Apoptosis Induced by the Nuclear Death Domain Protein p84N5 Is Associated with Caspase-6 and NF-κB Activation*

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Although the mechanisms involved in responses to extracellular or mitochondrial apoptotic signals have received considerable attention, the mechanisms utilized within the nucleus to transduce apoptotic signals are not well understood. We have characterized apoptosis induced by the nuclear death domain-containing protein p84N5. Adenovirus-mediated N5 gene transfer or transfection of p84N5 expression vectors induces apoptosis in tumor cell lines with nearly 100% efficiency as indicated by cellular morphology, DNA fragmentation, and annexin V staining. Using peptide substrates and Western blotting, we have determined that N5-induced apoptosis is initially accompanied by activation of caspase-6. Activation of caspases-3 and -9 does not peak until 3 days after the peak of caspase-6 activity. Expression of p84N5 also leads to activation of NF-κB as indicated by nuclear translocation of p65RelA and transcriptional activation of a NF-κB-dependent reporter promoter. Changes in the relative expression level of Bcl-2 family proteins, including Bak and Bcl-Xs, are also observed during p84N5-induced apoptosis. Finally, we demonstrate that p84N5-induced apoptosis does not require p53 and is not inhibited by p53 coexpression. We propose that p84N5 is involved in an apoptotic pathway distinct from those triggered by death domain-containing receptors or by p53.

Programmed cell death (PCD) is essential for normal development, tissue homeostasis, and host defense mechanisms. PCD is recognized by a collection of distinctive morphological and biochemical characteristics termed apoptosis (1). An intrinsic genetic program that is conserved through evolution controls PCD in mammals (2). For example, the ced-3 gene that is required for PCD in Caenorhabditis elegans encodes a protein that is a functional homologue of the family of mammalian cysteine proteases termed caspases (3). Activation of these caspases by proteolytic processing is required for the execution of apoptosis. Members of the mammalian Bcl-2 family of apoptotic regulatory proteins also have a homologue in C. elegans, the ced-9 gene (4). Some members of this family, like Bcl-2 and Bcl-XL, inhibit apoptosis, whereas others like Bax, Bak, or Bcl-Xs promote apoptosis (5).

The pathways leading to apoptosis in response to extracellular stimuli, like tumor necrosis factor, or in response to mitochondrial apoptotic signals, such as cytochrome c release, are well characterized (6, 7). Initiator caspases are typically recruited to protein complexes composed of death receptors and/or adapter molecules. These proteins contain signature protein interaction motifs like the death domain, the death effector domain, or the CARD domain. The locally high concentration of recruited caspase proenzyme presumably leads to proteolytic processing to more active forms, thereby triggering a caspase proteolytic cascade. Apoptotic signals can also originate from within the nucleus. For example, DNA damage caused by radiation triggers a stress response that can result in apoptotic cell death (8). The mechanisms utilized by nuclear apoptotic signals to initiate caspase proteolytic cascades are not well understood.

A number of nuclear transcription factors can induce apoptosis, including p53. Although p53 has a well-documented role in the cell’s response to DNA damage, the mechanism used by p53 to initiate apoptosis is controversial. Although it is presumed to trigger apoptosis from within the nucleus by altering the expression of genes more directly involved in the execution of apoptosis, non-nuclear mechanisms unrelated to transcriptional regulation have also been proposed (12). Few proteins other than transcription factors have been documented to initiate apoptosis from within the nucleus. Nuclear localization of expanded polyglutamine repeat proteins is required for their ability to induce apoptosis that causes progressive neurodegenerative diseases like Huntington’s disease or spinocerebellar ataxia (9, 10). Activation of caspase-8 is a required step in this process (11). Activation of caspase-8 apparently occurs by a novel mechanism involving recruitment of the proenzyme to characteristic protein aggregates that are associated with these diseases.

