The Inhibition of the Nuclear Transport of Caspase-7 by its Prodomain

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ABSTRACT

Apoptosis is a major form of cell death, characterized by a series of the morphological changes induced by cleaving cytoplasmic and nuclear proteins via active caspases. The data presented here show, by the fluorescence microscopic and the immunoblotting analyses, that a prodomain of caspase-7 inhibits its nuclear translocation and apoptosis-inducing activity. This nuclear localization is dependent on the presence of a basic tetrapeptide that is conserved in mammalian and *Xenopus* caspase-7 and that is located downstream of a cleavage site between a prodomain and a catalytic protease domain. Furthermore, an attachment of caspase-7 prodomain (31 amino acids) represses the nuclear transport of a fusion protein of a heterologous protein and the caspase-7 nuclear localization signal (19 amino acids), suggesting that the inhibition of the nuclear localization by the prodomain is mediated by the interaction of these short peptides.

Key Words: caspase; nuclear localization; prodomain; apoptosis; cytotoxic activity.

Introduction

Apoptosis is mediated by the sequential and coordinated activation of several caspases to cleave a variety of intracellular polypeptides such as major structural elements and functional molecules in the cytoplasm and the nucleus (1), contributing to the stereotypic morphological and biochemical changes that characterize apoptotic cell death. Many nuclear proteins are cut by caspases to disassemble the nuclear structure (2) and to degrade chromosomal DNA (3). Caspases-1 and -2 are shown to be translocated to the nucleus in a prodomain-dependent manner. A cell death stimulus induces the translocation of pro-caspase-1 to the nucleus, where release of its prodomain and proteolytic activation occur (4). Pro-caspase-2 is localized to the nucleus even in healthy cells (5). On the other hand, caspase-3 passes through the nuclear pores during apoptosis by diffusion, because activated caspase-9 inactivates nuclear transport and increases the diffusion limit of the nuclear pores (6).

In response to the apoptotic stimulus, caspase-7 is activated and accumulated in the nucleus, where it cleaves one of the nuclear proteins, poly(ADP-ribose)polymerase (7), but the molecular mechanism on the nuclear transport of caspase-7 remains obscure. The cloning of *Xenopus* caspase genes enabled to delineate evolutionarily conserved regions by comparison with mammalian caspase sequences (8), indicating the presence of a putative nuclear localization signal in caspase-7. In this study, to examine whether this nuclear localization signal is functional, various N-terminal truncated caspase-7-GFP fusion constructs were generated and transfected into cultured cells. The data presented here demonstrate that a prodomain of caspase-7 inhibits both the apoptosis-inducing activity and the nuclear localization.

Materials and methods

Mutagenesis and Expression Constructs

The active site pentapeptide QACRG of *Xenopus* caspase-7 gene (8) was changed to QAARG by site-directed mutagenesis using the mutagenic oligonucleotide (5'-CATTCAGGCCGCCAGAGGGCATGA) encoding the peptide IQAARGHE and Sculptor *in vitro* mutagenesis system (Amersham, England) according to the manufacturer's recommendation. This catalytically inactive mutation was verified by DNA sequencing. The caspase-7 coding region was amplified by polymerase chain reaction using T7 primer (Stratagene) and C7-Sal I primer (5'-CCGTCGACAACAGGATTTAAAGTAG) from pcDNA1/Amp plasmid (Invitrogen) containing wild-type or mutant caspase-7 gene. GFP fusion constructs, C7-1W and C7-1M, were generated by digesting with EcoR I and Sal I the amplified DNA fragments from wild-type and mutant caspase-7 genes, respectively, and inserting into the EcoR I and Sal I sites upstream of GFP coding region in pEGFP-N2 vector (Clontech). Similarly, C7-22W and -22M, C7-32W and -32M, C7-47W and -47M, and C7-51W and -51M were constructed by amplifying N-terminal truncated coding regions with C7-Sal I primer as a 3' primer and 22-primer (5'-CCGAATTCGCCATGGAGGAAGAGGAGGGT), 32-primer (5'-CCGAATTCGCCATGGCGAAACCTGACAGAA), 47-primer (5'-CCGAATTCGCCATGGCCAAGAAGAAAAAGG), and 51-primer (5'-CCGAATTCGCCATGGTAGAAGACAAGCCC) as a 5' primer from wild-type and mutant caspase-7 genes, respectively. Caspase-7-GFP fusion constructs, $C7_{1\text{-}50}$ and $C7_{32\text{-}50}$, were generated by amplifying amino terminal coding regions of caspase-7 (residues 1-50 and 32-50) using T7 primer and 32-primer as a 5' primer, respectively and $C7_{\text{-}50}$-Sal I primer (5'-CCGTCGACCCTTTTTCTTCTTGGTGCT) as a 3' primer and inserting into pEGFP-N2 vector after the digestion with EcoR I and Sal I. The nucleotide sequences of these N-terminal truncated caspase-7 genes were confirmed by DNA sequencing.

