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Inhibition of Alzheimer's amyloid-beta peptide-induced reduction of mitochondrial membrane potential and neurotoxicity by gelsolin

Hongjiang Qiao, Richard C. Koya, Koji Nakagawa, Hiroki Tanaka, Hisakazu Fujita, Masato Takimoto, Noboru Kuzumaki*

The Division of Cancer Gene Regulation, Research Section of Disease Control, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-Ku, Sapporo 060-0815, Japan

*Corresponding author: Tel: +81-11-706-5020, Fax: +81-11-706-7869, E-mail address: kuzumaki@igm.hokudai.ac.jp (N. Kuzumaki)

Abstract

Amyloid-beta (Abeta) peptides play a central role in the development of Alzheimer's disease. They are known to induce mitochondrial dysfunction and caspase activation, resulting in apoptosis of neuronal cells. Here we show that human cytoplasmic gelsolin inhibits Abeta peptide-induced cell death of neuronally differentiated rat pheochromocytoma (PC-12) cells. We also show that the segment 5 but not 6 of human cytoplasmic gelsolin is the important region responsible for inhibition of Abeta-induced cytotoxicity. Mitochondrial dysfunction associated with cell death, membrane potential loss and the release of cytochrome c are all abrogated in the presence of human full-length or segment 5 cytoplasmic gelsolin. Furthermore, RNA interference to reduce expression of endogenous gelsolin in PC12 cells shows that rat gelsolin act as an inhibitor of Abeta cytotoxicity. These results demonstrate that cytoplasmic gelsolin plays a important role in inhibiting Abeta-induced cytotoxicity by inhibiting apoptotic mitochondrial changes. The segment 5 of human cytoplasmic gelsolin is sufficient for the function.

Keywords: gelsolin, Alzheimer disease, amyloid-beta, rat pheochromocytoma, mitochondrial membrane potential, cytotoxicity, cytochrome c

1. Introduction

Characteristic events associated with Alzheimer's disease (AD) include progressive neuronal dysfunction and loss of neurons in specific regions (hippocampus, amygdala and others) of the brain [5]. The beta-amyloid peptide (Abeta) is a product formed within cells through the proteolytic processing of the amyloid precursor protein (APP). APP is present in soluble form in plasma and cerebrospinal fluid (CSF), and is constitutively expressed in many cells of normal individuals [1,19]. Delineating the cascade of antecedent events leading to the final

common path of neurodegeneration in AD can lead to the implementation of an effective therapeutic strategy. Neuronal cell death is associated with the accumulation of amyloid plaques formed by aggregates of fibrillar Abeta peptides [1,19]. In its soluble form, Abeta is known to bind several circulatory proteins. These include apolipoprotein (apo) E, apo J and transthyretin [22,28]. The binding of these proteins to Abeta may either prevent its fibrillization or, alternatively, promote its polymerization [11,22,28]. Neuronal cell death occurs through a mitochondrial-dependent pathway. This involves opening of the permeability transition pore, cytochrome c release, and increasing caspase activity and DNA damage [3,18,27].

Gelsolin, an actin-regulatory protein that modulates actin assembly and disassembly, is found as both an intrinsic cytoplasmic protein and as a secreted plasma protein [15,16]. Immunocytochemical studies using anti-gelsolin antibody revealed that the protein is localized in filopodia and the body part of growth cones of neuronally differentiated rat pheochromocytoma (PC12) cells treated with nerve growth factor (NGF) [31]. Gelsolin was also found in rat dorsal root ganglion neurons. Gelsolin-null neurons display enhanced cell death and a rapid, sustained elevation of Ca²⁺ levels following glucose/oxygen deprivation [7]. These cells also showed augmented cytosolic Ca²⁺ levels in nerve terminals following depolarization *in vitro*. Plasma gelsolin was found to bind Abeta, inhibiting the fibrillization of Abeta, and to defibrillize preformed fibrils of the peptides [4, 24]. We have previously shown that cytoplasmic gelsolin is also present in the mitochondrial fraction of cells, and that full-length gelsolin can inhibit apoptosis of human Jurkat T cells [13,14,20]. The overexpression of gelsolin can inhibit the loss of mitochondrial membrane potential and cytochrome c release from mitochondria, resulting in the lack of activation of caspase-3, -8, and -9 in Jurkat cells treated with staurosporine, thapsigargin, and protoporphyrin IX [13]. In this study, our efforts were directed in establishing whether cytoplasmic gelsolin can inhibit Abeta-induced cytotoxicity in rat neuronal PC12 cells.

