FEBS Letters 580 (2006) 5275–5282

Huntingtin-interacting protein 1-mediated neuronal cell death occurs through intrinsic apoptotic pathways and mitochondrial alterations

Shin Ae Choi[a], Steven J. Kim[b], Kwang Chul Chung[a,*]

[a] *Department of Biology, College of Science, Yonsei University, Shinchon-dong 134, Seodaemun-gu, Seoul 120-749, Republic of Korea*
[b] *Department of Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA 90095, USA*

Received 24 April 2006; revised 30 August 2006; accepted 31 August 2006

Available online 11 September 2006

Edited by Berend Wieringa

Abstract Huntingtin interacting protein-1 (Hip1) is known to be associated with the N-terminal domain of huntingtin. Although Hip1 can induce apoptosis, the exact upstream signal transduction pathways have not been clarified yet. In the present study, we examined whether activation of intrinsic and/or extrinsic apoptotic pathways occurs during Hip1-mediated neuronal cell death. Overexpression of Hip1 induced cell death through caspase-3 activation in immortalized hippocampal neuroprogenitor cells. Interestingly, proteolytic processing of Hip1 into partial fragments was observed in response to Hip1 transfection and apoptosis-inducing drugs. Moreover, Hip1 was found to directly bind to and activate caspase-9. This promoted cytosolic release of cytochrome *c* and apoptosis-inducing factor via mitochondrial membrane perturbation. Furthermore, Hip1 could directly bind to Apaf-1, suggesting that the neurotoxic signals of Hip1 transmit through the intrinsic mitochondrial apoptotic pathways and the formation of apoptosome complex.
© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Huntington disease; Huntingtin; Hip1; Caspase; Mitochondria; Apoptosome

1. Introduction

Huntington disease (HD) is an autosomal dominant disease characterized by selective neuronal loss primarily in the striatum and cortex. HD results in the expansion of the polyglutamine tract in the huntingtin (HT) protein [1–3]. Although HT was discovered 10 years ago, its function is still not fully understood [4]. However, many proteins that interact with HT have been identified [5,6]. These HT-interacting proteins provide valuable clues into elucidate the function of HT and the pathogenesis of HD.

Huntingtin-interacting protein 1 (Hip1) is one protein known to interact with HT. Hip1 interacts with the amino terminus of HT, weakly binds to the mutant HT, and is highly expressed in neurons [7]. Hip1 is an ortholog of the yeast protein Sla2p, which is important for the assembly and function of the cytoskeleton and endocytosis in *Saccharomyces cerevisiae* [7,8]. Several studies have demonstrated that Hip1 binds to clathrin, α-adaptin, and AP-2 [9,10]. Hip1 likely interacts with these proteins to form a protein complex that is involved in clathrin-mediated endocytosis [11]. Hip1 knockout mice show neurological deficits. Cultured neurons from Hip1 knockout mice have a defect in clathrin-mediated internalization of AMPA receptors [12].

DNA fragmentation has been observed in neurons in vulnerable regions of HD brains, suggesting that cell death in HD occurs through apoptosis [13]. This apoptotic cascade can be triggered through two major pathways: the receptor-mediated extrinsic pathway and the mitochondrial intrinsic pathway [14,15]. The receptor-mediated extrinsic pathway is activated by extracellular signals such as members of the tumor necrosis factor family. The mitochondrial intrinsic pathway can be activated by stress such as DNA damage, hypoxia, and loss of survival signals. Mitochondrial damage results in cytochrome *c* release and formation of the apoptosome, a multimeric protein complex containing Apaf-1, cytochrome *c*, and caspase-9 [16,17]. Once bound to the apoptosome, caspase-9 is activated, which triggers a cascade of effector caspase activation and proteolysis, leading to apoptotic cell death.

There are conflicting data concerning the influence of Hip1 on cellular survival (see review [18]). However, overexpression of Hip1 results in rapid caspase 3-dependent cell death in certain cells [19]. Upstream events leading to Hip1-induced neuronal cell death have not been clarified and the reported pro-apoptotic activity of Hip1 is independent of caspase-8, but is significantly inhibited by the anti-apoptotic protein Bcl-x_L[19]. This implicates the involvement of the intrinsic apoptotic pathway in Hip1-induced cell death. Our recent report that dual specificity protein kinase Dyrk1A selectively phosphorylates Hip-1 during neuronal differentiation of embryonic hippocampal neuroprogenitor (H19-7) cells, which results in the blockade of Hip-1-mediated neuronal cell death [20], also supports the pro-apoptotic role of Hip1 in neuronal cells. In the present study, we examined the signal transduction pathways leading to Hip1-induced neuronal cell death. Our study focuses on the functional relationship between Hip1 and intrinsic/extrinsic apoptotic pathways, and the alteration of mitochondrial apoptotic effectors. Overexpression of Hip1 induced cell death via the activation of intrinsic apoptotic pathways and

*Corresponding author. Fax: +82 2 312 5657.
E-mail address: kchung@yonsei.ac.kr (K.C. Chung).