Transient Transfection Assay

The cell line XLT-15-11 (8) was maintained in 67% L-15 medium supplemented with 10% thyroid hormone-depleted fetal calf serum (9) at 20˚C. Cells were allowed to grow to 30-40% confluence in Primaria 6-cm dishes (Becton Dickinson), and transfected with 1 µg of pEGFP-N2 or one of caspase-7-GFP fusion constructs using 3 µl of FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the instructions of the manufacturer. The culture medium was exchanged 8 h post transfection. Apoptotic cells were identified after transfection by visual inspection of GFP-positive cells with a Zeiss Axiovert 135 TV inverted fluorescence microscope as described (10).

Cellular Subfractionation and Immunoblotting

Cytoplasmic and nuclear extracts from transfected cells were prepared three days after transfection, and examined by immunoblot analysis as described previously (5).

Results

Intracellular distribution of N-terminal truncated wild-type caspase-7-GFP fusion proteins

The comparison of N-terminal 80 amino acid sequences between mammalian and *Xenopus* pro-caspase-7 revealed two conserved regions, a region containing the cleavage site between a prodomain and a catalytic protease domain, and a basic tetrapeptide downstream of the prodomain (Fig. 1). To investigate whether this basic motif could act as a nuclear localization signal, wild-type full-length and prodomain-deleted caspase-7 coding regions were fused at their C-termini in-frame with GFP gene in pEGFP-N2 to construct C7-1W and -32W, respectively, which were transfected into a myoblastic cell line derived from tadpole tail of *Xenopus laevis*, XLT-15-11 (9). The subcellular localization of GFP-tagged caspase-7 was examined using a fluorescence microscope. The GFP fusion protein was distributed mainly in the cytoplasm of cells introduced with C7-1W construct, whereas the transfection of C7-32W construct showed a strong cytotoxic activity (Fig. 2) and the nuclear localization in surviving cells (Fig. 3), suggesting that the basic tetrapeptide is functional as a nuclear localization signal and the prodomain inhibits the nuclear localization of caspase-7.

To confirm this idea, various truncated caspase-7-GFP fusion constructs (C7-22W, -47W and -51W) were generated from wild-type caspase-7 gene (Fig. 1). C7-47W fusion protein was localized relatively in nucleus, whereas C7-51W protein was distributed diffusely in transfected cells (Fig. 3). This observation supported the idea that the basic tetrapeptide act as a nuclear localization signal, because C7-47W protein contained this tetrapeptide, but C7-51W protein didn't. Diffuse distribution or, in some cases, nuclear localization of GFP fusion protein was observed in cells transfected with C7-22W construct lacking the N-terminal 2/3 of the prodomain (Fig. 3). This result showed that deletion of the N-terminal 21 amino acid residues was not enough to completely relieve the inhibition of nuclear localization by the prodomain, and that the residues 22-31 contributed to the inhibitory activity, while this region (22-31) suppressed the cell death-inducing activity.

The enforced expression of C7-32W, -47W or –51W fusion protein induced the cell death, whereas the transfection of C7-1W or -22W construct displayed a similar degree of cytotoxic

activity compared with the overexpression of GFP protein alone (Fig. 2). This observation is consistent with the report that the overexpression of full-length human caspase-7 fails to induce apoptosis, but the expression of a truncated derivative of caspase-7, which lacks N-terminal 53 amino acids including a prodomain and a part of a large subunit (30 amino acids), caused cell death (11). These data altogether show that the prodomain of caspase-7 inhibits not only its cell killing activity but also the function of a nuclear localization signal in the large subunit.