2. Materials and methods

2.1. Plasmids, Cells and Transfection

The LKCG expression plasmid containing human cytoplasmic gelsolin was a kind gift from D. Kwiatkowski (Harvard Medical School). Gelsolin segment 5 (amino acid sequence 516-618), segment 5-6 (amino acid sequence 516-731) and segment 6 (amino acid sequence 640-731) [15,16,29] were obtained by PCR amplification with LKCG as a template. Each PCR product was subcloned into the LK444 expression plasmid (a kind gift from P. Gunning) [13, 20] after insertion of a FLAG tag sequence at the 5' termini or into EcoRI-XhoI site of pCMV-FLAG expression plasmid which consists of pcDNA3.1(+) (Invitrogen, Carlsbad, CA) and 3xFLAG tag.

The rat pheochromocytoma cell line (PC-12) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated horse serum (Gibco BRL, Gaithersburg, MD) and 5% fetal calf serum (FCS) (Gibco BRL) at 37° C in a 5% CO₂ humidified atmosphere. After reaching 50-60% cell confluence, PC-12 cells were induced to undergo neuronal differentiation by NGF treatment (100 ng/ml)

for 5 days, followed by maintenance with a low dose of NGF (1 ng/ml). For cell death assays, the concentration of FCS in the media was decreased to 3% in the absence of horse serum. Stable clones overexpressing gelsolin (full length, gelsolin segments 5, 5-6 or 6) and control neo clones were obtained by lipofection using Lipofectamin (Gibco BRL) and selection with neomycin (G418, 0.8 mg/ml). The production of gelsolin was monitored by Western blot analysis as previously described [13]. The anti-mouse gelsolin antibody which reacts with rat gelsolin [8] and the anti-human gelsolin antibody (2C4, Sigma) were used for detection of endogenous rat gelsolin and ectopic human gelsolin, respectively. The latter antibody does not react with rat gelsolin of PC12 cells. To detect production of segments 5, 5-6 or 6 in the clones (GS5: 2, 8, 12, GS56: 1, 2, 3, GS6: 1, 7, 12), anti-FLAG M2 monoclonal antibody (Sigma) was used. The prestained SDS-PAGE standards (Board Range, #161-0318) was used for molecular weight markers (BIO-RAD, Hercules, CA).

2.2. Assays for Cytotoxicity and Mitochondrial functions

Amyloid-beta (A β) (1-40). HCl (AnaSpec, San Jose, CA)(1 mg) was dissolved in distilled water, sonicated and made up as a 200 microM stock solution. The solution was allowed to age at room temperature for 7 days [17]. Cells were treated with various doses (0.5 to 10 microM) of the aggregated A β (1-40).

Cell viability and death were assessed using propidium iodide (PI) and Hoechst 33342 (Sigma, St. Louis, MO) staining and counting the PI stained non-viable cells and the Hoechst dye monitored chromatin condensation and nuclear fragmentation under an inverted fluorescence microscope (Olympus, IX-70, Tokyo, Japan). Five hundreds cells were counted per well and the experiments were repeated at least three times.

Mitochondrial membrane potential was assayed by the addition of Rhodamine 123 to the culture medium. Following a 10 min. incubation, PC-12 cells were washed with PBS and then subjected to flow cytometric analysis (FACScalibur, Becton Dickinson, San Jose, CA) as previously described [13].

Cytochrome C release from mitochondria into the cytosol of PC-12 cells was evaluated by SDS-polyacrylamide gel electrophoresis followed by immunoblotting of the cytosolic fraction as previously described [13]. All steps were carried out at 4° C. Cells (1×10^7 cells) were washed with PBS and suspended in an isosmotic buffer (0.3 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). After 5 min of incubation, cells were Dounce homogenized using a type B (loose) pestle and centrifuged at 1,000 g for 10 min to separate nuclei and unbroken cells. Then the supernatant was centrifuged at 8,000 g for 10 min to pellet heavy membranes (mitochondrial fraction). The pellet was washed five times with the isosmotic buffer to eliminate contamination by other subcellular fractions. The supernatant from the 8,000 g spin fraction was further centrifuged at 100,000 g to produce a supernatant corresponding to the cytosolic fraction (S100). Aliquots from each subcellular fraction in the same proportion to the initial number of cells harvested were loaded for SDS-polyacrylamide gel electrophoresis and immunoblotting.