Abbreviations: AIF, apoptosis-inducing factor; HD, Huntington disease; Hip1, huntingtin interacting protein 1; Hippi, Hip1-protein interactor; HT, huntingtin; PBS, phosphate-buffered saline

0014-5793/$32.00 © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2006.08.076

the formation of apoptosome complex, concomitantly with the generation of its digested fragment, in H19-7 cells.

2. Materials and methods

2.1. Cell culture and DNA transfection

All culture media and antibiotics were purchased from Invitrogen. Conditionally immortalized H19-7 cells originated from rat embryonic hippocampal neurons were generated and maintained as described previously [20,21]. DNA transfection was performed using LipofectAMINE plus reagent (Invitrogen), according to the manufacturer's instructions. The total amount of DNA transfected in each individual sample was adjusted using parental empty vector DNA.

2.2. Plasmids and antiserum

The mammalian expression constructs encoding Hip-1 (pCMV-Hip-1), wild type huntingtin with 16 CAG repeats and polyclonal anti-Hip-1 antibodies were provided by M.R. Hayden (University of British Columbia, Vancouver, Canada). The mammalian construct encoding Myc-tagged Hip1 was prepared by PCR of the coding sequence of Hip1 and insertion into pcDNA3.1-Myc-His-(+) vector, and confirmed by DNA sequencing. A prokaryotic expression plasmid encoding bacterially recombinant GST-Hip1 was constructed by inserting the PCR product of wild type Hip1 sequence into pGEX-4T-1 vector (Amersham Biosciences). Bacterially expressed GST and GST-Hip1 were prepared by using Bulk GST purification module (Amersham Biosciences).

2.3. Assessment of cell viability

Quantification of cell survival was performed using the tetrazolium salt extraction method, as described previously [22]. Statistical analyses were performed with the aid of the StatView II program (Abacus Concepts, Berkeley, CA). Data were analyzed by one-way analysis of variance. Preplanned comparisons with controls were performed using the Dunnett's *t*-test.

2.4. Western blot analysis and immunoprecipitation

To prepare the cell lysates, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and then solubilized in lysis buffer (20 mM Tris, pH 7.9, containing 1.0% Triton X-100, 1 mM Na_3VO_4, 137 mM NaCl, 1 μg/ml leupeptin, 200 μM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 mM, 1 mM EGTA, 10 mM NaF, 1 mM tetrasodium pyrophosphate, 5 mM Na_2EDTA, 10% glycerol and 1 mM β-glycerophosphate). The cells were removed by scraping, and the supernatants were collected after centrifugation for 15 min at $14000 \times g$ at 4 °C. The immunoprecipiatation and Western blot analyses were performed as described previously [20].

2.5. Caspase-3 assay

The caspase assay was performed using the ApoAlert Caspase-3 Assay Protocol (Clontech). After 2 h incubation at 37 °C, the samples were measured using a fluorescence plate reader FL 600 (Bio-Tek Instruments, Winooski, VT) at an excitation wavelength of 390 nm and an emission of 505 nm in a 96-well plate.

2.6. Analysis of mitochondrial membrane potential

The mitochondrial membrane potential was assessed by the JC-1 staining method. The dye, existing as a monomer in solution and emitting green fluorescence (525 nm), can assume a dimeric configuration emitting red fluorescence (590 nm) in a reaction driven by the mitochondrial membrane potential. Thus, red fluorescence from JC-1 indicates intact mitochondria, whereas green fluorescence shows monomeric JC-1 that remained unprocessed due to the breakdown of membrane potential. H19-7 cells were adjusted to a density of 0.2×10^6/ml, trypsinized, washed in PBS, resuspended in 1 ml medium, stained with 5 mg/ml JC-1 (Invitrogen) for 30 min at 37 °C (with 5% CO_2 in the dark). Cells were then washed twice in PBS and resuspended in 0.5 ml PBS. Analysis of fluorescence was performed by FACS scan (FASC Calibur, BD Biosciences). The change of mitochondrial potential was assessed by measuring the amounts of JC-1 green (uncoupled mitochondria and detector FL-1) or red (intact mitochondria and detector FL-2) fluorescence.