Recently, we demonstrated that the nuclear protein encoded by the N5 gene (p84N5) was capable of initiating apoptosis (13). The N5 gene was originally isolated based on the ability of p84N5 to bind an amino-terminal domain of the retinoblastoma tumor suppressor protein (pRb) (14). Association of p84N5 with pRb inhibits p84N5-induced apoptosis, identifying p84N5 as a potential mediator of pRb’s inhibitory effects on apoptosis (15). Because p84N5 is unique among nuclear proteins in containing a death domain that is required for its ability to induce apoptosis (13), it may participate directly in a nuclear apoptotic pathway. In the current study, we characterize this pathway by analyzing caspase activation, Bcl-2 family member expression, the effects of p53, and NF-κB activation during p84N5-induced apoptosis.

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MATERIALS AND METHODS

Cell Culture—SAOS-2, 293, MCF-7, and C-33A cell lines were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin), in a 5% CO2 incubator at 37 °C. Growth curves with these cell lines were assayed by plating 500,000 cells per well of a 60-mm tissue culture dish, treating with indicated virus at a multiplicity of infection (m.o.i.) of 10, harvesting cells by trypsinization at the indicated time after infection, and counting the number of cells that exclude trypan blue with a hemacytometer.

The Colo357 X cell line was obtained from Dr. Keping Xie (M.D. Anderson Cancer Center) and was grown as above. Growth assays of virally infected Colo357 X cells were performed by plating at a density of 20,000 cells/well in 24-well plates in triplicate. One day later, the cells were infected with the indicated adenoviruses at a total m.o.i. for all viruses of 50. An equal number of cells were treated with PBS as a control. Cells were harvested at different time intervals and viable cells, as indicated by trypan blue exclusion, were counted using a hemacytometer.

Plasmids and Adenovirus—The full-length p84N5 cDNA was subcloned into pCEP4 (Invitrogen, Carlsbad, CA) expression vector to create the expression vector pCMVN5. The Bcl-2 (16), p53 (17), and pRb (18) expression vectors were previously described. The NF-kB luciferase reporter plasmids (19) and RelA expression plasmid (20) were described previously. To generate the recombinant p84N5 expressing adenovirus, the p84N5 cDNA was subcloned into the pAdCMV (AS)-BGHpA vector (Dr. T. J. Liu, M.D. Anderson Cancer Center). The resulting plasmid, was co-transfected with pCM17, the adenovirus backbone plasmid, into 293 cells and recombinant N5 adenovirus (AdN5) identified by polymerase chain reaction using primers specific for N5 essentially as described (21). Recombinant adenovirus was purified by CsCl equilibrium density gradient centrifugation, and viral particle numbers were estimated by phosphate precipitation (23) using 6–30 μg of total DNA. To analyze transfectants or infected cells for DNA fragmentation, cells were collected in a buffer containing 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin on ice for 10 min. The total protein concentration of the soluble extract was determined by Bradford assay according to the manufacturer’s recommendations (Amersham Pharmacia Biotech).

For Western analysis, transfected cells were extracted in a buffer containing 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin on ice for 10 min. The total protein concentration of the soluble extract was determined by Bradford assay according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). 70 μg of total protein for each sample was resolved by 10% SDS-polyacrylamide gel electrophoresis, blotted, and stained as described previously (13). Antibodies directed against p84N5 (14) and pRb (24) were described previously. All other primary antibodies were used as directed by the manufacturer. Antibodies directed against caspases were obtained from Oncogene Research Products (Cambridge, MA); all other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies were detected using a peroxidase-conjugated secondary antibody and enhanced chemiluminescence according to the manufacturer’s recommendations (Amersham Pharmacia Biotech).

RESULTS

Expression of p84N5 Induces Apoptosis through Activation of Caspase-6—The full-length, wild type N5 cDNA was used to generate a recombinant, E1-deleted, replication-defective adenovirus (AdN5) that expressed wild-type p84N5 under control of the cytomegalovirus early promoter and the bovine growth hormone polyadenylation signal. This adenovirus was used to drive expression of p84N5 in infected cells. The proliferation of cells infected with AdN5 at a multiplicity of infection (m.o.i.) of 10 slowed significantly by the third day after infection (Fig. 1A). Nearly all AdN5-infected cells died by 6 days after infection. The proliferation of cells infected with a similarly constructed GFP-expressing recombinant adenovirus (AdGFP) was similar to uninfected cells. Both AdGFP-infected and untreated cells reached confluency by day 4, explaining the decrease in proliferation rate observed at the later stages of the

