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Identification of NRF2, a member of the NF-E2 family of transcription factors, as a substrate for caspase-3(-like) proteases

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Abstract

Apoptosis is mediated by members of the interleukin-1β converting enzyme (ICE) family of proteases (caspases), which are activated by diverse stimuli, although the downstream molecular targets of caspases are still poorly understood. Using the modified yeast two-hybrid system, which we recently established to clone genes for caspase substrates, we identified NRF2 as a novel caspase substrate. NRF2 is a member of the NF-E2 family of basic region leucine-zipper transcription factors and has been shown to induce phase II detoxifying enzymes through anti-oxidant response elements. NRF2 was cleaved at two sites by recombinant caspase-3 *in vitro* as well as in HeLa cells during TNFα-mediated apoptosis. Overexpression of the C-terminal cleavage fragment containing the DNA binding and leucine-zipper domains induced apoptosis in HeLa cells. These observations suggest that NRF2 might have some role in the induction of apoptosis after cleavage by caspases.

Keywords: apoptosis; caspases; NRF2; transcription factor; substrates

Abbreviations: AREs, anti-oxidant response elements; FMK, fluoromethylketone; ICE, interleukin-1β converting enzyme; IL-1β, interleukin-1β; MAREs, Maf recognition elements; MCA, α-(4-methyl-coumaryl-7-amide); PAGE, polyacrylamide gel electrophoresis; TNF, tumor necrosis factor

Introduction

Apoptosis is an active form of cell death that plays an essential role in the development, homeostasis, and defense of multicellular organisms.[1] Disruption of the regulation of apoptosis underlies the pathogenesis of many human diseases, including autoimmune diseases, neurodegenerative disorders, and cancer.[2] The process of apoptosis can be initiated by a variety of stimuli and finally generates morphologically defined changes such as nuclear condensation, nuclear fragmentation, and membrane blebbing. Analysis of the apoptotic pathway in the nematode *Caenorhabditis elegans* has defined several genes that play essential roles in the execution of this process.[3] The protein encoded by one of these genes, CED-3, is homologous to ICE family of cysteine proteases,[4,5] now called the caspase family.[6] More than ten caspases have been identified in mammals and have been shown to induce apoptosis when overexpressed in cells.[6,7] The caspase family of proteases has been suggested to constitute a protease cascade.[8–11] Since caspase-mediated proteolysis is a critical and central element of the apoptotic process,[12–14] identification of the crucial downstream molecular targets of these proteases is necessary for better understanding of apoptotic signal transduction. Although a number of structural and signaling proteins have been shown to be cleaved by caspases during apoptotic cell death[12–14] including ICAD, an inhibitor of caspase-activated DNase (CAD), which is essential for internucleosomal DNA degradation,[15,16] the crucial target proteins for which cleavage triggers the execution of apoptosis are still poorly understood.

Transcription factor NF-E2 is composed of p45, a large subunit belonging to the CNC ('cap 'n' collar') family of basic region leucine-zipper transcription factors, and a small Maf subunit, and binds to Maf recognition elements (MAREs, NF-E2 binding sites) to activate the transcription of a set of genes.[17–20] Erythroid-specific p45-NF-E2 has been implicated in globin gene regulation, and p45-NF-E2 null mice succumb to bleeding due to lack of platelets while those that survive exhibit mild anemia.[21,22] MAREs also bind various basic leucine-zipper (bZip) proteins including other CNC proteins, Nrf1, and Nrf2, sometimes as obligate heterodimers with a small Maf protein.[23–25] NRF2 is ubiquitously expressed and is capable of transactivating genes through MAREs,[24] but unlike p45-NF-E2, disruption of the nrf2 gene does not cause any hematological abnormalities,[26] suggesting that NRF2 deficiency might be compensated for by other related proteins. Recently, it was reported that NRF2 was involved in the transcriptional complex mediating the induction of genes for phase II detoxifying enzymes through the anti-oxidant response element (AREs).[27,28] However, the biological role of NRF2 is still unclear.

We recently established a method of cloning the genes for caspase substrates using the yeast two-hybrid system.[29] Using this method, we identified NRF2 as a substrate for caspases and obtained evidence that NRF2

may function as an effector molecule downstream of caspases during apoptosis.