2.7. Subcellular fractionation

Cells were washed with ice-cold PBS and resuspended in isotonic buffer (10 mM HEPES, pH 8.0, 250 mM sucrose, 1 mM EDTA, and 1 mM EGTA,) supplemented with protease inhibitors containing 2 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin, and homogenized in a Dounce homogenizer on ice. Cells were then centrifuged at $13000 \times g$ for 20 min at 4 °C. Cellular fractionation analysis was performed to detect the cytosolic release of cytochrome *c* from mitochondria, as previously described [23]. Protein contents were measured using a Bio-Rad protein assay kit.

3. Results

3.1. The overexpression of Hip1 induces neuronal cell death

According to a previous report, the overexpression of Hip1 induced cell death in HEK 293 cells [19]. In order to confirm

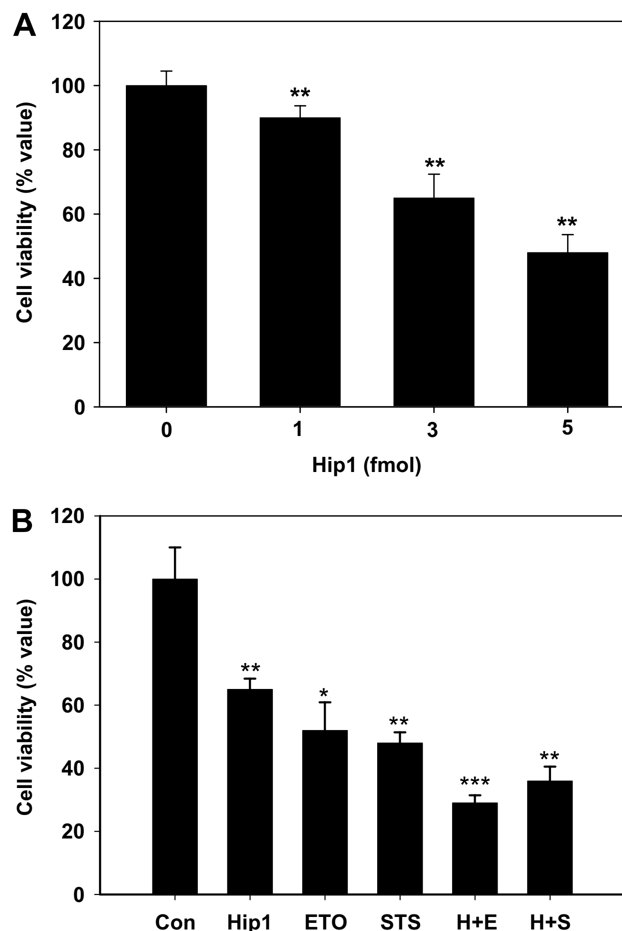

Fig. 1. Transfection of Hip1 induced neuronal cell death. (A) H19-7 cells were transiently transfected for 24 h with the indicated amounts of Hip1 DNA. Cell viability was measured using the MTT extraction assay. (B) Cells were mock-tranfected or transfected for 24 h with 3 μg of plasmid encoding Hip1. The cells were either left untreated or treated with 1 μM staurosporine (STS or S) or 100 μM etoposide (ETO or E) for 12 h. Cell viability of each sample was measured by using MTT extraction assay (CON- mock-transfected; Hip1, Hip1 alone; ETO, ETO-treated mock-transfected cells; STS, STS-treated mock-transfected cells; H + E, ETO-treated Hip1 cells; H + S, STS-treated Hip1 cells; $^{***}P < 0.001$ vs. control, $^{**}P < 0.01$, $^{*}P < 0.05$).

that this also occurs in neuronal cells conditionally immortalized hippocampal H19-7 cells were transiently transfected with a plasmid encoding Hip1 and the change of cell viability was measured after 24 h by means of MTT extraction method. As shown in Fig. 1A, overexpression of Hip1 decreased cell viability compared with mock-transfected cells.

We then examined effects of apoptosis-inducing drugs etoposide and staurosporine on the survival of Hip1-transfected cells. These drugs caused cell death in non-transfected cells (Fig. 1B). Treatment of Hip1-transfected cells with etoposide and staurosporine augmented the amount of cell death caused by Hip1 transfection alone (Fig. 1B). This indicates that upregulation of intracellular Hip1 levels leads to cell death in neuronal H19-7 cells.

3.2. Digestion of Hip1 occurs during cell death

Next, we examined changes in endogenous Hip1 levels during neuronal cell death caused by apoptosis-inducing drugs.