FIG. 1. AdN5-mediated p84N5 expression induces apoptosis. A, equal numbers of SAOS-2 cells were infected with the indicated virus at a m.o.i. of 10 or left untreated. On subsequent days, the number of viable cells in each aliquot was counted. The data are from a single representative experiment that has been repeated five times. B, SAOS-2 cells were infected at an m.o.i. of 10 with the indicated virus and 72 h later harvested and assayed for fragmented DNA by TUNEL. The percentage of TUNEL-positive cells was determined by flow cytometry. The data presented are the mean of three infections for each virus. C, C-33A cells were infected with the indicated virus at a m.o.i. of 10 and harvested 2 days later for annexin V staining. The percentage of annexin V-positive cells was determined by flow cytometry. The data are the mean of three infections. D, Hoechst 33342 was added to the culture media 3 days following infection of SAOS-2 cells with the indicated virus (10 m.o.i.). The cells were photographed at 100× under a fluorescent microscope with a UV filter. The results are representative of multiple experiments. E, extracts prepared from SAOS-2 infected with the indicated virus were analyzed by Western blotting. Blots were stained with monoclonal antibody directed against p84N5. Staining with anti-β-actin served as a protein loading control. The position of molecular weight markers is shown at left for the p84N5 blot.
Characterization of p84N5-induced Apoptosis

To confirm that caspase-6 was involved in the increase in VEID cleavage activity upon AdN5 infection, extracts were analyzed for proteolytic processing of caspases-6 and -9 by Western blotting. Proteolytically processed caspase-6 was detected in extracts prepared 2 or 3 days after infection with AdN5, but not in extracts prepared from AdGFP-infected cells (Fig. 2B). Processing of caspase-9 was also detected in extracts prepared 4 days after infection. These observations indicated that the activation of VEID cleavage activity detected was likely due, at least in part, to activation of caspase-6.

Activation of NF-κB during p84N5-induced Apoptosis—Activation of NF-κB serves as a survival signal in response to varied apoptotic stimuli, including genotoxic agents like ionizing radiation (26). Activation of NF-κB involves translocation of the protein from the cytoplasm to the nucleus where it functions as a transcription factor to regulate the expression of target genes. To examine possible activation of NF-κB by p84N5, nuclear or cytoplasmic extracts prepared from 293 cells transfected with pCMVN5 or empty vector have been analyzed by Western blotting using an anti-p65RelA monoclonal antibody. In comparison to cells transfected with empty vector, the level of p65RelA in the cytoplasm of pCMVN5-transfected cells decreases while the relative level of p65RelA in the nucleus increases (Fig. 3A). To test whether the nuclear translocation of p65RelA coincides with increased transcriptional activity, a luciferase reporter gene driven by an artificial NF-κB-responsive promoter was cotransfected with pCMVN5 or empty vector, and the levels of luciferase activity in the extracts of transfected cells were assayed. Expression of p84N5 potently increases luciferase activity generated from the NF-κB-responsive promoter construct but not from a reporter construct containing a mutant promoter that is insensitive to NF-κB (Fig. 3B). The level of reporter gene induction by p84N5 expression was similar to that achieved by expression of exogenous p65RelA.

Changes in Bel-2 Family Member Gene Expression during p84N5-induced Apoptosis—Bel-2 and its homologues are critical regulators of cell death. Changes in the expression of either pro-apoptotic or anti-apoptotic family members can facilitate apoptosis. We have examined the expression of Bak, Bax, Bad, Bel-2, and Bel-3 during p84N5-induced apoptosis by Western blot analysis. Transfection of pCMVN5 into 293 cells or SAOS-2 cells causes detectable apoptosis within 48 h (13). An increase in relative protein expression of Bak and Bel-2 was detected in similarly transfected 293 cells relative to cells transfected with empty vector (Fig. 4A). No change in the relative level of expression of Bad, Bax, or Bel-2 was detected in N5-transfected cells. Nor were there changes in MAD protein expression.

