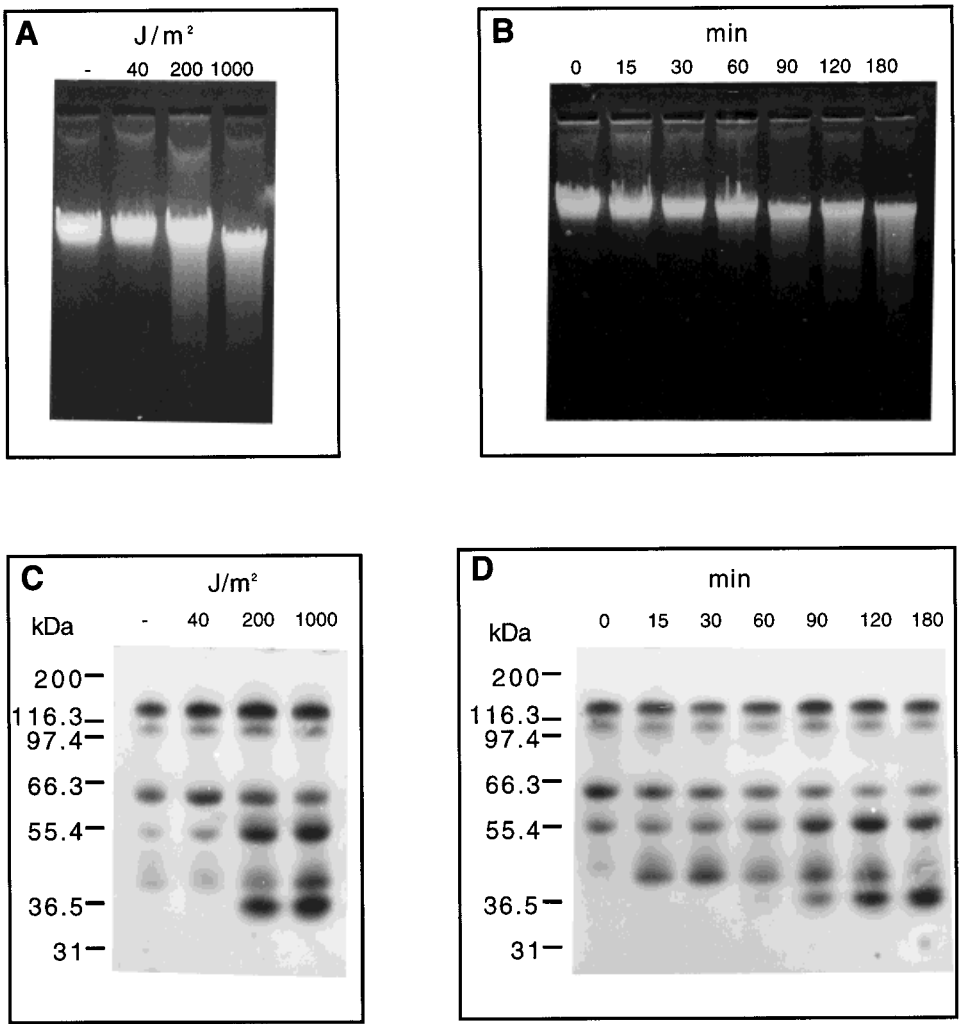

Fig. 1. Activation of a 36-kDa kinase during UV irradiation-induced apoptosis in A431 cells. DNA fragmentation in A431 cells induced by UV irradiation. Genomic DNA of A431 cells (~1 x10^6 cells) was prepared at 2 h later after the cells were exposed to various doses of UV light (**A**) or prepared at various time intervals after the cells exposed to 200 J/m^2 of UV light (**B**) as described under Experimental Procedures. Twenty microliters of prepared genomic DNA from each sample was subjected to 2% agarose gel electrophoresis for DNA fragmentation analysis. In-gel kinase activity assay of extracts of A431 cells exposed to UV light. A431 cells (~1 x10^7 cells) were exposed to various doses of UV light and cell extracts were prepared at 2 h later (**C**) or the cells were exposed to 200 J/m^2 of UV light and cell extracts were prepared at various time intervals later (**D**). Forty micrograms of cell extracts were separated on a 10% SDS-gel containing MBP and the in-gel kinase activities were then assayed as described under Experimental Procedures.