Intracellular distribution of N-terminal truncated inactive caspase-7-GFP fusion proteins

The observation that cell death was induced by the enforced expression of truncated wild-type caspase-7-GFP fusion proteins (C7-32W, -47W and -51W) supports the idea that GFP fusion at the C-terminus did not affect enzyme activity of caspase-7. However, it points out a possibility that the distribution of GFP fusion proteins did not reflect the physiological cellular localization, since surviving cells represented a minor population. To eliminate the influence of cell death on the distribution of GFP fusion proteins, N-terminal truncated caspase-7-GFP fusion constructs were generated using a mutant caspase gene whose active site pentapeptide was changed from QACRG to QAARG by site-directed mutagenesis. Inasmuch as high viability was observed in transfected cells overexpressing truncated mutant caspase-7-GFP fusion proteins (Fig. 2), these proteases appeared to be inactive. C7-32M and -47M fusion proteins containing the basic tetrapeptide were localized mainly in the nucleus, whereas C7-51M fusion protein lacking only the tetrapeptide in comparison with C7-47M fusion protein was excluded from the nucleus and distributed in the cytoplasm, suggesting that the basic tetrapeptide is a functional nuclear localization signal. The full-length mutant caspase-7 fusion protein (C7-1M) showed the diffused cellular localization (Fig. 3). C7-22M fusion protein lacking the N-terminal 2/3 of the prodomain was localized in the nucleus in some cells like C7-22W protein. The obvious differences of the distribution between active and inactive N-terminal truncated caspase-7-GFP fusion proteins were the strong nuclear localization of C7-47M protein and nuclear exclusion of C7-51M protein, maybe due to the high viability of the transfected cells. The results of the transfection experiment using mutant caspase-7-GFP fusion proteins unambiguously corroborated that the prodomain of caspase-7 inhibited the nuclear localization by the basic tetrapeptide in a large subunit, and that even the

C-terminal 10 amino acid sequence in the prodomain (C7-22W and C7-22M) has a partial activity to hinder caspase-7 from localizing in the nucleus.

Intracellular distribution of inactive caspase-7-GFP fusion proteins shown by immunoblotting analysis

To ensure an inhibition of the nuclear localization by the prodomain, N-terminal truncated mutant caspase-7-GFP fusion proteins were analyzed by subcellular fractionation and immunoblotting using an anti-GFP antibody (Fig. 4). Unprocessed caspase-7-GFP fusion protein was observed in both cytoplasmic and nuclear fractions of cells transfected with C7-32M (61 kDa) or -47M (60 kDa) expression construct, but localized mainly in the cytoplasm of cells transfected with C7-51M (59 kDa) construct. These results were consistent with the fluorescence microscopic analysis (Fig. 3). A band of 60 kDa present in the cytoplasmic fraction of cells transfected with C7-M1 expression construct might be a degradation product during the preparation. A small amount of GFP-tagged caspase-7 proteins seemed to be cleaved to 40 and 41 kDa polypeptides that might correspond to fusion proteins of a small subunit and GFP. The existence of these processed forms was unexpected, since mutant caspase-7 was supposed to be inactive by a substitution of an active site from QA<u>C</u>RG to QA<u>A</u>RG. However, the processed fragments were also detected in COS cells transfected with GFP-tagged mutant inactive caspase-2 expression construct (5). The endogenous proteinase might be partially activated by the overproduction of an artificial protein and cleave mutant caspase-GFP fusion proteins, which was supported by an observation that the enforced expression of GFP or mutant caspase-GFP fusion proteins led to 20 % cell death (Fig. 2). Another possibility is that mutant caspase-7-GFP fusion protein was degraded during the subcellular fractionation.

The effect of caspase-7 prodomain on the nuclear translocation of GFP induced by the nuclear localization signal of caspase-7

To investigate whether the caspase-7 prodomain inhibits the nuclear transport which is conferred to a heterologous protein by the nuclear localization signal of caspase-7, DNA fragments encoding the first 50 amino acids (the prodomain and nuclear localization signal) and the residues

32-50 (the nuclear localization signal) of caspase-7 were inserted at the amino terminus of GFP gene in-frame to generate expression constructs, $C7_{1-50}$ and $C7_{32-50}$, respectively. The $C7_{1-50}$ fusion protein showed diffused cellular localization similar to that of GFP (Fig. 5). However, deletion of the prodomain resulted in the nuclear transport of GFP fusion protein ($C7_{32-50}$), which indicates that an attachment of the prodomain (31 amino acid residues) reduces the function of the nuclear localization signal (19 amino acid residues) of caspase-7.