Bak and Bel-2 are apoptotic agonists that heterodimerize with Bel-2 and inhibit its ability to protect cells from cell death (27–29). The increase in expression of these genes may facilitate p84N5-induced apoptosis. If true, a compensating increase in Bel-2 expression would be predicted to inhibit p84N5-induced apoptosis. To test this possibility, we coexpressed Bel-2 with p84N5 and measured the effect on apoptosis using the TUNEL assay. More than 90% of transfected cells express-

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2 J. Doostzadeh-Cizeron, unpublished observation.
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N5-induced Apoptosis Does Not Require and Is Not Inhibited by p53—We have demonstrated that expression of p84N5 in p53 null SAOS-2 cells (30) induces apoptosis (Fig. 1A), indicating that p53 was not required for p84N5-induced apoptosis. We wished to explore the possibility that p53 may inhibit p84N5-induced apoptosis. To this aim, p84N5 and wild-type p53 were expressed in SAOS-2 cells and the cells assayed for apoptosis by TUNEL. Coexpression of wild-type p53 did not affect the percentage of cells containing fragmented DNA (Fig. 5A). Consistent with our previous results (13), expression of a constitutively active pRb mutant (RbΔCDK) decreased the percentage of cells containing fragmented DNA. Expression of p53 and RbΔCDK upon transfection of the appropriate expression vectors was confirmed by Western blotting.2 We also compared the effects of pCMVN5 transfection in wild-type p53 expressing MCF-7 cells or SAOS-2 cells. MCF-7 and SAOS-2 cells were equally sensitive to p84N5-induced apoptosis as indicated by the similar percentage of transfected cells containing fragmented DNA (Fig. 5B). MCF-7 and SAOS-2 cells were also equally sensitive to apoptosis induced by AdN5 (32). Finally, we checked the effect of p84N5 expression on the relative level of endogenous p53, p110Rb, p16, or p27 expression by Western blotting. The expression level of each of these proteins was similar in CMVN5-transfected cells and cells transfected with empty vector (Fig. 5C).

These observations suggested that N5 and p53 function in different apoptotic pathways. If true, the effects of each gene on the inhibition of cell growth would be expected to be at least additive. To test this prediction, the effect of AdN5 infection, Adp53 infection, or a combination of both on the growth of Colo357 X cells was determined. These cells are relatively resistant to the effects of AdN5,3 allowing any increase in the effect of infection on cell growth to be determined. Both AdN5 infection and Adp53 infection decreased the growth rate of these cells. Treatment with either virus caused the number of cells to decrease by day 6. The kinetics of cell accumulation upon AdN5 or Adp53 infection appeared similar; AdN5 had a slightly greater effect on cell growth than did Adp53. Interestingly, infection with a combination of both viruses had an even greater effect on cell growth (Fig. 6). The number of cells infected with AdN5 and Adp53 at any one time were typically 2- to 4-fold less than the number of cells infected with either AdN5 or Adp53 alone. This difference is likely an underestimate, because the m.o.i. of each virus in the combination infection is half that of the m.o.i. in the single infections.

DISCUSSION

Our data indicate that forced expression of p84N5, either by transfection or adenovirus-mediated gene transfer, renders cells inviable. Loss of viability is due to induction of apoptotic cell death, because p84N5-expressing cells exhibit many of the typical characteristics of this process, including fragmentation of DNA, exposure of phosphatidylserine on the outer leaflet of the plasma membrane, changes in membrane permeability, and changes in cellular and nuclear morphology. The p84N5 protein has significant sequence similarity to the death domains of other proteins involved in apoptosis (31), and characteristic point mutations known to inactivate other death do-

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2 S. Yin, unpublished observation.

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main proteins also compromise p84N5-induced cell death (13). Interestingly, p84N5 is unique among death domain-containing proteins in its nuclear localization (14) and nuclear localization is required for p84N5-induced cell death. There is a relatively small number of proteins that are known to initiate apoptosis from within the nucleus, including the expanded polyglutamine repeat proteins and possibly transcription factors like p53. To test whether the apoptotic pathway triggered by p84N5 expression is similar to that triggered by these other nuclear proteins, we have examined the activation of NF-κB, changes in Bcl-2 family protein expression, the effects of p53, and caspase activation during p84N5-induced apoptosis.