pig liver is in fact a catalytic fragment of PAK2 (or γPAK) [Yu J-S, Chen W-J, Chan W-H, Yang S-D, unpublished communications]. As PAK2 has been recently shown to be cleaved and activated during apoptosis of Jurkat T cells treated with tumor necrosis factor-α (TNF-α) or anti-Fas antibodies [Rudel and Bokoch, 1997], it is possible that the UV irradiation-activated 36-kDa MBP kinase in A431 cells may represent the catalytic fragment of PAK2. To test this possibility, the bands corresponding to the 36-kDa MBP kinase detected in dried gels by autoradiography from extracts of cells irradiated with and without UV light as described in Figure 1 were excised, rehydrated in SDS-containing buffer, and subjected to 10% SDS-PAGE followed by immunoblotting with a commercial αPAK (C19) antibody, which is known to recognize all three PAK isoforms. It was found that the UV irradiation-activated 36-kDa MBP kinase in A431 cells could indeed be recognized by the αPAK (C19) antibody and inclusion of the C-terminal 19-amino acid peptide of αPAK could prevent this recognition (Fig. 2),

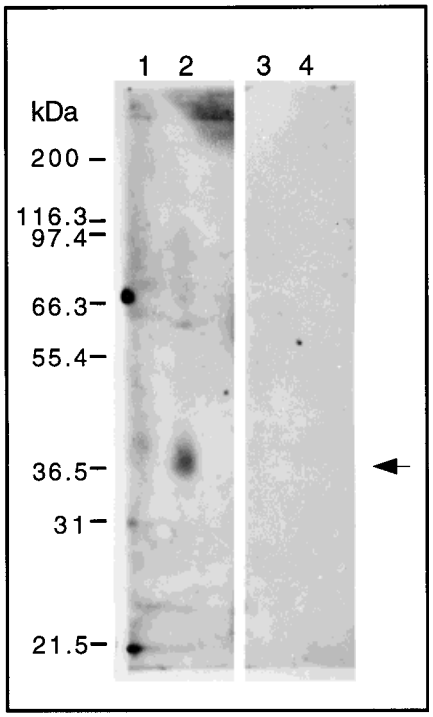

Fig. 2. Immunoblot analysis of the UV irradiation-activated 36-kDa kinase by an antibody against PAK. The bands corresponding to the 36-kDa kinase in control and UV (200J/m^2)-irradiated cells as described in Figure 1A were excised from the dried gel and rehydrated in buffer (125 mM Tris-HCl at pH 6.8 and 1 mM EDTA) containing 0.1% SDS for 1 h. The rehydrated gel slices were inserted into the wells of stacking gel of another 10% SDS-gel and subjected to electrophoresis followed by immunoblotting with αPAK (C19) antibody in the absence **(lanes 1 and 2)** and presence **(lanes 3 and 4)** of 1 μg/ml C-terminal 19-amino acid peptide of αPAK. Lanes 1 and 3, excised samples from control cells; lanes 2 and 4, excised samples from UV-irradiated cells.

indicating that this 36-kDa MBP kinase belongs to a member of PAK family and may be a catalytic fragment of PAK2.

Proteolytic Cleavage of PAK2 to Generate a 36-kDa Catalytic Fragment Induced by UV Irradiation in A431 Cells