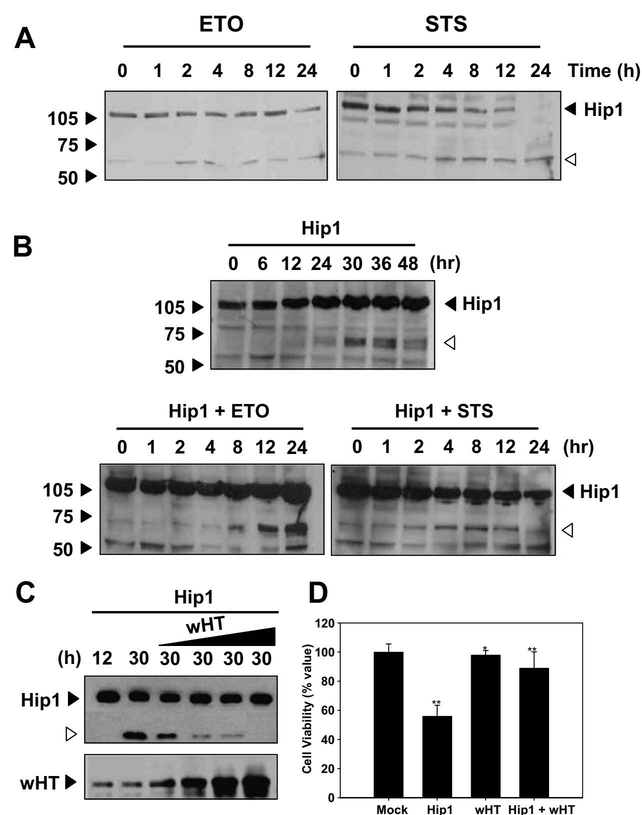

Fig. 2. Proteolytic processing of Hip1 into the smaller fragment occurs in response to toxic stimuli and/or Hip1 transfection. (A) After cells were treated with either 100 μM etoposide or 1 μM staurosporine for the indicated times, Western blot analysis of cell lysates was performed with anti-Hip1 antibodies. A filled arrow head denotes the intact Hip1 while as empty arrow head marks its fragment. (B) After transient transfection with 1 μg Hip1 plasmid for the indicated times, cells were stimulated with etoposide (ETO) or staurosporine (STS) for the indicated times. (C) Where specified, cells were transfected with 1 μg of Hip1 plasmid alone for 12 h or co-transfected with the increasing dose of plasmid encoding non-toxic huntingtin (wHT) for 30 h. The levels of Hip1 and HT in each sample were analyzed by Western blotting with anti-Hip1 and anti-HT antibodies, as indicated. (D) Where indicated, cells were either mock-transfected or transfected with 1 μg of Hip1 plasmid, 5 μg of wild type HT plasmid alone, or both together for 24 h. The cell viability in each sample was then measured by MTT extraction method ($^{**}P < 0.01$ vs. control, $^{*}P < 0.05$).

The mitochondrial apoptotic inducer staurosporine, a potent protein kinase inhibitor, causes apoptosis by directly stimulating cytochrome *c* release from mitochondria. Etoposide is a DNA-damaging anticancer drug. Etoposide-induced apoptosis is also executed through the mitochondrial apoptotic pathway. Levels of Hip1 were assessed using Western blot analyses. In the absence of apoptosis-inducing drugs, the levels of Hip1 are high (Fig. 2A). This is consistent with a previous finding that Hip1 expression is detected very early in undifferentiated embryonic stem cells prior to neuronal differentiation [7].

Application of apoptosis-inducing drugs did not result in a significant change of Hip1 expression. Interestingly, 4–24 h after stimulation with the drugs, wild type 110 kDa Hip1 was digested proteolytically into 65 kDa fragment (Fig. 2A). We then examined fragmentation of Hip1 still occurs after transfection of Hip1 in the absence or presence of apoptosis-inducing drugs. As shown in Fig. 2B, Hip1 overexpression resulted in proteolysis of Hip1 in the presence or absence of toxic stimuli. The presence of toxic stimuli caused fragmentation of Hip1 to occur more rapidly (Fig. 2B). This suggests that overexpression of Hip1 can augment the neurotoxic effects of apoptosis-inducing agents.

Transfection of Hip1 or stimulation with toxic drugs can vary the levels of unbound Hip1 from the soluble huntingtin complexes without altering the total amount of Hip1 We examined the upregulation and fragmentation of unbound Hip1 in response to apoptotic-inducing drugs, and the contribution of unbound Hip1 to the occurrence of Hip1-induced cell death. To verify the accumulation of 'free' Hip1 indirectly and to estimate its consequential toxic effect on cell viability, Hip1 was co-transfected into cells along with increasing doses of wild type nontoxic huntingtin with 16 CAG repeats. This nontoxic huntingtin could recruit endogenously free Hip1 into its binding complexes (Fig. 2C).