2.3. RNA interference

We designed siRNA to interfere with gelsolin expression essentially as described in the technical information from Ambion (Austin, TX, USA). A set of 19-mer oligonucleotides (CGGTGACTGCTTCATTCTG) corresponding to nucleotides 640 downstream of the transcription start site was selected from the rat gelsolin open reading frame (ORF) sequence [33]. We confirmed that the selected oligonucleotide set did not have homology to any other genes by a BLAST search, so that they would not interfere with other genes. The oligonucleotides were synthesized and column-purified at Hokkaido System Science Co., Ltd. (Sapporo, Japan). The 19-mer sense siRNA sequence and antisense siRNA sequence were linked with a nine nucleotide spacer (TTCAAGAGA) loop. Six T bases and 6 A bases were added as a termination signal to the 3' end of the forward oligonucleotides and 5' end of the reverse oligonucleotides, respectively. Then five nucleotides corresponding to the BamHI (GATCC) and HindIII (AGCTT) restriction sites were added to the 5' end of the forward oligonucleotides and 3' end of the reverse oligonucleotides, respectively. Forward and reverse oligonucleotides were incubated in annealing buffer (100 mM K-acetate, 30 mM HEPESKOH (pH 7.4) and 2 mM Mg-acetate) for 3 min at 90° C, followed by incubation for 1 h at 37° C. The annealed DNA for siRNA was ligated with linearized pSilencer 3.1-H1 hygro siRNA expression vector (Ambion) at BamHI and HindIII sites and a gelsolin siRNA vector (pS-RGSN1) was constructed. A non-related control siRNA vector (pS-GFP), which targeted GFP DNA sequence 5'-GGTTATGTACAGGAACGCA-3' with no significant match in the complete human genome was also prepared under similar conditions.

PC12 cells were freshly passaged overnight at a concentration of 5×10^4 /ml to 1×10^5 /ml in 6-well plates under standard conditions. Fifteen minutes prior to transfections, medium was changed to quiescent medium (no FCS). Transfections were carried out using SuperFect transfection reagent (Qiagen, Tokyo) according to the manufacturer's instructions. Highest efficiency of transfection was reached using 10 microliter of SuperFect transfection reagent and 2 microgram of DNA per 6 well (2-ml medium). Four hour after transfection, 10% FCS was added. The reduction of gelsolin expression by siRNA was analyzed by immunoblotting using anti-mouse gelsolin antibody which reacts with rat gelsolin [8]. Experiments were repeated at least twice and performed for each set in duplicate or triplicate.

2.4. Statistical Analysis

The data shown were the mean value of at least three different experiments and expressed as mean \pm SE. Student's *t* test or ANOVA followed by a post hoc Scheffe *F*-test (StatView, Berkeley, CA) were used to compare the data. A *p* value of less than 0.05 was considered as statistically significant.

3. Results

In an effort to study the effect of cytoplasmic gelsolin on cell death induced by Amyloid-beta (A β) peptides, we transfected an expression vector encoding the cytoplasmic form of human gelsolin (HGSN) into rat pheochromocytoma (PC-12)

cells. Stable clones overexpressing human full-length gelsolin (GF-1, GF-2) or control neo genes (N) were obtained by selection in medium containing neomycin (Fig.1a). Expression of endogenous rat gelsolin (RGSN) showed no significant change among all cell lines (GF-1, GF-2, parental PC-12 wild-type (W) and N). These cells differentiated into neuronal-like cells five days after addition of NGF in the medium. These cells were then treated with different concentrations of an Abeta peptide (a fragment representing amino acids 1-40) to induce cell death. At 48 hours after treatment, cells were assayed for viability and cell death using PI and Hoechst 33342 staining. Human full-length gelsolin overexpressing cells (GF-1 and GF-2) were significantly more resistant to cytotoxicity induced by Abeta compared to control cells (W and N) (Fig.1b). Abeta-induced cell death in control N transfectants or parental W cells was not accompanied by changes in nuclear morphology such as nuclear condensation or fragmentation. This is in agreement with findings of previous reports [2, 30].

We have previously shown that the carboxy-terminal segment of gelsolin represents an important regulatory region in determining its inhibitory effect on apoptosis [14]. In the present study, our efforts were directed towards investigating the importance of the carboxy-terminal segments of gelsolin in Abeta-induced cell death. PC12 cell clones stably expressing segment 5 (GS5-2, GS5-8, GS5-12), segment 5-6 (GS56-1, GS56-2, GS56-3) or segment 6 (GS6-1, GS6-7, GS6-12) (Fig. 2a) were obtained. Following NGF-induced differentiation, Abeta (1-40) (10 microM) was added to induce cell death, and cell viability was tested. Segment 5 gelsolin inhibited Abeta cytotoxicity in all three clones as full-length gelsolin did in GF-1 clone in comparison with W and N control cells (Fig. 2b). On the other hand, segment 6 gelsolin did not inhibit Abeta cytotoxicity. Segment 5-6 resulted in inhibition but weaker than that observed with the full-length and segment 5 gelsolin proteins.