Discussion

The data presented in this study demonstrate that the prodomain of caspase-7 suppressed the nuclear localization and the cytotoxic activity. However, the nuclear transport of caspase-7 is unnecessary to the death-inducing activity, because a high rate of cell death was also observed in cells overexpressing C7-51W fusion protein that lacked the basic tetrapeptide and distributed diffusely. The different mechanisms mediate the inhibition of the nuclear localization and the repression of the cytotoxic activity by the prodomain.

During apoptosis, several proteins are reported to translocate into the nucleus. Apoptosis-inducing factor loses the mitochondrial localization sequence, when imported into mitochondria in healthy cells, and translocates to the cytosol and the nucleus after induction of apoptosis (12). Granzyme B secreted by cytotoxic T cells and natural killer cells is taken up by target cells and is translocated into the nucleus in a perforin-dependent manner (13). Pro-caspase-1 migrates to the nucleus in a prodomain-dependent manner after death stimuli and is processed to release the prodomain that has a nuclear localization signal (4), while procaspase-2 is localized to the nucleus by its prodomain even in nonapoptotic cells (5). In the case of caspase-7, the removal of the prodomain induces both the nuclear import of the catalytic protease and the cell killing activity. It is interesting that different apoptosis-related proteins accumulate in the nucleus through different molecular mechanisms.

The presented data clearly show that caspase-7 prodomain (31 amino acid residues) reduces the function of the nuclear localization signal (19 amino acid residues) attached to a heterologous protein, indicating that this 31 amino acid sequence is enough to inhibit the nuclear transport. The prodomains of mammalian and *Xenopus* caspase-7 contain many acidic amino acids (35-48 %). It is tempting to speculate that negative charges in the prodomain neutralize positive charges in the nuclear localization signal, leading to the inhibition of the nuclear transport.

Some proteins are known to move into the nucleus following to the cleavage. Sterol regulatory element-binding protein (14), Ire1 (15) and Notch (16) are transmembrane proteins that reside on the nuclear envelope, endoplasmic reticulum, or plasma membrane, are proteolytically processed after a proper signal, and release a cytoplasmic domain into the nucleus to regulate the

gene expression. Interleukin-1α precursor is cleaved by calpain into two components. The C-terminal fragment is secreted and binds to the plasma membrane-localized interleukin-1 receptor to act as a classical cytokine, whereas the overexpressed N-terminal propiece is concentrated within the nucleus by its own nuclear localization signal to induce malignant transformation (17). The molecular mechanism of nuclear accumulation of processed caspase-7 is similar to that of interleukin-1α. In both cases, the activity of the nuclear localization signal is latent in the intact precursor and proteolytic processing results in the development of nuclear localizing activity.

A putative nuclear export signal (LXXXFXXLXF), that contains four hydrophobic residues present at a typical and characteristic spacing (18), is conserved among human (11, 19), mouse (20), rat (DDBJ/EMBL/GenBank accession number AF072124-1), golden hamster (21) (residues 97-106), and *Xenopus* caspase-7 (8) (residues 108-117). A similar sequence is also observed in the corresponding regions of caspase-6 (22), -9 (23, 24), and -10 (25) in human and *Xenopus* (8). This signal might explain why C7-51M fusion protein that did not have the basic tetrapeptide was excluded from the nucleus and located in the cytoplasm. It is plausible that pro-caspase-7 which contains both the nuclear localization and nuclear export signals distributes mainly in the cytoplasm by masking the nuclear localization sequence via the prodomain and excluding pro-caspase-7 from the nucleus, and that the removal of the prodomain restores the function of the nuclear localization signal to a maximal activity, leading to the nuclear accumulation of the processed caspase-7.