Transfection of normal huntingtin did not produce any toxic effect in H19-7 cells (Fig. 2D). DNA fragmentation was not detectable using TUNEL staining (data not shown). Furthermore, co-transfection of Hip1 with wild type huntingtin significantly blocked the occurrence of cell death (Fig. 2D) as well as the generation of cleaved Hip1 proteins in a dose-dependent manner (Fig. 2C). This effect could be caused by free wild type huntingtin binding to Hip1, thereby decreasing Hip1 cytotoxicity. Taken together, these findings indicate that overexpression of Hip1 results in neuronal cell death, and its toxic effect could be transmitted via the upregulation of intracellular free Hip1 levels, which leads to the subsequent fragmentation of Hip1.

3.3. Hip1-mediated cell death occurs through the activation of caspase-3 in H19-7 cells

To determine whether Hip1-induced neuronal cell death occurs through the activation of caspase-dependent pathways, we firstly examined the activation of caspase-3 in H19-7 cells. We chose to investigate caspase-3 since it is the most important effector caspase. Western blot analysis performed with anti-procaspase-3 and anti-caspase-3 IgGs indicates that overexpression of Hip1 induced the activation of caspase-3 in H19-7 cells (Fig. 3A). In addition, stimulation of H19-7 cells with etoposide also significantly increased caspase-3 activity. This was similar to the effect of ectopic Hip1 expression (Fig. 3A). Moreover, to confirm the activation of the cas-

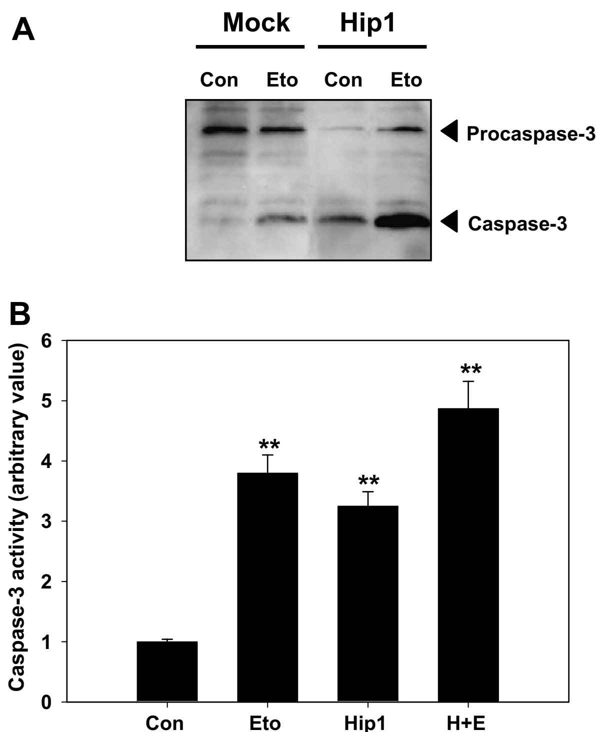

Fig. 3. Transfection of Hip1 results in the activation of caspase-3 in H19-7 cells. (A) Cells were mock-transfected or transfected with 3 μg of Hip1 plasmid. After 24 h, the cells were left untreated (Con) or stimulated with 100 μM of etoposide for 3 h. Total cells were harvested and the cell lysates were immunoblotted with anti-cleaved caspase-3 antibodies. (B) Fifty microgram of cell lysates were incubated with 25 μM of DEVD-AFC for the assay of caspase-3 activity for 2 h at 30°C in a reaction buffer, and fluorogenic caspase activity was measured ($^{**}P < 0.01$ vs. control).

pase-3-dependent pathway, the proteolytic activity of caspase-3 was assessed in vitro by using the fluorogenic caspase-3 substrate. After transfection of the plasmid encoding Hip1 into H19-7 cells, a ~3.2-fold increase in caspase-3 activity was observed, compared with mock-transfected cells (Fig. 3B). This suggests that Hip1 induces neuronal cell death through the activation of caspase-3. Furthermore, the transfection of Hip1 plus etoposide potentiated the activation of caspase-3 in a synergistic way (Fig. 3B). Hip1 plus staurosporine produced a similar stimulatory effect on caspase-3 (data not shown).