Results

Identification of NRF2 as a substrate for caspases using the yeast two-hybrid system

We recently reported a cloning method for caspase substrates using the yeast two-hybrid system.[29] This method involves two major modifications of the conventional yeast two-hybrid system: (1) both large and small subunits of active caspases are separately expressed in yeast under the ADH1 promoter and the small subunit is fused to the LexA DNA binding domain, (2) a point mutation was introduced into the active site cysteine to prevent proteolytic cleavage of the substrate, possibly stabilizing the enzyme-substrate complex in yeast. Using this method, we attempted to identify substrates for caspase-1 in the present study. The bait plasmid for caspase-1, pBTM-casp1-p10p20^{m} [29], was cotransfected into yeast with prey plasmids containing a human thymus cDNA expression library fused to the Gal4 activation domain. One hundred and thirty-one His^{+} colonies were obtained by screening 5.1×10^{7} transformants, and finally one candidate clone was obtained. DNA sequencing analysis revealed that this plasmid (pGAD$_{10}$ICE204) encoded part of human NRF2 (amino acid residues 1–247), with two substitution mutations (threonine 56 to alanine and threonine 75 to isoleucine) relative to the published sequence.[24] We also confirmed the interaction between the full length NRF2 and caspase-1 using the yeast two-hybrid system (data not shown), indicating that NRF2 might be a substrate for caspase-1. To directly assess the ability of caspase-1 to cleave NRF2, purified His$_6$-tagged NRF2 was incubated with purified active caspase-1 (Figure 1A). The 90 kDa NRF2 was cleaved to produce a 30 kDa C-terminal fragment and cleavage was inhibited in the presence of 10 μM Ac-YVAD-CHO, suggesting that NRF2 is a candidate substrate for caspase-1. Since caspase-3 has been implicated as a key mediator of apoptosis in mammalian cells,[30,31] we also examined whether NRF2 was cleaved by recombinant caspase-3 *in vitro*. As shown in Figure 1B, NRF2 was cleaved to produce 50 and 30 kDa fragments both containing the C-terminal epitope, and cleavage was completely inhibited in the presence of 100 nM Ac-DEVD-CHO. These results suggested that NRF2 was a possible substrate for both caspase-1 and caspase-3, and has at least two cleavage sites.

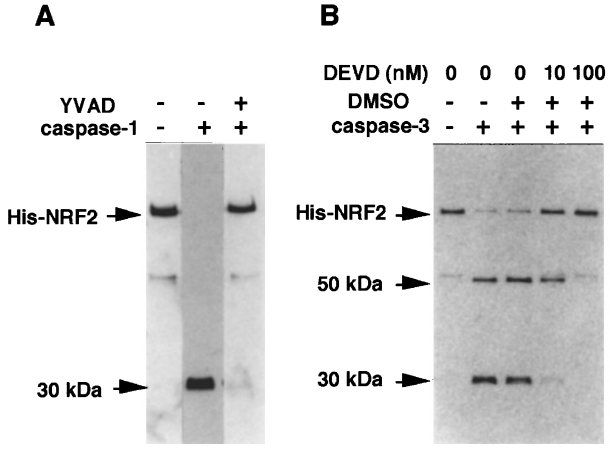

Figure 1 *In vitro* cleavage of NRF2 by purified caspases. (**A**) Purified His$_6$-tagged NRF2 was incubated with purified active caspase-1 (95 units) with or without 10 μM Ac-YVAD-CHO for 2 h at 30°C. The reaction product was analyzed by 10–20% gradient SDS–PAGE followed by immunoblotting with anti-NRF2 antibodies that recognized the C-terminal epitope. (**B**) Purified His$_6$-tagged NRF2 was incubated with 0.8 μg of purified recombinant active caspase-3 with or without the indicated amount of Ac-DEVD-CHO for 2 h at 30°C. The reaction product was analyzed as described in (**A**). Intact NRF2 (90 kDa) and its cleavage fragments (50 and 30 kDa) are indicated by arrows

Cleavage of NRF2 *in vivo*

To examine the cleavage of NRF2 during apoptosis *in vivo*, HeLa cells that were transiently transfected with the NRF2 expression plasmid (pCAGGS-NRF2) were treated with TNFα in the presence of cyclohexamide. As shown in Figure 2A, we detected, in addition to a full length NRF2 protein, two fragments of 50 kDa and 30 kDa corresponding to the NRF2 fragments generated by caspase-3 *in vitro* (Figure 1B). The full length NRF2 protein and the 50 kDa fragment decreased to undetectable levels within 4 h, and the 30 kDa fragment increased as cell viability decreased and caspase-3(-like) protease activity increased during apoptosis (Figure 2A,B). The 50 kDa and 30 kDa fragments shown by non-treated cells were probably produced by cleavage of NRF2 with caspases which were transiently activated by transfection procedure. These results suggested the NRF2 protein is a substrate of caspases *in vivo*.

Cleavage of NRF2 by caspase-3(-like) proteases in apoptotic HeLa cell lysate

To characterize the proteases responsible for the cleavage of NRF2 in apoptotic cells, we used an *in vitro* cleavage assay in which ^{35}S-methionine-labeled NRF2 was added to a lysate prepared from apoptotic cells. Incubation of ^{35}S-methionine-labeled NRF2 with the lysate from TNFα-treated apoptotic HeLa cells resulted in appearance of an about 30 kDa fragment, whereas the control lysate from untreated cells had no effect (Figure 3). Iodoacetamide inhibited the cleavage of NRF2, indicating that this cleavage was catalyzed by cysteine proteases including members of the caspase family.[32] Addition of Ac-DEVD-CHO, a potent inhibitor of caspase-3(-like) proteases, but not Ac-YVAD-CHO, an inhibitor of caspase-1, abolished the NRF2 cleavage activity of apoptotic cell extract, indicating that NRF2 was catalyzed by caspase-3(-like) proteases in apoptotic HeLa cell lysate. The failure of inhibition of NRF2 cleavage by the Ac-YVAD-CHO was simply because of no or very low levels of caspase-1(-like) proteases in apoptotic HeLa cell extract, as shown by no cleavage of *in vitro*-translated pro-IL-1β in the extract (data not shown), consistent with our previous observations.[33]