NF-κB activation during p84N5-induced apoptosis is indicated by the change in localization of p65RelA from the cytoplasm to the nucleus in AdN5-infected cells but not in AdGFP-infected cells. The change in subcellular localization is accompanied by an increase in NF-κB-dependent transcriptional activation as determined by increased levels of a luciferase reporter gene whose transcription is dependent on NF-κB transactivation. In contrast to p84N5, expression of p53 inhibits NF-κB activation (33, 34). Activation of NF-κB is observed during apoptosis initiated by DNA-damaging agents like ionizing radiation (35, 36), among others (37, 38). Although similar mechanisms may be utilized to activate NF-κB, p84N5-induced apoptosis is unlikely to be mediated by NF-κB-mediated Fas ligand expression, and CD95 ligation as has been proposed for some DNA-damaging agents (37). Fas-mediated apoptosis requires activation of caspase-8 and is CrmA-sensitive, whereas p84N5-induced apoptosis does not require caspase-8 activation and is insensitive to CrmA (see below).

Our results also demonstrate that apoptosis induced by p84N5 is accompanied by an increase in the relative expression of pro-apoptotic Bcl-2 family members Bak and Bel-Xs. The expression of Bcl-2, Bak, and Bad are unchanged during p84N5-induced apoptosis. In contrast, p53-induced apoptosis involves an increase in the relative expression of Bax (39). Alteration in the levels of Bcl-2 family protein expression can change mitochondrial membrane permeability allowing release of cytochrome c. Cytoplasmic cytochrome c participates in a protein complex composed of caspase-9 and Apaf-1 that leads to caspase activation (40). For example, Bak can accelerate release of cytochrome c through mitochondrial porin channels (41). Bak can also disrupt association of Apaf-1 and the anti-apoptotic Bcl-2 family protein Boo (42). Hence, p84N5-induced apoptosis may be facilitated by the increase in Bak expression observed. Consistent with this hypothesis, we demonstrate that coexpression of Bcl-2 can inhibit p84N5-induced apoptosis. Furthermore, like p84N5-induced apoptosis, apoptosis induced by forced expression of Bak is insensitive to CrmA (43). However, because p84N5-induced apoptosis is initiated by caspase-6 activation rather than caspase-9, Bak either facilitates apoptosis by a different mechanism or it participates in the later execution stages of the process. Whether p84N5 can directly influence transcription is unknown. However, NF-κB can regulate the transcription of some Bcl-2 family genes (44, 45), so the effect of p84N5 on the expression of Bcl-2 family proteins may be indirect.

Our data indicate that p84N5-induced apoptosis does not require p53. Cells with wild-type p53 (MCF-7) or null for p53 (SAOS-2) are equally sensitive to p84N5-induced cell death. Furthermore, we show that p84N5-induced apoptosis does not alter the level of p53 expression and that coexpression of p53 does not inhibit p84N5-induced apoptosis. For a number of reasons we believe it likely that N5 and p53 function in different apoptotic pathways. As described above, p53 and p84N5 have different effects on NF-κB activation and Bcl-2 family protein expression. Furthermore, p53-induced apoptosis is dependent on caspase-3 and/or caspase-9 in many cases (46–48). These caspases are not required for p84N5-induced apoptosis. Finally, we show that infection with both AdN5 and Adp53 causes a more dramatic reduction of Colo357 X cell growth than infection with either one alone. Genes that participate in or activate the same apoptotic pathway would not be expected to have such an additive or synergistic effect. Consistent with our hypothesis that N5 and p53 function in different pathways, N5-induced apoptosis is preceded by a G1/M cell cycle arrest while expression of p53 typically induces a G1 cell cycle arrest.

The pattern of caspase activation during p84N5-induced apoptosis is characterized by the activation of caspase-6 and the absence of activation of caspase-8 or caspase-9.

**Fig. 5.** p84N5-induced apoptosis is independent of p53 status. A. SAOS-2 were cotransfected with indicated expression vectors and analyzed for apoptosis by TUNEL 24 h later as in Fig. 4B. The results are the mean of at least three experiments. B. MCF-7 cells (wild-type p53) or SAOS-2 cells (p53 null) were cotransfected with pCMV5 and pEGFP-c1. Transfected cells were analyzed for apoptosis by TUNEL 24 h later as in Fig. 4B. The data are the mean of at least three experiments. C. 293 cells were transfected with pCMV, pCMV5. Extracts were prepared 24 h later, and equal quantities of total protein analyzed by Western blotting using antibodies directed against the indicated proteins. Staining with anti-β-actin served as a protein loading control.