LEGENDS TO FIGURES

Fig. 1. The comparison of the N-terminal sequences of pro-caspase-7 in different species and the N-terminal truncated caspase-7-GFP fusion proteins used in this study. The N-terminal amino acid sequences of human (11, 19), mouse (20), rat (DDBJ/EMBL/GenBank accession number AF072124-1), and golden hamster caspase-7 (21) are aligned with that of *Xenopus* caspase-7 (8). Amino acids identical to *Xenopus* caspase-7 sequence are shown by dots, basic tetrapeptides are boxed, and Asp cleavage sites are indicated by a vertical arrow. Dashes mean gaps. It is worth noting that acidic amino acids, aspartic and glutamic acids, are abundant in prodomains of mammalian and *Xenopus* caspase-7 (35-48 %).

Fig. 2. The cell death-inducing activity by the overexpression of the N-terminal truncated caspase-7-GFP fusion protein in XLT-15-11 cultured cells. XLT-15-11 cells were transfected with an expression construct encoding GFP , C7-1W, C7-22W, C7-32W, C7-47W, C7-51W, C7-1M, C7-22M, C7-32M, C7-47M, or C7-51M fusion protein, and two days later, the percentages of apoptotic round cells were determined.

Fig. 3. A fluorescence microscopic study showing the localization of various GFP fusion proteins in transiently transfected XLT-15-11 cells. XLT-15-11 cells were transfected with an expression construct, C7-1W, C7-1M, C7-22W, C7-22M, C7-32W, C7-32M, C7-47W, C7-47M, C7-51W, or C7-51M, and two days later, photographed. Apoptotic cells expressing GFP fusion protein are indicated by arrow heads. Bar, 20 μm.

Fig. 4. An immunoblotting study showing the localization of various GFP fusion proteins in transiently transfected XLT-15-11 cells. XLT-15-11 cells were transfected with an expression construct encoding GFP, C7-1M, C7-22M, C7-32M, C7-47M, or C7-51M fusion protein. Three days later, cytoplasmic (C) and nuclear (N) extracts from transfected cells were prepared and analyzed by immunoblotting using an anti-GFP antibody.

Fig. 5. A fluorescence microscopic study showing that caspase-7 prodomain inhibits the nuclear transport of a GFP fusion protein containing the nuclear localization signal of caspase-7. XLT-15-11 cells were transfected with an expression construct, pEGFP-N2, $C7_{1\text{-}50}$ or $C7_{32\text{-}50}$, and one day later, photographed. Bar, 20 μm.