Identification of NRF2 cleavage sites

To identify the cleavage sites of NRF2 by caspase-3, we employed both mutational analysis and mass spectro-

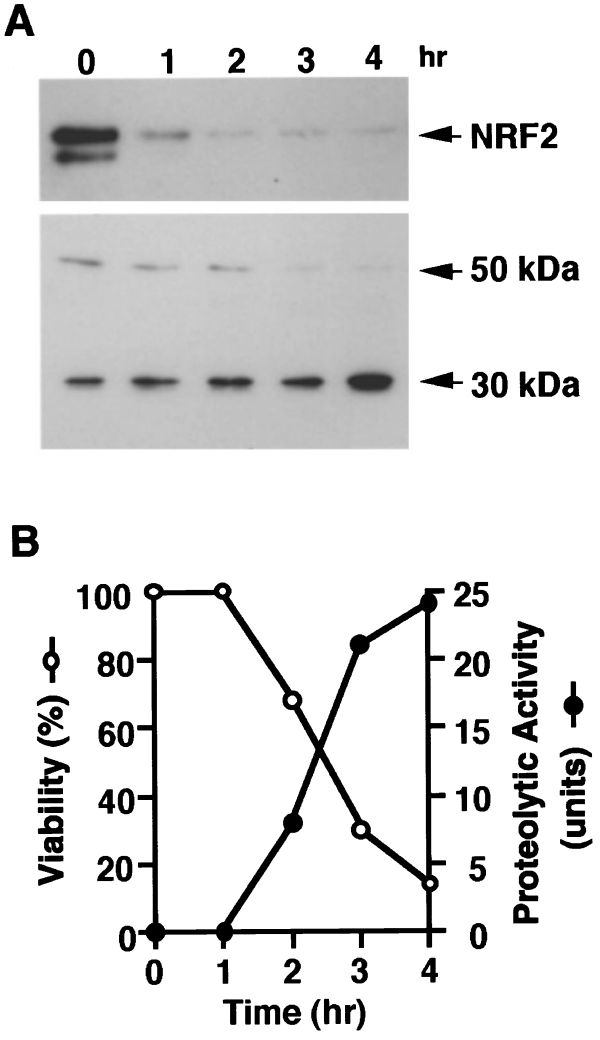

Figure 2 *In vivo* cleavage of NRF2 in apoptotic HeLa cells. (**A**) Cleavage of NRF2 after TNFα stimulation. HeLa cells were transfected with pCAGGS-NRF2 for 15 h, and then treated with 20 ng/ml of recombinant hTNFα in the presence of 30 μg/ml of cyclohexamide for the indicated periods. The cells were harvested and the lysates were subjected to SDS–PAGE followed by immunoblot analysis with anti-NRF2 antibodies. Intact NRF2 (90 kDa) and its cleavage fragments (50 and 30 kDa) are indicated by arrows. (**B**) Cell viability and proteolytic activity of caspase-3(-like) proteases. HeLa cells were treated with TNFα as described in (**A**). Cell viability (%) was assessed morphologically by counting flat and well attached cells as viable cells and round or fragmented cells as dead cells. Three independent experiments were carried out and more than 200 cells were counted in each experiment. The proteolytic activity of caspase-3(-like) proteases in HeLa cell extract was measured with Ac-DEVD-MCA as described in Materials and Methods

metry. Since pGAD$_{10}$ICE204 encodes amino acids 1–247 of human NRF2, at least one of the two potential cleavage sites must exist in this region. We introduced mutations into the N-terminal fragment, replacing each of the aspartates at five putative cleavage sites with alanines. Among these mutants (D96A, D116A, D162A, D208A and D236A), only D208A resisted cleavage to yield the 50 kDa fragment (Figure 4A). This indicated that aspartate 208 was one of the potential cleavage sites for caspase-3.