3.4. *Apoptosis induced by Hip1 occurs through the activation of caspase-9*

Two major pathways of the initiation of apoptotic cell death have been well characterized. While the signals of the extrinsic pathway transmit via the activation of caspase-8, the intrinsic pathway depends on local events in mitochondria. The intrinsic pathway then results in the cytosolic release of cytochrome *c* from mitochondria, Apaf-1 binding, and activation of caspase-9. To determine which pathway is contributing to Hip1-dependent activation of caspase-3, Hip1 plasmid was transfected into cells. Immunoprecipitation was performed using either anti-procaspase-8 or anti-procaspase-9 antibodies (Fig. 4A). Western blot analysis of immunocomplexes using anti-Hip1 antibodies showed that Hip1 selectively binds to procaspase-9, but not to procaspase-8 (Fig. 4A). Moreover, we examined whether overexpression of Hip1 causes the activation of caspase-8 and/or -9 (Fig. 4B). Western blot analysis with their antibodies, respectively, showed that caspase-9 was significantly activated by Hip1 transfection, while caspase-8 was not (Fig. 4B). These data show that neuronal cell death induced by Hip1 occurs via the sequential activation of caspase-9 and caspase-3, and that Hip1 directly binds to caspase-9.

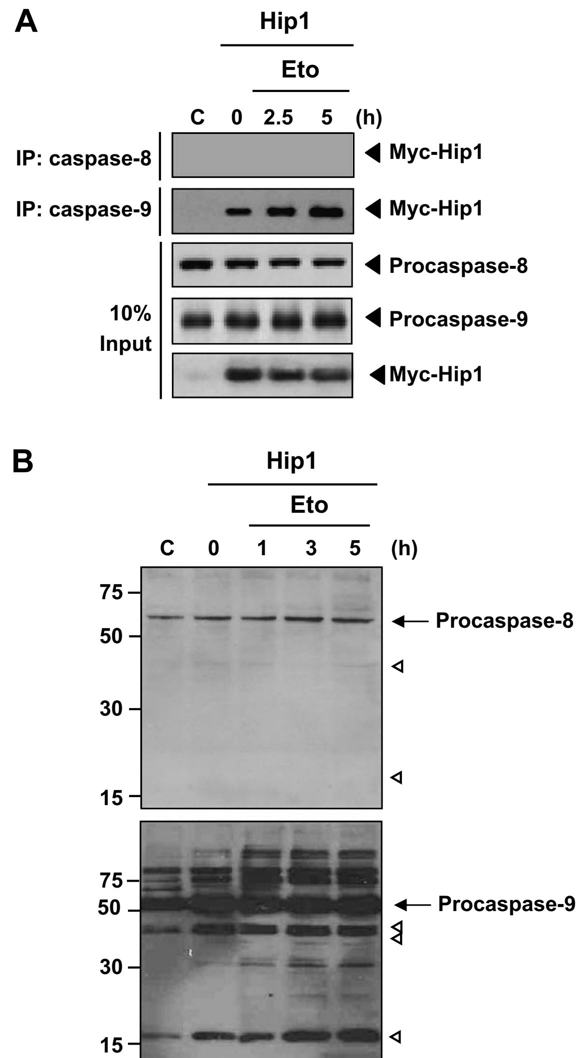

Fig. 4. Hip1 interacts with and activates caspase-9, but not caspase-8. (A) Where indicated, H19-7 cells were mock-transfected (C) or transfected with 6 μg of plasmid encoding Myc-Hip1 for 24 h. The cells were left untreated or treated with 100 μM of etoposide for the indicated times. After total cells were harvested, the lysates were immunoprecipitated with either anti-caspase-8 or anti-caspase-9 IgG, followed by immunoblotting with anti-c-Myc antibodies, as indicated. The proper expression of transiently transfected proteins in cell lysates was identified with Western blot analysis with anti-Myc IgGs. (B) After transfection with 6 μg of plasmid encoding Hip1 for 24 h, cells were stimulated with 100 μM of etoposide for the indicated times. Total cells were harvested and the lysates were immunoblotted with either anti-procaspase-8 or anti-procaspase-9 antibodies, as indicated. The intact procaspase-8 and -9 are denoted as filled arrow, whereas the cleaved procaspase-8 and -9 are marked as empty arrow head, as indicated.