To further address this issue, we examined whether PAK2 can be proteolytically cleaved into 36-kDa fragment in A431 cells when irradiated with UV light. Immunoblotting analysis of extracts of A431 cells without any treatment using the αPAK (C19) antibody revealed two blotted protein bands at ~68 kDa (αPAK or PAK1) and ~62 kDa (γPAK or PAK2) with approximately equal amount (Fig. 3A, lane 1). In addition to the two bands, however, a ~36 kDa blotted band was found to appear in the extracts of cells irradiated with UV light in both dose- and time-dependent manners (Fig. 3A,B). When the same cell extracts were further probed with PAK2 (N17) antibody produced here, it was found that only one predicted 62-kDa band corresponding to PAK2 could be detected in the control cell extracts (Fig. 3C, lane 1), demonstrating the immuno-specificity of this antibody. Furthermore, the intensity of the 62-kDa PAK2 band decreased significantly in extracts of cells exposed to UV light and this decrease of 62-kDa band correlated well with the appearance of another faintly blotted band at ~30-kDa (Fig. 3C,D). This ~30-kDa band was detected more clearly after prolonged chemiluminescent detection of the same blotted membranes (Fig. 3E,F). Because this antibody is produced against the N-terminal region of PAK2, the ~30-kDa blotted band detected in the extracts of UV-irradiated cells appears to be the N-terminal fragment of PAK2. More importantly, it was noted that the appearance of the 36-kDa blotted band paralleled exactly with the activation of the 36-kDa MBP kinase detected by in-gel kinase assays in UV-irradiated cells (Figs. 1 and 3). The results taken together demonstrate that UV irradiation can induce cleavage of PAK2 to generate a 36-kDa catalytic fragment as well as a N-terminal ~30-kDa fragment in A431 cells and the 36-kDa catalytic fragment can be detected by the MBP in-gel kinase assay.

Correlation of UV Irradiation-Induced Activation of CPP32/caspase-3 With Cleavage/activation of PAK2 in A431 Cells

As activation of ICE/CED-3 family cysteine proteases termed caspases is known to occur during apoptosis of various cell types [Martins and Earnshaw, 1997; Nicholson and Thornberry, 1997], we therefore evaluated the relationship between this event and the cleavage/activation of PAK2 in A431 cells upon UV irradiation. By using the anti-CPP32/caspase-3 antibody as studying tool, we found that UV irradiation also induced cleavage and activation of CPP32/caspase-3 in A431 cells as the p17 subunit of the protease, which corresponds to one of the two subunits forming the active protease after processing of the 32 kDa proenzyme [Fernandes-Alnemri et al., 1994; Nicholson et al., 1995] could be clearly detected in extracts of cells exposed to UV light (Fig. 4A,B). However, UV irradiation had no effect on another ICE/CED-3 family cysteine protease, ICH-