**Fig. 6.** Infection with both AdN5 and Adp53 reduces cell growth to a greater extent than treatment with either virus alone. Aliquots of 20,000 Colo357 X cells were infected with the indicated viruses, or PBS, and the number of viable cells remaining at the indicated times after infection determined. Each data point is the mean of three infections. The standard deviation for each data point is smaller than the size of the symbol representing that point. The total m.o.i. of all viruses combined in each sample is 50. At this m.o.i., more than 90% of AdGFP-infected cells fluoresce green.

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4. R. Evans and D. W. Goodrich, unpublished observation.
apoptosis is atypical. Caspase-6 is the first caspase activated, and it makes up the majority of caspase activity observed in extracts of AdN5-infected cells. This conclusion is based upon cleavage of a peptide substrate, VEID, that is prefered by caspase-6 as well as by detection of proteolytically processed caspase-6. The pattern of caspase activation is unlikely to be influenced by adenosine infection, because caspases are not activated in AdGFP-infected cells. Because activation of caspase-6 precedes or is coincident with DNA fragmentation, annexin V staining, and loss of cell viability, we conclude that it may mediate p84N5-induced apoptosis. Activation of caspase-9 and caspase-3 activity is also detected in AdN5-infected cells. However, activation of these caspases is unlikely to mediate p84N5-induced apoptosis. First, activation of caspase-9 or -3 occurs after execution of apoptosis has already begun. The activation of a caspase-3, for example, is not detected until 4 days after infection, well after the time when DNA fragmentation can be detected. This observation suggests activation of caspases-9 and -3 is a consequence of earlier caspase-6 activation. Second, MCF-7 cells, which lack caspase-3 (49), are sensitive to AdN5. Hence, caspase-3 is not required for p84N5-induced apoptosis. This pattern of caspase activation suggests p84N5 triggers an apoptotic pathway distinct from those triggered by expanded polyglutamine repeat proteins or death domain-containing receptors. Apoptosis induced by these proteins require caspases-3, -8, or -9, whereas p84N5-induced apoptosis involves caspase-6.

Short prodomain caspases are presumed responsible for the execution phase of apoptosis and typically rely on long prodomain caspases for initial proteolytic activation. Caspase-6 is a short prodomain caspase yet is activated before activation of any other caspase, including long prodomain caspase-8 or -9, during p84N5-induced apoptosis. The activation of caspase-6 is not likely to depend on caspase-9, because caspase-6 is a poor substrate for caspase-9 in vitro (50). Caspase-8 is also unlikely to be involved, because an efficient caspase-8 peptide substrate is not cleaved by extracts of AdN5-treated cells and because p84N5-induced apoptosis is insensitive to CrmA (19), a potent inhibitor of caspase-8. Finally, caspase-3 is unlikely to be involved, because it is not required for p84N5-induced apoptosis. Caspase-6 is either activated by a protease whose activity has not been detected in our experiments or is activated by a novel mechanism during p84N5-induced apoptosis. By analogy to other death domain-containing proteins, p84N5 may play an important part in caspase-6 activation.

In this study, we have characterized the apoptotic pathway triggered by the expression of the nuclear death domain-containing p84N5. Our data indicate that p84N5-induced apoptosis is characterized by activation of caspase-6, increases in the expression of Bcl-2 pro-apoptotic family members Bak and Bcl-Xs, and activation of NF-kB. Furthermore, this pathway does not require p53, nor does the presence of p53 affect the efficiency of cell killing. Expression of Rh, however, does inhibit p84N5-induced apoptosis, presumably mediated by physical association between the two proteins (13, 14). These observations, coupled with the fact that p84N5 is a death domain-containing protein localized within the nucleus, suggest that p84N5 participates in an apoptotic pathway that is different from those triggered by p53, death domain-containing receptors, or expanded polyglutamine repeat proteins.

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