We demonstrated that gelsolin can inhibit apoptosis by blocking signal transduction at the mitochondrial level upstream of the caspase cascade in human T lymphocytes [13, 19]. To analyze the alteration in mitochondrial membrane potential that follows Abeta apoptotic stimulation, we incubated cells treated with 10 microM Abeta with the cationic dye Rhodamine 123, and subsequently analyzed the cells using a flow-cytometer. Unlike the W and N control cells, human gelsolin (full-length or segment 5) overexpressing clones (GF-1, 2 or GS5-2, 8, 12) did not display any loss in mitochondrial potential, while GS6 clones (GS6-1, 7, 12) showed the loss (Fig. 3a). GS5-6 clones (GS56-1, 2, 3) demonstrated weaker inhibitory activity. Of note, gelsolin did not affect mitochondrial potential in cells not challenged with insult (data not shown). Another change observed in the mitochondria of dying cells is the translocation of cytochrome c from its intermembrane location to the cytosol. Immunoblot analysis of cytosolic fractions revealed that, in contrast to the W and N control cells, there was almost no cytochrome c release in GF-1, 2 and GS5-2, 8, 12 clones, while the release was shown in GS6-1, 7, 12 clones. GS56-1, 2, 3 clones showed partial cytochrome c release (Fig. 3b).

Then, in order to assess the role of the endogenous gelsolin in the neurocytotoxicity protection, we used RNA interference to reduce the expression of gelsolin. PC12 cells were transfected with a construct expressing gelsolin-interfering RNAs (pSRGSN1) or control RNA (pSGFP), subcloned, and proteins were harvested

and analyzed by Western blot experiments. The results, shown in Fig. 4a, demonstrate that gelsolin protein levels of 3 S-RGSN1-1, -2, -3 clones were significantly reduced by nearly 75% without detectable change in actin expression by RNA interference using pSRGSN1, in comparison with W and N control cells while a SGFP clone did not lead to significant inhibition of gelsolin expression. These cells were then treated with 10 microM Abeta peptide (1-40) to induce cell death. At 48 hours after treatment, cells were assayed for viability and cell death using PI and Hoechst 33342 staining (Fig. 4b). All three gelsolin reducing S-RGSN-1, -2, -3 clones were significantly more sensitive to cytotoxicity induced by Abeta compared to the W and N control cells and an S-GFP-1 clone. These studies clearly show that in PC12 cells, rat endogenous gelsolin act as an inhibitor for Abeta cytotoxicity as well.

Taken together, all these data indicate that cytoplasmic gelsolin can effectively inhibit Abeta-induced cell death at a point concomitant with, or upstream to, the mitochondrial events.

4. Discussion

Rational neuroprotective approaches have led to recent trials of estrogen, antioxidants and anti-inflammatory medications in AD, and to the development of anti-amyloid strategies for delaying the progression, or preventing the development, of AD [5, 32]. The presence of several intracellular Abeta-binding proteins suggests that these proteins may promote the polymerization or prevent the fibrillization of Abeta [21, 25]. Plasma gelsolin was found to bind Abeta, inhibit the fibrillization of Abeta, and defibrillize preformed fibrils of the peptides [4, 24]. We previously reported that the C-terminal half of human cytoplasmic gelsolin, like Bcl-xL, could prevent apoptotic mitochondrial changes such as mitochondrial membrane potential loss and cytochrome c release by binding and closing the voltage-dependent anion channel (VDAC) in isolated mitochondria [14]. This indicates that human gelsolin targets the mitochondria to prevent apoptosis via its C-terminal half. In this report, we showed that segment 5 is an important region for the anti-apoptotic property of gelsolin, and could sufficiently inhibit Abeta-induced cell death. Segment 6 is not associated with the inhibition, and segment 5-6 had weaker effects. Structural analysis of gelsolin segments 5-6 demonstrates that G5 does not bind to actin, while there are a few contacts between G6 and actin [26]. G5-6 binds a single calcium ion, but there is no binding by G5 [12]. The C-terminal phosphatidyl inositol-binding sites are located within residues 515–545 of G5 but not G6 [34]. Whether these differences between G5 and G6 segments explain the protective function on apoptosis remains to be investigated.