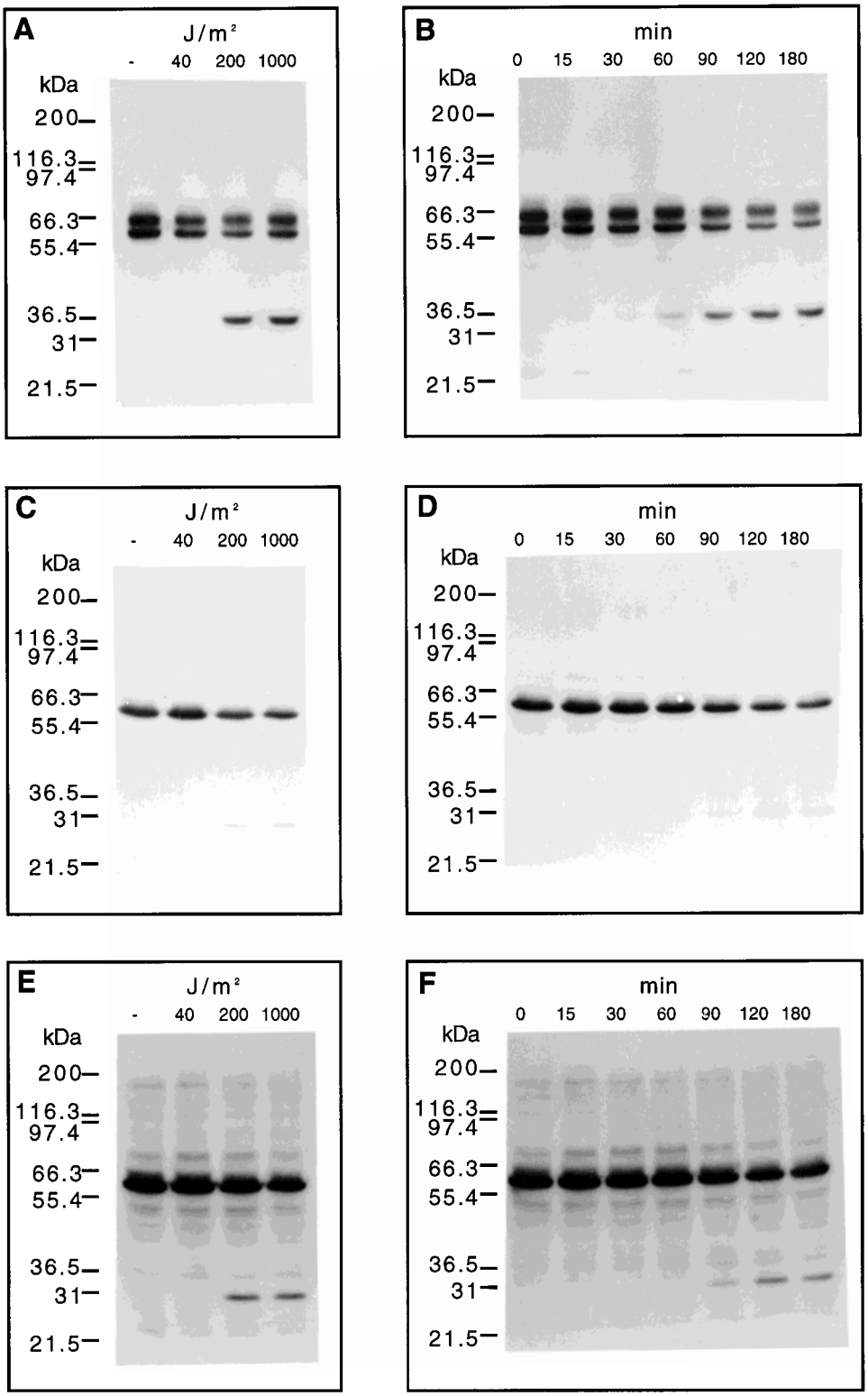

Fig. 3. Cleavage of PAK2 in A431 cells exposed to UV light. Forty (**A** and **B**) or 80 (**C** and **D**) microgram extracts of A431 cells prepared under the conditions as described in Figure 1 were separated on 10% SDS-gels followed by immunoblotting with αPAK (C19) antibody (A and B) or with PAK2 (N17) antibody (C and D) as described under Experimental Procedures. **E** and **F** represent blotting patterns obtained from prolonged exposure of C and D, respectively, during chemiluminescent detection of blotted proteins.

1L/caspase-2 [Wang et al., 1994] under the same condition as evidenced by immunoblotting analysis with antibody against this protease (Fig. 4C,D). Immunoblotting analysis also revealed that A431 cells contain little amount of ICE/caspase-1 as compared to the two caspases mentioned above and UV irradiation had no effect on this caspase (result not shown). The results indicate that selective activation of distinct subtype of caspase can occur in A431 cells upon UV irradiation. Furthermore, it is interesting to note that the kinetics of UV irradiation-induced activation of CPP32/caspase-3 are quite similar with that of UV irradiation-induced cleavage/activation of PAK2 (see Figs. 3 and 4 for comparison). This observation together with a recent report that PAK2 could be cleaved by CPP32/caspase-3 in vitro [Rudel and Bokoch, 1997] suggest that CPP32/caspase-3 may represent the protease responsible for the UV irradiation-induced cleavage of PAK2 in A431 cells.