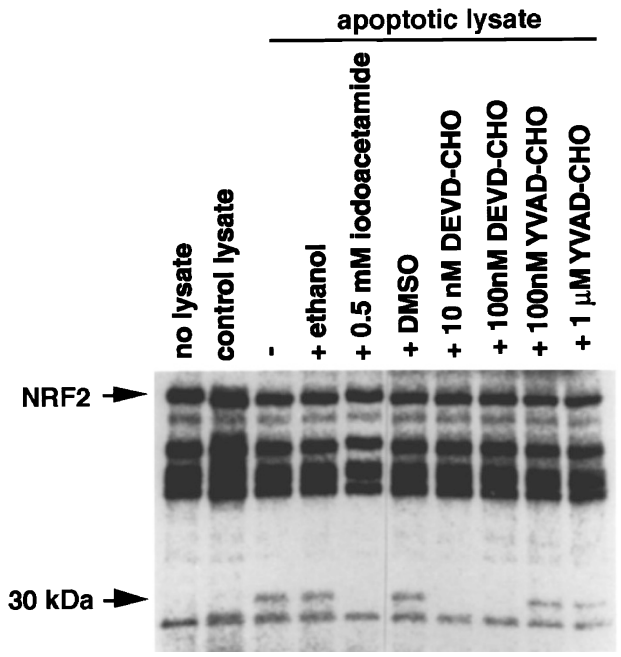

Figure 3 Inhibition of NRF2 cleavage in apoptotic HeLa extract by various protease inhibitors. The cytosolic fraction of HeLa cells treated with or without 20 ng/ml of recombinant hTNFα in the presence of 30 μg/ml of cyclohexamide for 3.5 h was incubated with ^{35}S-methionine-labeled NRF2 and various protease inhibitors as indicated. Each reaction mixture was separated by 10–20% gradient SDS–PAGE and analyzed with a BAS2000 Bio-Imaging analyzer (Fuji film). Intact NRF2 (90 kDa) and its cleavage fragment (30 kDa) are indicated by arrows

To find the other caspase-3 cleavage site of NRF2, which was responsible for production of the 30 kDa fragment, we purified the 30 kDa fragment from the *in vitro* cleavage reaction mixture by HPLC and directly measured the precise molecular weight of this fragment using electro-spray ionization mass spectrometry (ESI-MS). The exact molecular weight was 25406 ± 8, indicating that the second cleavage site of NRF2 is likely to be aspartate 366. We replaced aspartate 366 with alanine and incubated this mutant protein with recombinant caspase-3, resulting in no production of the 30 kDa cleavage fragment (Figure 4B), indicating that an aspartate 366 was the other caspase-3 cleavage site. Consistent with this finding, NRF2 with both the D208A and D366A mutations was not cleaved at all by caspase-3 (Figure 4B), indicating that NRF2 is cleaved by caspase-3 at aspartate 208 and aspartate 366 (Figure 4C).

Overexpression of the C-terminal cleavage fragment of NRF2 induces apoptosis

We next studied the biological role of NRF2 in apoptosis. As shown in Figure 4C, NRF2 was cleaved into three fragments by caspase-3(-like) proteases and the C-terminal fragment contained the DNA binding and leucine-zipper domains. Since the leucine-zipper domain of transcription factors can function as a dominant negative effector,[34] we examined

whether the expression of the C-terminal fragment of NRF2 inhibits the transcriptional activity of NRF2 through NF-E2 binding site. As shown in Figure 5A, the C-terminal fragment inhibited the transcriptional activity of NRF2 in a dose-dependent manner. We then examined the effect of over-expression of the C-terminal fragment of NRF2 on apoptosis. Although none of the wild-type, double mutant (D208AD366A), or C-terminal fragment (bZip) induced apoptosis at 24 h after transfection, the C-terminal fragment induced apoptosis at 30 h (Figure 5B). Since the C-terminal fragment was stably expressed after transfection (Figure 5C), the apoptosis-inducing activity seemed to be mediated by the C-terminal fragment. These results indicated that NRF2 might act as one of the effector molecules downstream of caspase-3 during apoptosis.