Abeta neurotoxicity was significantly prevented by repetitive treatments with TGF-beta 1 via upregulation of bcl-xL and bcl-2 gene expression [10, 23]. The protective effects of TGF-beta 1 were associated with a preservation of mitochondrial potential and function [23]. Bcl-2 protects against carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP)-induced apoptosis in NGF-differentiated PC12 cells by stabilizing the mitochondrial membrane potential [6]. Bcl-xL plays a regulatory role in controlling the membrane potential of mitochondria [10]. Tauroursodeoxycholate abrogated Abeta-induced cell death by preventing

mitochondrial membrane perturbation [27]. Furthermore, in this report, we observed that inhibition of gelsolin gene expression was associated with an increased apoptotic response in the presence of Aβ, indicating that the interference with gelsolin leads to a sensitization to proapoptotic stimuli. This observation is in line with experiments showing that gelsolin-null neurons have enhanced cell death [7] and vulnerability of cultured hippocampal neurons to glutamate toxicity was greater in cells lacking gelsolin [9].

In this study, we showed that cytoplasmic gelsolin effectively inhibit the cytotoxicity induced by the Aβ at a point in the mitochondrial pathway in PC12 cells. The segment 5 of human cytoplasmic gelsolin is sufficient for the function, suggesting this segment of human cytoplasmic gelsolin is one candidate for use in the anti-amyloid therapy of Alzheimer's disease.

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Figure legends

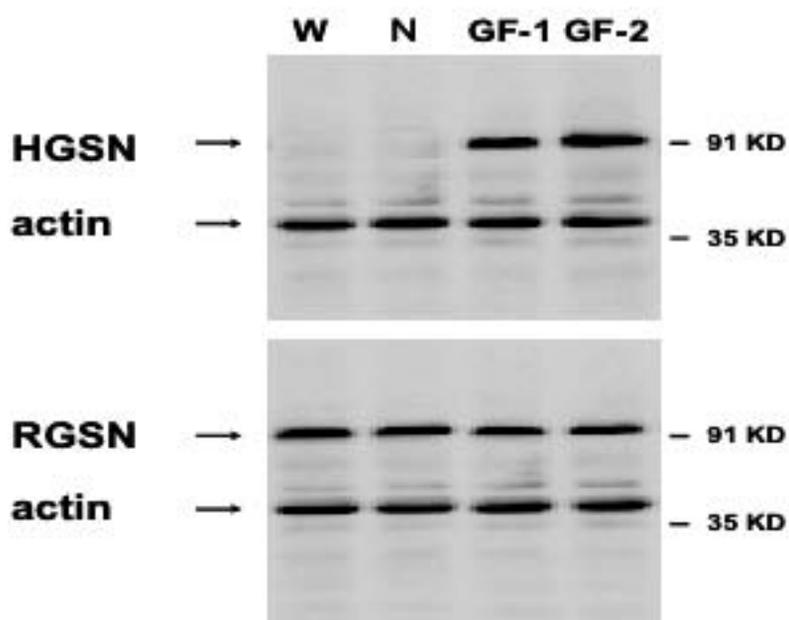
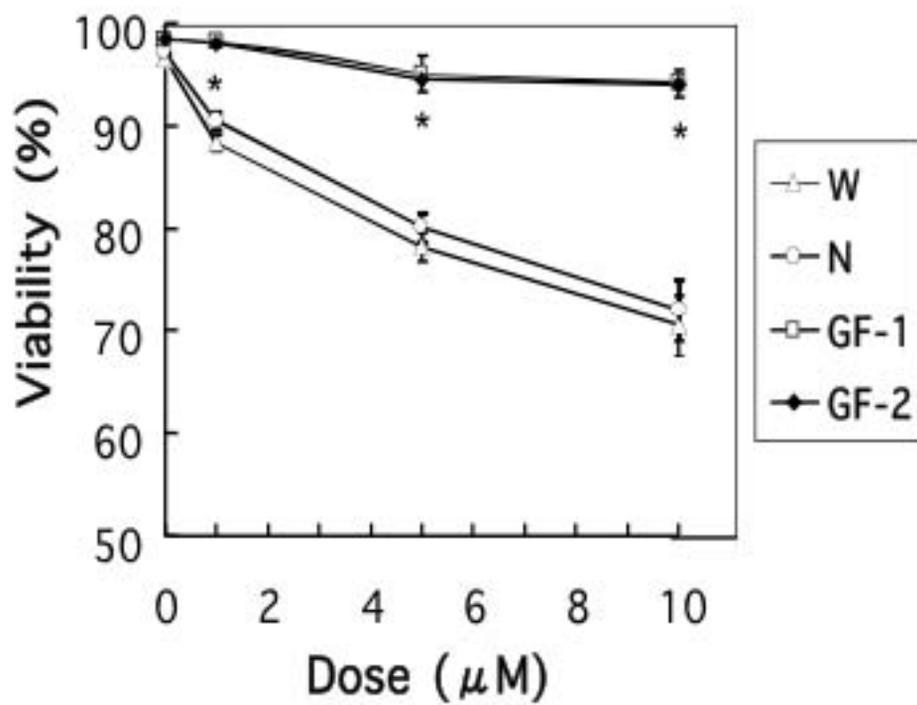
Fig. 1. Growth curve of PC12 cells treated by Abeta (1-40) in gelsolin-overexpressing PC-12 cells. a, Expression of endogenous rat gelsolin (RGSN) and ectopic human gelsolin (HGSN) as assessed by immunoblotting in wild-type PC-12 cells (W), a neo control clone (N) and two full-length gelsolin transfectants (GF-1 and GF-2). The molecular weight markers (35 KD: carbonic anhydrase, 91 KD: bovine serum albumin) are indicated beside the panel. b, Cell viability at 48 hours after treatment with various doses of Abeta (1-40) on W, N, GF-1, and GF-2 cells. Cell viability was calculated as the percentage of viable cells compared to total cells using propidium iodide and Hoechst 33342. Each data point of three experiments was measured in duplicate, and values represent the mean + S.E. An *asterisk* indicates a significant difference ($p < 0.05$) between gelsolin-overexpressing cells and control cells.