Inhibitors of Caspases Attenuate UV Irradiation-Induced DNA Fragmentation, Activation of CPP32/caspase-3, and Cleavage/Activation of PAK2 in A431 Cells

A series of tetrapeptidic inhibitors for distinct types of caspase have been developed and

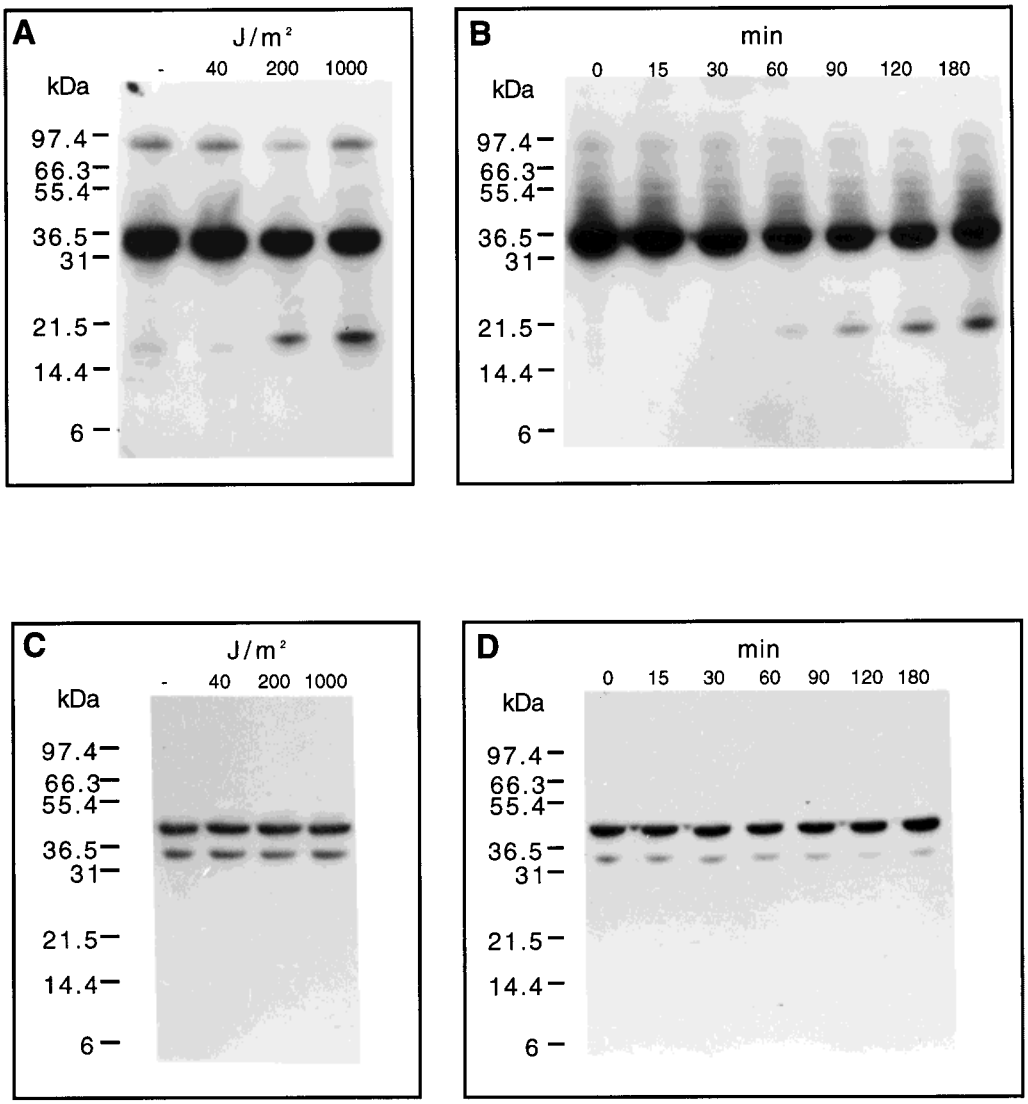

Fig. 4. Effects of UV irradiation on the activation of caspases in A431 cells. Forty microgram extracts of A431 cells prepared under the conditions as described in Figure 1 were separated on 15% SDS-gels followed by immunoblotting with anti-CPP32/caspase-3 antibody (**A**), (**B**) or with anti-ICH-1L/caspase-2 antibody (**C**), (**D**) as described under Experimental Procedures.