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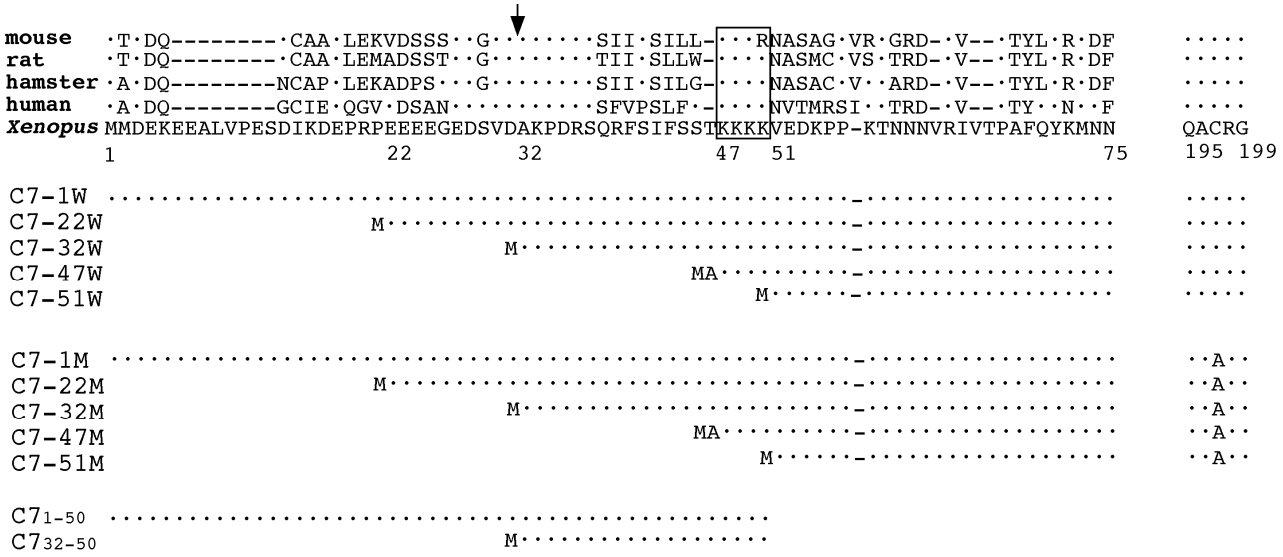
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human ·A·DQ--------GCIE·QGV·DSAN·····SFVPSLF·-····NVTMRSI··TRD--V--·TY··N··F
Xenopus MMDEKEEALVPESDIKDEPRPEEEEGEDSVDAKPDRSQRFSIFSSTKKKKVEDKPP-KTNNNVRIVTPAFQYKMNN QACRG
1 22 32 47 51 75 195 199
C7-1W
C7-22W M
C7-32W M
C7-47W MA
C7-51W M
C7-1M
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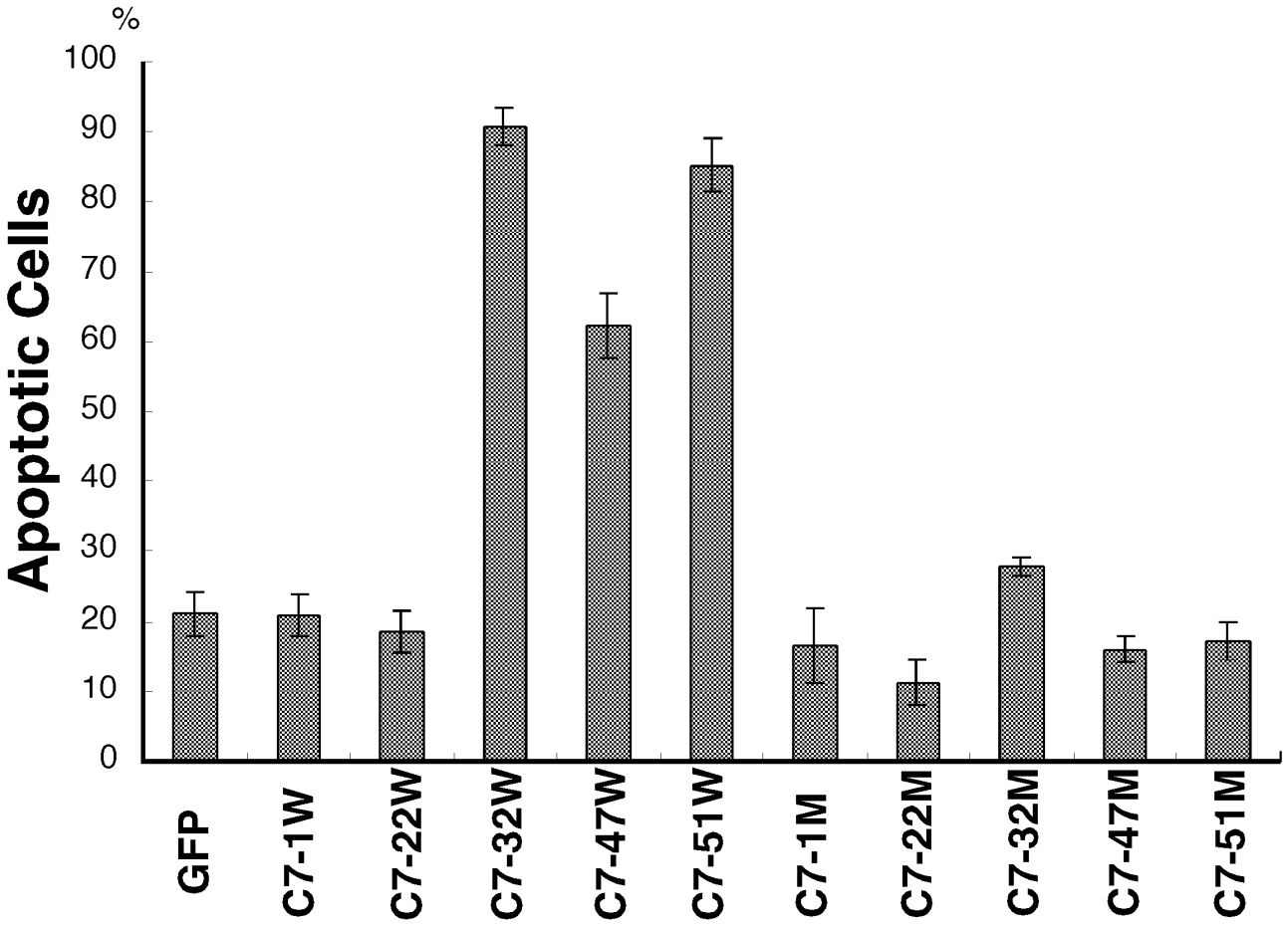
C7 1-50
C7 32-50 M