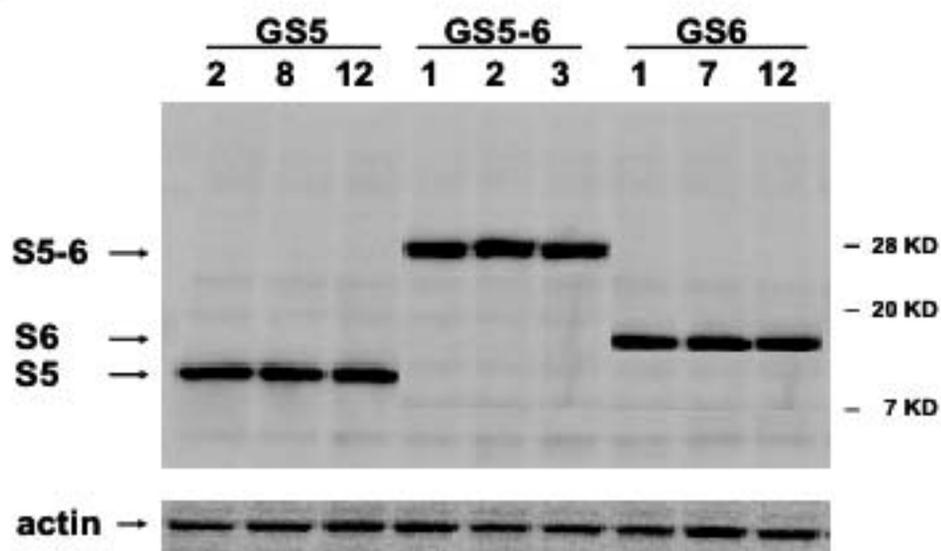
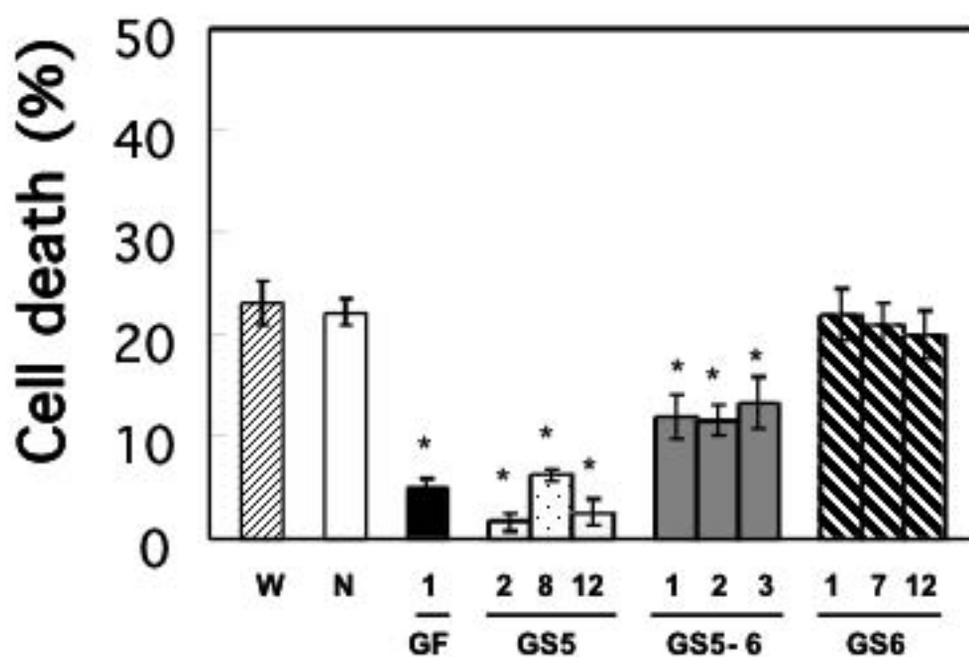
Fig. 2. Effect of gelsolin segments 5, 5-6 or 6 on cell death of PC12 induced by Abeta (1-40). a, Immunoblots using anti-FLAG antibody show the expression of gelsolin segments 5, 5-6 or 6 in the clones (GS5: 2, 8, 12, GS5-6: 1, 2, 3, GS6: 1, 7, 12). The molecular weight markers (7 KD: aprotinin, 20 KD: lysozyme, 28 KD: soybean trypsin inhibitor) are indicated beside the panel. b, Cell-death assay using propidium iodide and Hoechst 33342 after treatment with 10 micromM of Abeta (1-40)