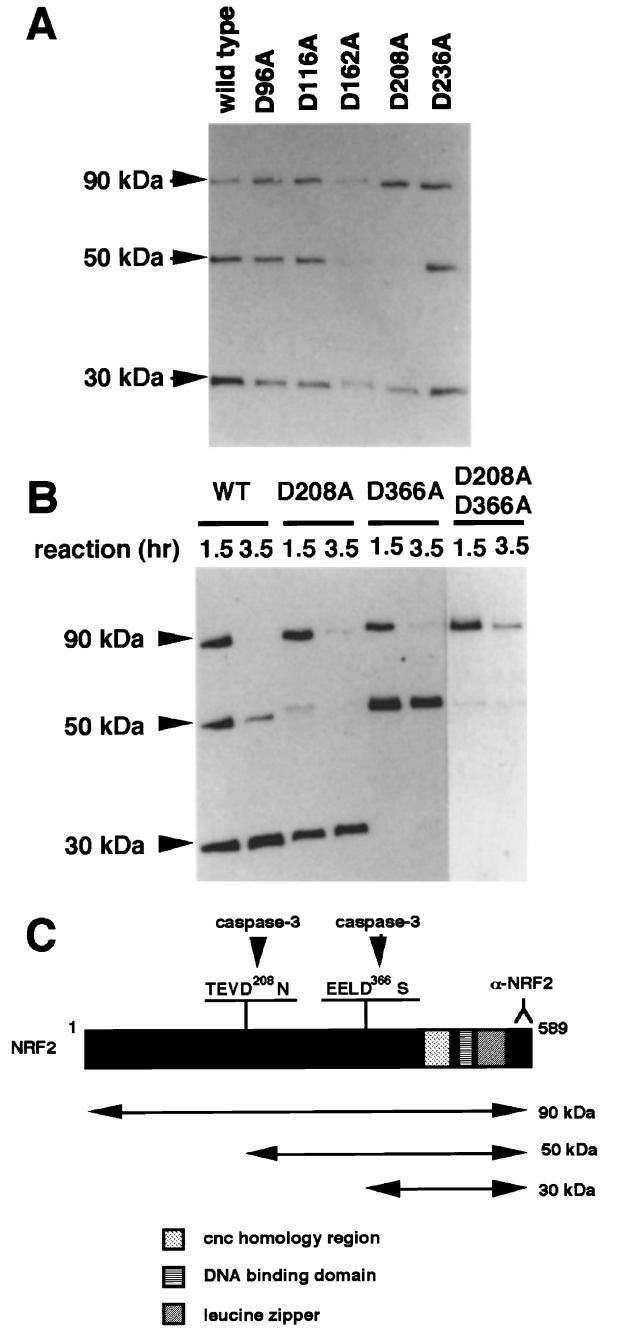

Figure 4 Determination of the cleavage sites of NRF2. (**A**) Purified His_6-tagged wild-type and mutant NRF2 proteins were incubated with 0.8 μg of purified recombinant caspase-3 for 2 h at 30°C. The reaction products were analyzed by 10–20% gradient SDS–PAGE followed by immunoblotting with anti-NRF2 antibodies. (**B**) Purified His_6-tagged wild-type and mutant NRF2 proteins were incubated with 0.8 μg of purified recombinant caspase-3 for the indicated periods at 30°C and the reaction products were analyzed as described in (**A**). Intact NRF2 (90 kDa) and its cleavage fragments (50 and 30 kDa) are indicated by arrows. (**C**) Diagram showing a summary of caspase-3 mediated cleavage of NRF2

Discussion

Using the modified yeast two-hybrid-based cloning mehtod,[29] we identified NRF2, a member of the NF-E2 family of transcription factors, as a substrate for caspases.

There are many proteins that are cleaved by caspases in response to various apoptotic stimuli. Some of these proteins are essential for cell survival and are activated by cleavage, such as poly(ADP-ribose) polymerase, lamin, the catalytic subunit of DNA protein kinase, the retinoblastoma tumor suppressor protein, GDP-dissociation inhibitor type D4 (D4-GDI), and focal adhesion kinase.[12] Other proteins are activated by caspase cleavage, such as MEKK1, p21-activated kinase 2 (PAK2), protein kinase Cδ (PKCδ), gelsolin, and the mammalian Ste20-like kinase Mst1.[12,29,35–39] After cleavage, each of these proteins contributes to the apoptotic response in various manners. The caspases seem to cleave proteins essential for cell survival in addition to activating death signaling molecules. Although NRF2 was originally identified as a member of the NF-E2 family of transcription factors,[24] it was recently reported to be an essential transcription factor for induction of phase II detoxifying enzymes through the anti-oxidant response elements (AREs).[27,28] The phase II detoxifying enzymes, such as glutathione S-transferase (GST) and NAD(P)H : quinone oxidoreductase (NQO1), are important for defense mechanisms against xenobiotics and oxidative stress. Thus, NRF2 is a survival factor mediating the induction of proteins that protect against oxidative stress, and the cleavage and inactivation of NRF2 by caspases may facilitate transduction of death signals.

We have shown here that the C-terminal cleavage fragment of NRF2 induce apoptosis, when overexpressed, suggesting a positive role of the C-terminal fragment in accelerating apoptosis. Although we cannot formally exclude the possibility that the N-terminal or internal fragment of NRF2, both of which are rich in aspartic acid and glutamic acid residues and therefore might represent an acidic type of transactivation domain,[40] might have a role in apoptosis, the possibility seems to be unlikely, given that transactivation domains function only when fused to a DNA-binding domain. How does the C-terminal fragment of NRF2 induce apoptosis? It has been reported that truncated variants of several transcription factors that lack the putative N-terminal transactivation domain can function as dominant negative regulators.[41,42] Since NRF2 is a member of the family of basic region leucine-zipper transcription factors and form heterodimers with MAF protein to bind MAREs (Maf recognition elements),[19,20,25]