used as tools to study the physiological functions of this protease family [Thornberry et al., 1992; Nicholson et al., 1995]. To evaluate the possible role of CPP32/caspase-3 in the UV irradiation-induced apoptosis and cleavage/activation of PAK2 in A431 cells, cells preincubated with or without caspase inhibitor (Ac-DEVD-cho or Ac-YVAD-cmk) were exposed to UV irradiation and genomic DNA and cell extracts were then prepared for analysis of DNA fragmentation and cleavage and activation of both CPP32/caspase-3 and PAK2, respectively. As shown in Figure 5A, both inhibitors prevented the UV irradiation-induced DNA fragmentation in A431 cells. Furthermore, it was found that the UV irradiation-induced formation of the active p17 subunit of CPP32/caspase-3 in A431 cells was also blocked by both inhibitors, although it appeared that the two inhibitors prevented CPP32/caspase-3 activation via different mechanisms as a ~21-22 kDa intermediate form of processed CPP32/caspase-3, which occurred in the UV-irradiated cells pretreated with Ac-YVAD-cmk, was not detected in the Ac-DEVD-cho-pretreated, UV-irradiated cells (Fig. 5B). When examining the effects of the two inhibitors on the cleavage and activation of PAK2 induced by UV irradiation, it was found that both inhibitors could significantly diminish the cleavage (Fig. 5C) and activation (Fig. 5D) of PAK2, indicating that CPP32/caspase-3 is involved in and can be the protease responsible for the UV irradiation-induced cleavage of PAK2 in A431 cells.

DISCUSSION

By an in-gel MBP kinase assay, we have shown here that a 36-kDa kinase is dramatically activated during UV irradiation-induced apoptosis in A431 cells. The result is consistent with a previous report [Lu et al., 1996] that a 36-kDa MBP kinase is activated by UV irradiation as well as other apoptosis-inducing agents including okadaic acid and retinotic acid in human promyelocytic leukemia HL-60 cells. To explore the molecular identity of this 36-kDa kinase, we demonstrate by immunoblot analysis that it is in fact a C-terminal catalytic fragment derived from proteolytic cleavage of PAK2 in apoptotic A431 cells (Figs. 1–3). These results together with the previous observation by Rudel and Bokoch [1997] that TNF-α and anti-Fas antibodies induce cleavage and activation of PAK2 in Jurkat T cells seem to indicate that cleavage and activation of PAK2 is a common signaling mechanism occurring in apoptosis of a variety of cell types induced by diverse apoptotic inducers. Indeed, this notion is further supported by our recent observations that osmotic stress and heat shock can also induce cleavage and activation of PAK2 in Balb/c 3T3 fibroblasts and human hepatoma 3B cells [Chan W-H, Yu J-S, Yang S-D, unpublished communications].

It appears that PAK2 is cleaved in apoptotic A431 cells by UV irradiation-activated protease(s) at a specific site as only one set of N- and C-terminal fragments of this kinase can be detected by immunoblotting (Fig. 3). Based on the known primary structure of PAK2 [Martin et al., 1995; Jakobi et al., 1996] and the relative molecular weights of the two cleavage products (~30 and 36 kDa for N- and C-terminal fragments, respectively), this cleavage site should locate at near central region connecting the N-terminal p21 binding domain and the C-terminal catalytic domain of this kinase. PAK2 has also been independently identified as a protease-activated kinase [Jakobi et al., 1996] and a S6/H4 kinase [Benner et al., 1995]. Removal of the N-terminal regulatory region of PAK2 by mild proteolysis in vitro renders this kinase able to autophosphorylate and activate itself in the absence of Cdc42 and Rac [Benner et al., 1995; Jakobi et al., 1996]. In apoptotic A431 cells triggered by UV irradiation, removal of the N-terminal regulatory region of PAK2 by protease action seems to cause the resulting 36-kDa C-terminal catalytic fragment to gain the capability to autophosphorylate and activate itself (Figs. 1 and 5). Furthermore, this 36-kDa catalytic fragment of PAK2, which is generated at the onset of apoptosis, can exist at least 2 h in apoptotic A431 cells (Fig. 3). In this situation, it is reasonable to speculate that this 36-kDa catalytic fragment of PAK2 may have a good chance to express its kinase activity in apoptotic cells and thereby act on, if any, downstream substrate protein(s) involved in the apoptotic signaling pathway. Although many proteins and enzymes can serve as substrates for PAK in vitro [Yang et al., 1987a, b; Benner et al., 1995; Yu and Yang, 1995; Jakobi et al., 1996; Brzeska et al., 1997], the physiological substrates for the 36-kDa catalytic fragment of PAK2 in apoptotic cells have not yet been iden-