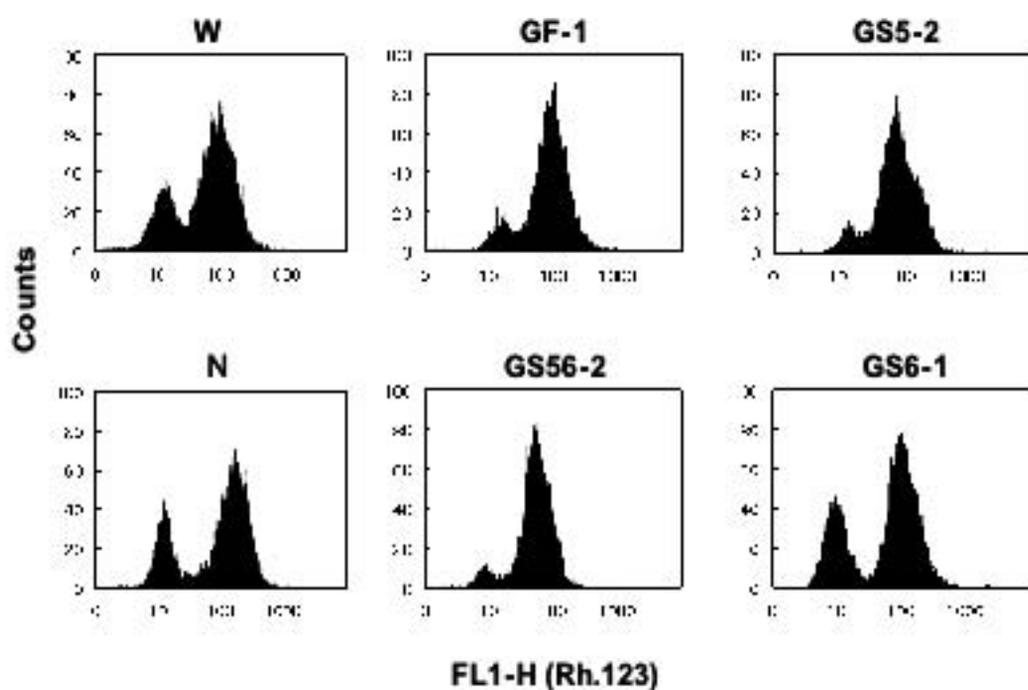
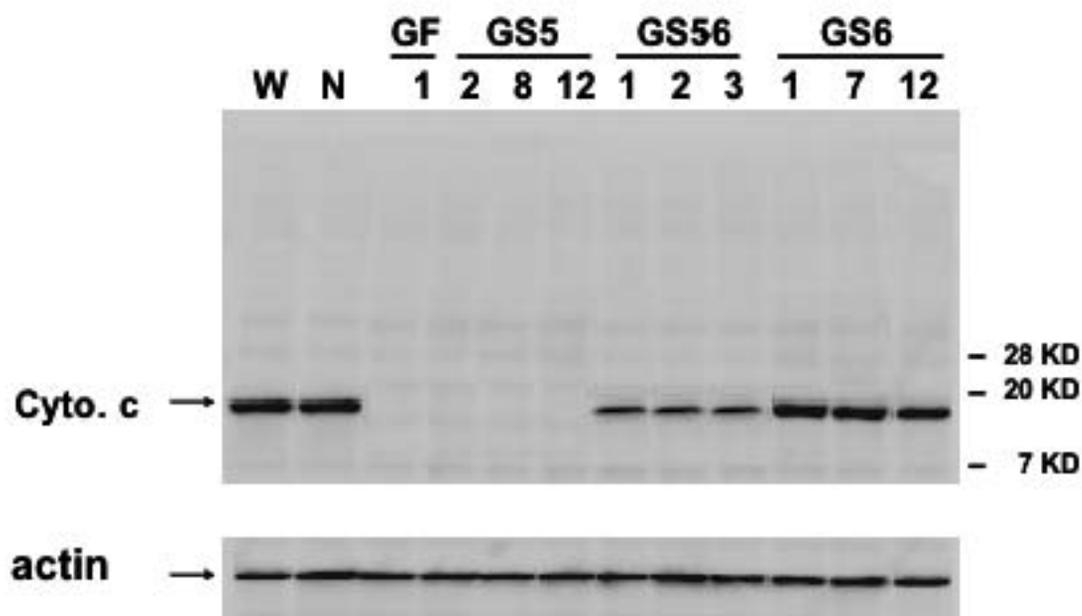
at 48 hours after induction. W: wild-type cells, N: neo control clone, GF1: full-length gelsolin-transfectant, GS5: segment 5 transfectants and GS5-6: segment 5-6 transfectants, GS6: segments 6 transfectants. Data are the mean values + S.E. of three experiments in duplicate. An *asterisk* indicates a significant difference ($p < 0.05$) between gelsolin-overexpressing cells and control cells.