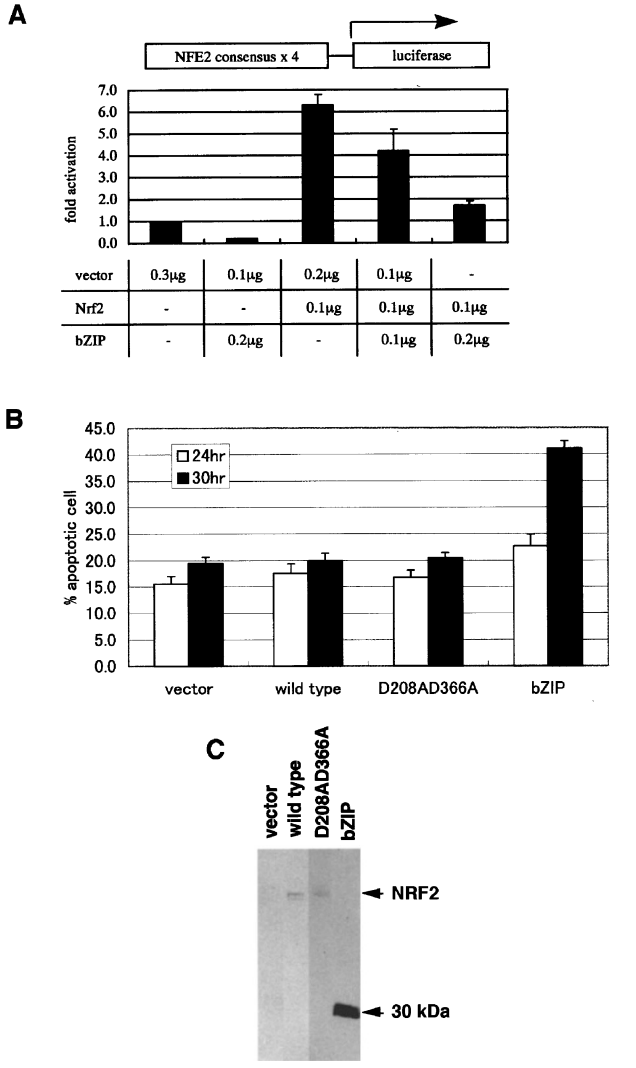

Figure 5 Function of the C-terminal cleavage fragment of NRF2. (**A**) C-terminal cleavage fragment of NRF2 functions as a dominant negative mutant. One day before transfection, NIH3T3 cells were seeded at a density of about 5×10^4 per well in 24-well dishes. In each well, 0.1 μg of pGL3-NFE2-Luc and 0.1 μg of pactβGal were cotransfected with the indicated amounts of pcDL-SRα296 (vector), pSRαNrf2 (Nrf2) and pSRαNrf2bZip (bZip). Cells were harvested at 24 h after transfection and luciferase activities were measured using PicaGene luminescence kit (Toyo Ink). Assay for β-galactosidase activities were performed by a standard colorimetric procedure with o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. All luciferase activities were normalized to β-galactosidase activities. The data (mean $\pm$ S.D.) were obtained from at least three independent experiments. (**B**) Induction of apoptosis in HeLa cells by expression of the C-terminal cleavage fragment of NRF2. One day before transfection, cells were seeded at a density of about 4×10^5 per well in 6-well dishes. In each well, 2.0 μg of pcDL-SRα296 (vector), pSRαNrf2 (wild-type), pSRαNrf2D208AD366A, or pSRαNrf2bZip were cotransfected with 0.2 μg of pactβGal plasmid DNA using 7 μl of lipofectAMINE reagent. The cells were incubated for 8 h in serum-free medium containing DNA and lipofectAMINE, an equal volume of DMEM containing 20% FBS was added, and further incubation was done for 16 or 22 h. Then the cells were stained with X-gal for 3 h as described in Materials and Methods. The ratio of round blue cells to total blue cells was determined. The data (mean $\pm$ S.D.) were obtained from at least three independent experiments. (**C**) Expression of wild-type, mutant (D208AD366A) and C-terminal fragment of NRF2. Cells were transfected and harvested at 30 h after transfection as described in (**B**), and subjected to SDS–PAGE followed by immunoblotting with anti-NRF2 antibodies. Intact NRF2 (90 kDa) and the C-terminal fragments (30 kDa) are indicated by arrows

this cleavage fragment may preferentially heterodimerize with MAF family proteins, and may interfere with the binding of endogenous NRF2/MAFs heterodimers to target DNA. NRF2 is an essential survival transcription factor for the induction of various proteins, which are a defense mechanism against some stress as described above. Therefore, overexpression of the C-terminal fragment of NRF2 may induce apoptosis by inhibiting the expression of proteins necessary for cell survival.

In conclusion, we found that NRF2 was a substrate for caspase-3(-like) proteases both *in vitro* and *in vivo*. We also found that overexpression of the C-terminal cleavage fragment induced apoptosis. These observations suggest that NRF2 is a target for proteolysis by caspase-3(-like) proteases and acts as an effector molecule downstream of caspases in the cell death program.

Materials and Methods

Yeast two-hybrid screening

Yeast two-hybrid screening using pBTM-casp1-p10p20$^{m\ 29}$ as the bait was performed with a human thymus cDNA expression library fused to the GAL4 activation domain in the pGAD$_{10}$ plasmid following the matchmaker Two-Hybrid System Protocol (Clontech) in L40 cells (*MATa trp1 leu2 his3 ade2 LYS2*::*lexA-HIS3 URA3*::*lexA-lacZ*).