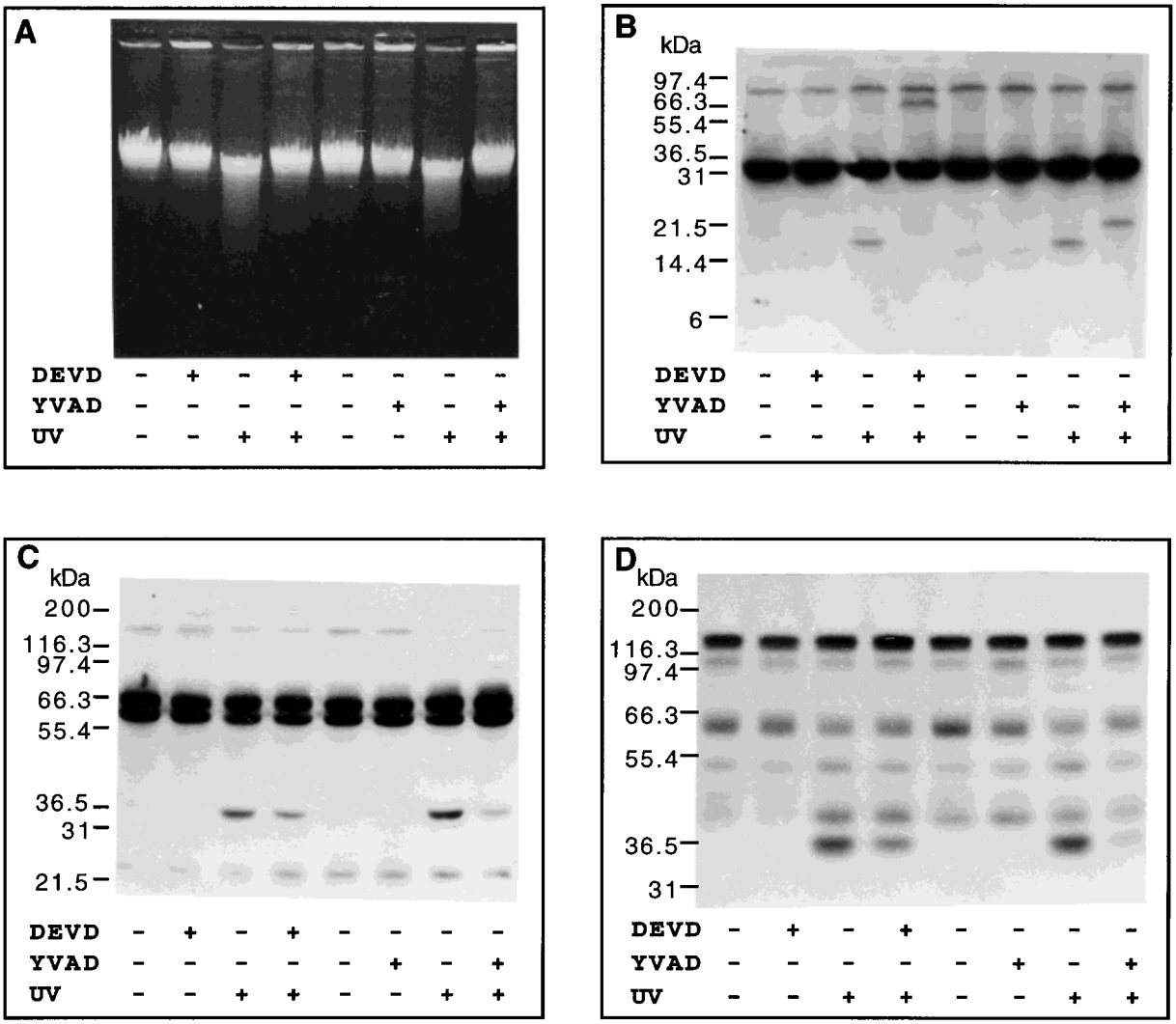

Fig. 5. Effects of caspase inhibitors on the UV irradiation-induced DNA fragmentation, activation of CPP32/caspase-3, and cleavage/activation of PAK2 in A431 cells. A431 cells were incubated with or without 300 μM Ac-DEVD-cho or Ac-YVAD-cmk at 37°C for 1 h and then exposed to UV light (200 J/m^2) and genomic DNA and cell extracts were prepared at 2 h later, respectively. The genomic DNA (20 μl/sample) was subjected to 2% agarose gel electrophoresis for DNA fragmentation analysis (**A**). Forty micrograms of the cell extracts were separated on a 15% SDS-gel followed by immunoblotting with anti-CPP32/caspase-3 antibody (**B**), or separated on a 10% SDS-gel followed by immunoblotting with αPAK (C19) antibody (**C**), or separated on a 10% SDS-gel containing MBP followed by in-gel kinase assay (**D**) as described under Experimental Procedures.

tified and the exact functional role of this 36-kDa catalytic fragment in apoptotic cell death remains to be further established.

By using recombinant PAK2 as substrate, Rudel and Bokoch [1997] reported that recombinant CPP32/caspase-3 could cleave PAK2 at Asp^{212} in vitro. They also showed that tetrapeptidic caspase inhibitors could block the TNF-α- and anti-Fas antibodies-induced cleavage and activation of PAK2 in Jurkat T cells. These observations implicate CPP32/caspase-3 as the candidate protease responsible for the TNF-α- and anti-Fas antibodies-induced cleavage and activation of PAK2 in Jurkat T cells. In the present study, several lines of evidence indicate that CPP32/caspase-3 can also involve in the UV irradiation-induced cleavage and activation of PAK2 in A431 cells. First, among the three caspases that we have examined in A431 cells, only CPP32/caspase-3 is activated by UV irradiation (Fig. 4 and results not shown). Second, the kinetics of activation of CPP32/caspase-3 is almost identical to that of the cleavage and activation of PAK2 (Figs. 3 and 4).