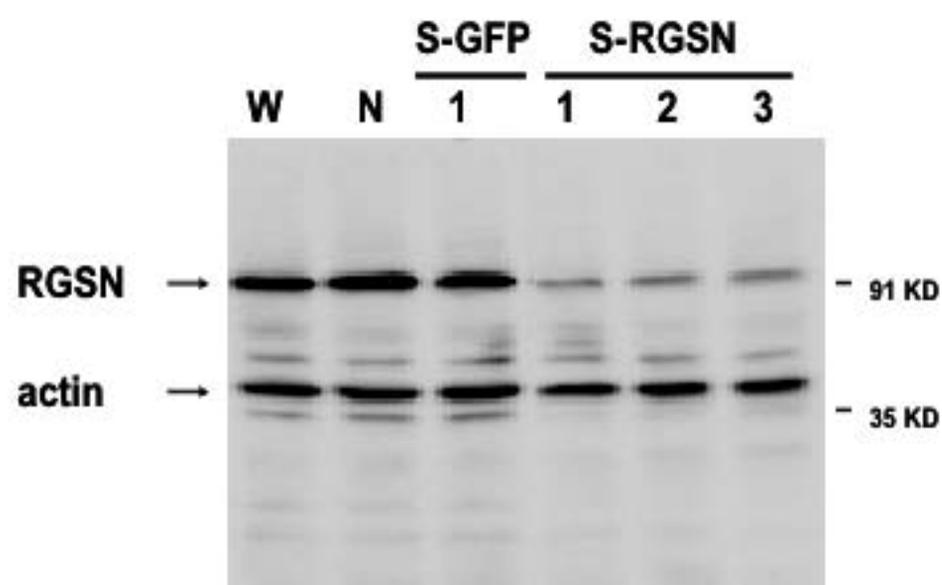
Fig. 3. Mitochondrial transmembrane potential and cytochrome c release from mitochondria induced by Abeta in gelsolin-overexpressing PC12 cells. a. Mitochondrial transmembrane potential assessed by flow-cytometry with Rhodamine 123. b. Cytochrome c release from mitochondria in gelsolin-overexpressing PC12 cells treated with Abeta. Cytochrome (Cyto.) c immunoblot of the cytosolic fraction from samples. W: wild-type cells, N: Neo control clone, GF-1: full-length gelsolin-overexpressing clone, GS5-2, GS56-2, GS6-1: gelsolin segment overexpressing clones, harvested at 48 hours after treatment with 10 microM of Abeta (1-40). The molecular weight markers (7 KD: aprotinin, 20 KD: lysozyme, 28 KD: soybean trypsin inhibitor) are indicated beside the panel.

Fig. 4. Vector-based siRNA targeting gelsolin. a. P12 cells were transfected with a rat gelsolin siRNA vector pS-RGSN1. After transfection, cells were harvested, and cell lysates were subjected to Western blotting using the antibodies as indicated on the left. W: wild-type cells, N: neo control clone, S-GFP-1:GFP-knockdown clone, S-RGSN:gelsolin-knockdown clones 1, 2, 3. The molecular weight markers (35 KD: carbonic anhydrase, 91 KD: bovine serum albumin) are indicated beside the panel. b. Cell viability at 48 hours after treatment with 10 microM Abeta (1-40) on W, N, S-GFP-1 or S-RGSN-1, -2, -3 cells. Each data point of three experiments was measured in duplicate, and values represent the mean + S.E. An *asterisk* indicates a significant difference ($p < 0.05$) between GFP- or gelsolin-knockdown cells and neo control cells.

a**b**

a**b**

a**b**

a**b**