Fig. 1.


%
Apoptotic Cells
100
90
80
70
60
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40
30
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GFP
C7-1W
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C7-32W
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C7-51M

Fig. 2.

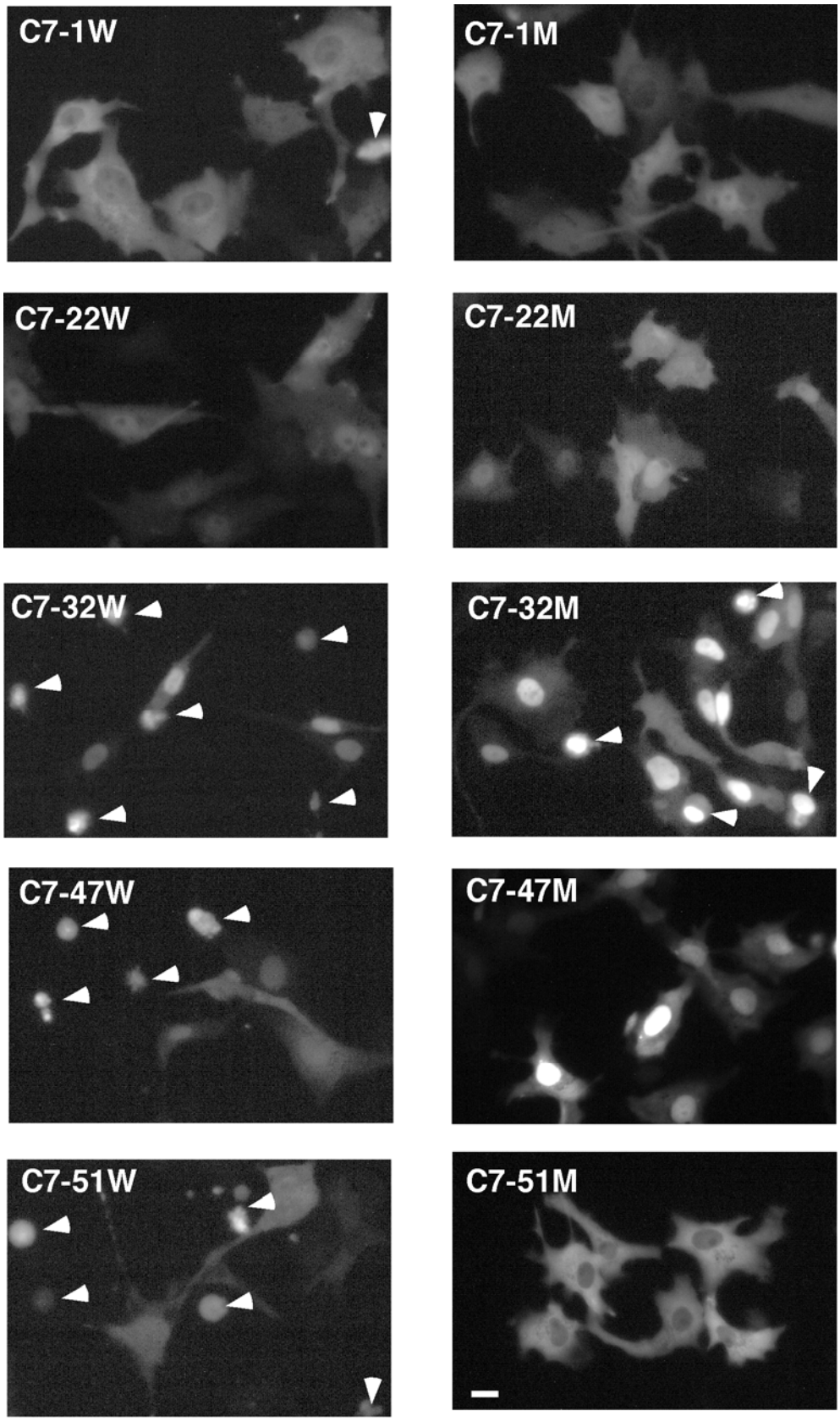

Fig. 3.