3.5. Overexpression of Hip1 induces the release of cytochrome c and apoptosis-inducing factor (AIF) as well as the depolarization of mitochondrial membrane potential

When cells die via intrinsic apoptotic pathways, mitochondria lose membrane integrity, which results in loss of mitochondrial membrane potential. Mitochondrial dysfunction is followed by the formation of apoptosome and activation of caspase-9 through the following mechanism. Loss of mitochondrial membrane integrity causes the release of pro-apoptotic proteins such as cytochrome *c* and AIF. The resultant apoptosome complex is comprised of cytochrome *c*, Apaf-1 and caspase-9. The complex induces the activation of caspase-9, and then activates caspase-3. In order to determine whether Hip1-induced apoptotic processes accompany the alteration of mitochondrial membrane integrity, the occurrence of cytosolic cytochrome *c* release from mitochondria was firstly examined. As shown in Fig. 5A, Western blot analysis with anti-cytochrome *c* antibodies showed a remarkable increase in the cytosolic levels of cytochrome *c* after 8–12 h, which is accompanied by a significant loss in mitochondrial fraction. Additionally, cytosolic AIF levels increased in a time-dependent manner (Fig. 5A). Moreover, when cells were stimulated with 1 μM of staurosporine after Hip1 transfection, the cytosolic release of cytochrome *c* and AIF occurred more rapidly, compared with the cells transfected with Hip1 alone (Fig. 5B). Etoposide (100 μM) had a similar effect on cytochrome *c* and AIF release (data not shown).

To determine the effect of Hip1 on the mitochondrial membrane potential ($\Delta\psi_m$), the fluorescent carbocyanine dye JC-1 was used. The lipophilic cation JC-1 accumulates in the mitochondrial matrix driven by the electrochemical gradient. The higher the $\Delta\psi_m$, the more polarized is the mitochondrial membrane, and the more JC-1 dye is taken up into the matrix. In the cytosol, the monomeric form of the dye fluoresces green (emissions read at 527 nm), whereas highly concentrated within mitochondrial matrix, JC-1 forms aggregates that fluoresce red (emissions read at 590 nm). To quantify the red fluorescence in cells that had taken up JC-1, FACS analysis has been performed. As shown in Fig. 5C, a two-dimensional display of JC-1 red fluorescence(*y* axis, 590 nm) versus green fluorescence (*x* axis, 527 nm) illustrates the quantitative changes in $\Delta\psi_m$ that occur following Hip1 transfection or the addition of staurosporine. At 0 h, cells display a baseline level of red fluorescence that would represent normal mitochondrial membrane polarization. However, by 2 h after treatment with 1 μM staurosporine, a rise in $\Delta\psi_m$ was observed. This was indicated by an increase in the number of cells in the upper two quadrants, totaling 18.8% at time 0, versus 62.8% at 12 h (Fig. 5C). In a similar way, transfection of Hip1 for 12 h also led to the increase of $\Delta\psi_m$, when compared with mock-transfected control cells (Fig. 5C). These results suggest that overexpression of Hip1 induces neuronal cell death via mitochondrial dysfunction and the cytosolic release of pro-apoptotic effectors, such as cytochrome *c* and AIF.