Third, the extent of cleavage and activation of PAK2 can be substantially inhibited by pretreating the cells with Ac-DEVD-cho and Ac-YVAD-cmk, two inhibitors for CPP32/caspase-3 (Fig. 5). Thus activation of caspase, most likely CPP32/caspase-3, apprears to be a converging point at which distinct signaling pathways elicited by different apoptotic inducers such as proteinous stimuli (e.g., anti-Fas antibodies and TNF-α) and environmental stress (e.g., UV irradiation) are integrated to trigger the cleavage/activation of PAK2 in cells. At present, how a physical perturbation provided by UV irradiation can result in activation of CPP32/caspase-3 in cells is unclear and obviously represents an intriguing issue deserving further investigation.

On the other hand, albeit CPP32/caspase-3 can be the candidate protease responsible for the cleavage of PAK2 in UV-irradiated A431 cells, it might not be the only one. It is interesting to note that even when the activation of CPP32/caspase-3 was completely blocked by the two caspase inhibitors in the UV-irradiated cells as evidenced by a total disappearance of the active p17 subunit of this caspase on immnoblot (Fig. 5B), the cleavage and activation of PAK2 remained detectable significantly (Fig. 5C,D). This finding together with the recent observations that multiple caspases can be selectively activated by a single apoptotic stimulus in different cell types [Eischen et al., 1997; Martins et al., 1997] and distinct subtypes of caspase can cleave the same substrate protein to generate identical proteolytic fragments [Orth et al., 1996; Wen et al., 1997] raise the possibility that in addition to CPP32/caspase-3, other members of the caspase family proteases might also be activated by UV irradiation and directly involve in the cleavage and activation of PAK2 in A431 cells. Further studies will be needed to clearify this point. In summary, our results demonstrate that cleavage and activation of PAK2 can be induced during the early stages of UV irradiation-triggered apoptosis and indicate the involvement of CPP32/caspase-3 in this process.

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