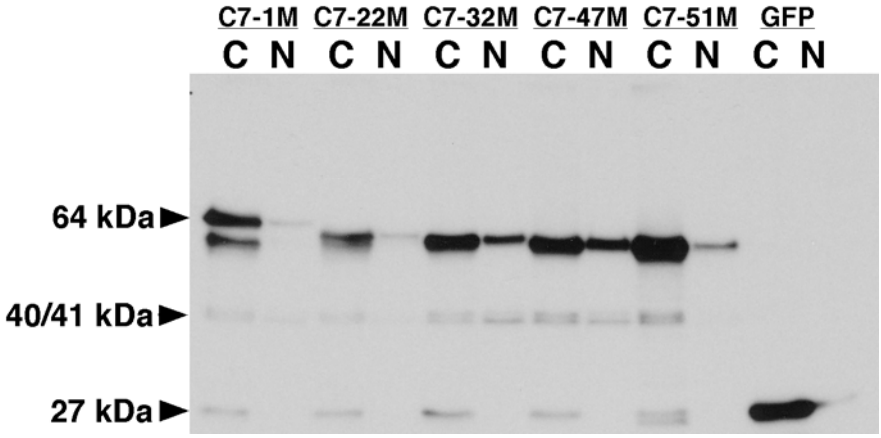
C7-1M C7-22M C7-32M C7-47M C7-51M GFP
C N C N C N C N C N C N
64 kDa
40/41 kDa
27 kDa

Fig. 4.

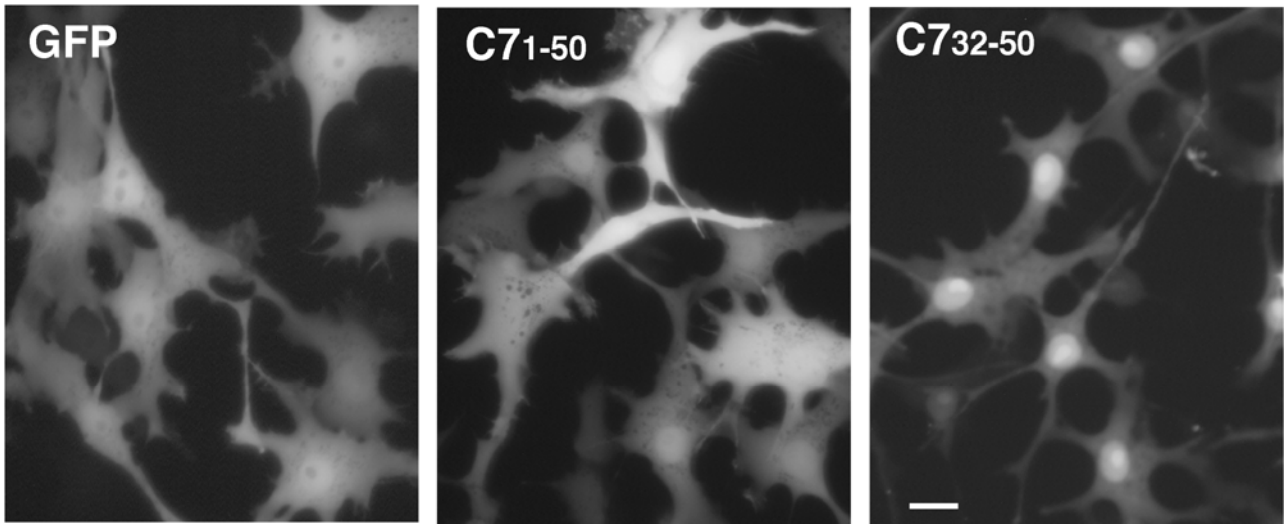
GFP
C71-50
C732-50

Fig. 5.