Plasmid construction

For construction of full length Nrf2 cDNA, the fragment encoding amino acids 195–589 was amplified by PCR using two primers [Cnrf-5′ (5′-CTACCATGGTTCCAAGTCCA-3′) and Cnrf-3′ (5′-GTCGGATCCTCC-TAAATCTAGT-3′)] and the human thymus cDNA library as a template. The Cnrf-5′ and Cnrf-3′ primers respectively had *Nco*I and *Bam*HI sites at the 5′ end. The amplified fragments were cloned into the *Hinc*II site of pUC118 and sequenced. The *Xho*I–*Nco*I fragment encoding amino acids 1–195 of NRF2 from pGAD$_{10}$ICE204 and the *Nco*I–*Bam*HI fragment encoding amino acids 195–589 were joined and inserted into pUC118 to generate pUC118Nrf2. The resultant full length Nrf2 cDNA was subcloned into pGAD$_{10}$, pQE-30 (QIAGEN), pBluescript II SK(−) (Stratagene), pUC-CAGGS,[43] and pcDL-SRα296[44] to generate pGAD$_{10}$Nrf2, pQE-Nrf2, pSK(−)Nrf2, pCAGGS-Nrf2, and pSRαNrf2.

Base substitution mutants of NRF2 were generated with a Mutan-Express Km kit (Takara Shuzo). The mutations were introduced using the following oligonucleotides: 5′-CCCAAATCAGCTGCTTTGTAC-3′ for D96A, 5′-GTTTGTAGATGCCAATGAGGTTTC-3′ for D116A, 5′-GTTGATTTAGCCGGTATGCAAC-3′ for D162A, 5′-GACA-GAAGTTGCCAATTATCAT-3′ for D208A, and 5′-GCTTTTGAGGCTTCCTTCAGC-3′ for D236A. To substitute Asp 366 for Ala, a PCR method employing these mutagenic oligonucleotide primers was used. The mutations in the final constructs were confirmed by DNA sequencing. The cDNAs containing mutations at D96A, D116A, D162A, D208A, D236A, D366A, and D208AD366A were inserted into the *Bam*HI site of pQE-30 (QIAGEN) to generate pQE-NrfD96A, pQE-NrfD116A, pQE-NrfD162A, pQE-NrfD208A, pQE-NrfD236A, pQE-NrfD366A and pQE-NrfD208AD366A, respectively, for production of bacterially expressed His-tagged proteins. The cDNA containing mutation D208AD366A was inserted into pcDL-SRα296 to generate pSRαNrf2D208AD366A.

A DNA fragment encoding the C-terminal cleavage fragment (amino acids 367–589) of NRF2, which included a basic region

leucine-zipper domain, was amplified by PCR using two primers: nrfc-5′ (5′-ATGCATGAGTGCCCCTGGAAGTGTC-3′) and Cnrf-3′, and the full length Nrf2 cDNA as a template. The nrfc-5′ and Cnrf-3′ primers respectively had *Eco*T22I site with ATG sequence and *Bam*HI site at the 5′ end. The amplified fragment was cloned into pUC118, sequenced, and inserted into pcDL-SRα296 to generate pSRαNrf2bZIP.

For luciferase assay, a reporter plasmid was constructed by inserting the NF-E2 response elements into pGL3 vector (Promega). The synthetic oligonucleotides containing NF-E2 response elements (underlined) are as follows: NFE2-U (5′-CGCACAGCAATGC-TGAGTCATGATGAGTCATGCTGGGATCCGCACAGCAATGC-TGAGTCATGATGAGTCATGCTGC-3′) and NFE2-D (5′-TCGAGCAG-CATGACTCATCATGACTCAGCATTGCTGTGCGGATCCCAGCA-TGACTCATCATGACTCAGCATTGCTGTGCGGTAC-3′). The NFE2-U and NFE2-D oligonucleotides were annealed and inserted into the *Kpn*I – *Xho*I site of pGL3 to generate pGL3-NFE2-Luc.

Purification of His_6-tagged NRF2 proteins

His_6-tagged wild-type and mutant NRF2 proteins (including D96A, D116A, D162A, D208A, D236A, D366A, and D208AD366A) were expressed in *E. coli* strain JM109 using plasmids pQE-Nrf2, pQE-NrfD96A, pQE-NrfD116A, pQE-NrfD162A, pQE-NrfD208A, pQE-NrfD236A, pQE-NrfD366A, and pQE-NrfD208AD366A, respectively. Induction and purification of recombinant His_6-tagged proteins by Ni^{2+} affinity chromatography was done according to the manufacturer's instructions (QIAGEN).

Cell culture and cell death assay

HeLa and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Transfection was performed using LipofectAMINE (GibcoBRL). For induction of apoptosis, HeLa cells were treated with 20 ng/ml of recombinant hTNFα (PeproTech Inc.) in the presence of 30 μg/ml of cycloheximide. Cell death after DNA transfection was assessed morphologically by co-transfecting pactβGal that produces bacterial β-galactosidase[45] to identify the DNA transfectants. For analysis of β-galactosidase expression, cells were rinsed once with phosphate-buffered saline (PBS), fixed with 1% glutaraldehyde for 5 min, rinsed twice with PBS, and stained in X-Gal buffer (0.5 mg/ml 5-bromo-4-chloro-3-indoxyl β-galactoside, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$-$3H_2O$, 1 mM $MgCl_2$, 10 mM KCl, and 0.1% Triton X-100 in 0.1 M sodium phosphate buffer, pH 7.5)[46] for 3 h at 37°C.

Preparation of cell extracts

Cell extracts were prepared essentially as described elsewhere.[32] Briefly, cells were washed twice with PBS and once with an extraction buffer (50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM cytochalasin B, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml antipain, and 1 μg/ml chymopapain). Cells were then resuspended in extraction buffer and lysed by five cycles of freezing and thawing. Cell extracts were centrifuged at $10\,000 \times g$ for 10 min and then at $100\,000 \times g$ for 90 min at 4°C. The final supernatant was used as the cytosolic extract.

In vitro cleavage assay

^{35}S-methionine-labeled NRF2 was prepared by *in vitro* transcription-translation using the TNT T7 coupled reticulocyte system (Promega) with ^{35}S-methionine (10 mCi/ml, Amersham) and pSK(−)Nrf2. *In vitro* translated ^{35}S-methionine-labeled NRF2 or His_6-tagged NRF2 purified from bacterial lysates was incubated with purified caspase-1 (a gift from Dr. N.A. Thornberry), recombinant caspase-3 prepared as described elsewhere,[46,47] or cytosolic extracts from control or apoptotic cells for 2.5 h at 30°C in 20 μl of reaction buffer (100 mM HEPES, pH 7.0, 20% glycerol, 5 mM dithiothreitol, and 0.5 mM EDTA). The reaction was stopped by addition of SDS – PAGE sample buffer and cleavage products were analyzed by 10 – 20% gradient SDS – PAGE.

Antibodies and Western blot analysis

Anti-NRF2 polyclonal antibodies raised against a peptide corresponding to amino acids 569 – 588 of human NRF2 were purchased from Santa Cruz Biotechnology. Western blot analysis was carried out essentially as described elsewhere.[48]

Assay of caspase-3(-like) protease activity

Cells were collected and suspended in buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA). After the addition of 0.3 mg/ml of digitonin, the cells were incubated at 37°C for 10 min. Lysates were centrifuged at 15 000 r.p.m. for 3 min and the cleared supernatants were collected. Aliquots (equivalent to 5×10^5 cells) were incubated with 1 μM Ac-DEVD-MCA (Peptide Institute) in 1 ml of buffer A at 37°C for 15 min, and the release of amino-4-methylcoumarin was monitored by a spectrofluorometer (Hitachi F-4500) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. One unit was defined as the amount of enzyme required to release 1 pmol of MCA per hour per 1×10^4 cells at 37°C.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

The C-terminal cleavage fragment of NRF2 was separated from 50 μl of *in vitro* cleavage reaction mixture by reversed-phase high-performance liquid chromatography (RP-HPLC). The HPLC system used was a Shimadzu LC10AD equipped with a gradient pump, UV detector, and integrator. Peptides were separated on a YMC-Pack-PROTEIN-RP column (2.1×150 mm) at a flow rate of 0.2 ml/min. The mobile phase system comprised mobile phase A [0.1% trifluoroacetic acid (TFA)-distilled water] and mobile phase B (0.1% TFA-acetonitrile). The column was equilibrated with 15% mobile phase B for 5 min, and then the sample was applied to the column. Peptides were eluted with a linear gradient of mobile phase B from 15% to 75% over 60 min, and were detected by monitoring the UV absorbance at 215 nm. The peak with a retention time of 33.6 min was identified as the C-terminal cleavage fragment of NRF2 by 10 – 20% gradient SDS – PAGE followed by immunoblot analysis with anti-NRF2 antibodies.

Liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS)

A YMC-PROTEIN-RP column (0.3×150 mm) was used at a flow rate of 4 μl/min with the same mobile phase system as that described above. The column was equilibrated with 20% mobile phase B for 5 min, and then the RP-HPLC fraction, which contained the C-terminal cleavage fragment of NRF2, was applied to the column. Peptides were eluted with a linear gradient of mobile phase B from 20% to 70% over 20 min and the column elute was directly injected into the electrospray interface of a Finnigan MAT TSQ700 mass spectrometer (San Jose, CA, USA) equipped with a Finnigan ESI ion source. The electrospray ion source was operated at a potential of 4.5 kV. Nitrogen was used for

the sheath gas and the auxiliary gas, while 2-methoxyethanol was used for the sheath liquid at a flow rate of 2 μl/min.

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