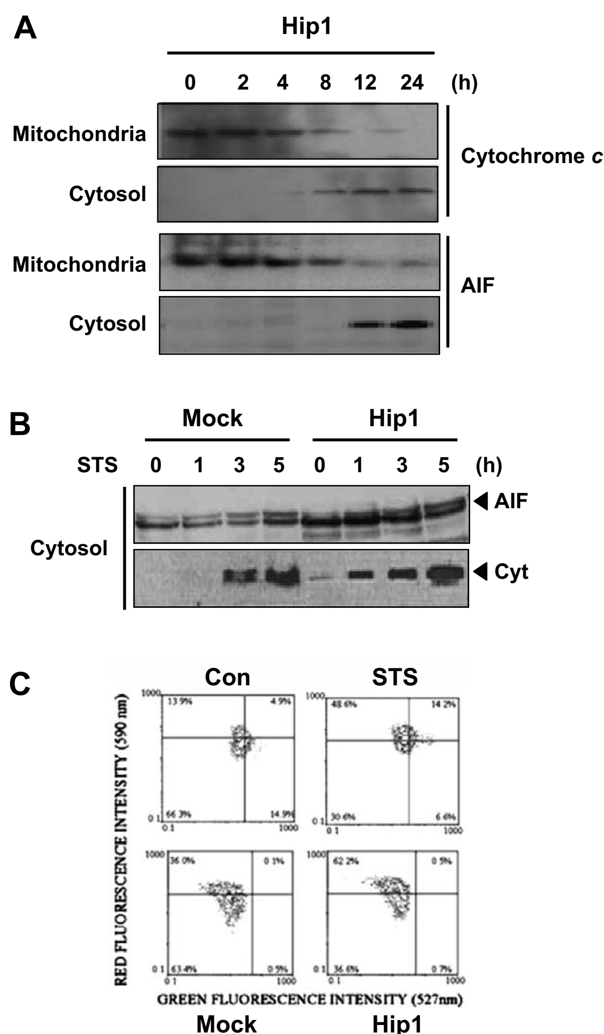

Fig. 5. Hip1 induces the cytosolic release of mitochondrial apoptotic factors, such as cytochrome *c* and AIF, and the depolarization of mitochondrial membrane potential. (A) Where specified, H19-7 cells were transfected with 6 μg of plasmid encoding Hip1 for the indicated times. The cells were then suspended in isotonic buffer, homogenized, and separated into soluble fraction (S), pellet fraction (P) by differential centrifugation. The fractions were analyzed by Western blot with anti-cytochrome *c* and anti-AIF antibodies, as indicated. The P1 pellet contains mitochondria and the soluble (S) fraction represents the cytosol. (B) Where indicated, cells were mock-transfected or transfected with 1 μg of plasmid Hip1 for 12 h. Then, the cells were untreated or treated with 1 μM staurosporine for the indicated times. The soluble fraction was prepared by differential centrifugation, and analyzed by Western blot with anti-cytochrome *c* and anti-AIF IgGs, as indicated. (C) Cells were incubated after treatment with 1 μM of staurosporine (STS) for 3 h (top panel) or transfection with 1 μg of Hip1 plasmid for 12 h (lower panel), and mitochondrial membrane potential was assayed with JC-1 fluorescence. Quantification was determined by FACS analysis, and the presented results are representative from three independent experiments. Lines defining quadrants were arbitrarily defined to highlight changes in the fluorescence of the cell populations, and percentages represent cell number in each quadrant.

3.6. Hip1 interacts with Apaf-1 and promotes the binding of Apaf-1 to cytochrome c

Based on the current finding that Hip1 interacts with caspase-9 and -3, along with the essential role of Apaf-1 for the activation of caspase-3 via the interaction of caspase-9, it could be speculated that Hip1 also directly interacts with Apaf-1. To validate this possibility, the plasmid encoding Myc-tagged Hip1 was transiently transfected into cells, followed by immunoprecipitation with an anti-Myc antibody. The immunoprecipitates were examined by Western blot analysis with anti-Apaf-1 antibodies. As shown in Fig. 6A, Hip1 directly bound

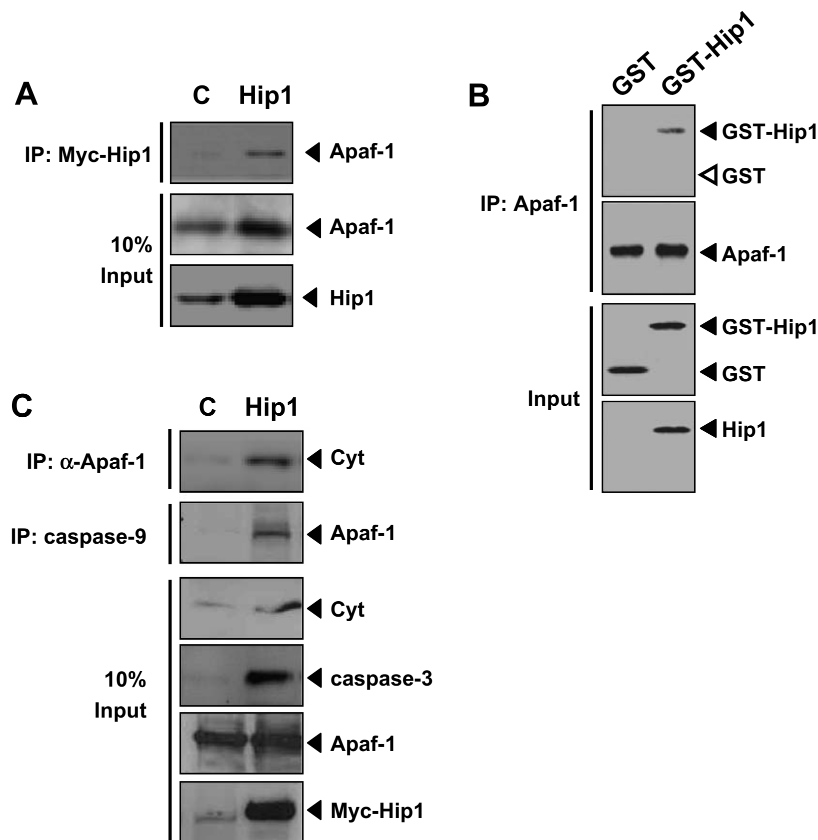

Fig. 6. Hip1 directly interacts with Apaf-1 and is involved in the formation of apoptosome. (A) H19-7 cells were mock-transfected or transfected with 6 μg of plasmid encoding Myc-Hip1, as indicated. After 24 h, total cells were fractionated and the cytosolic fraction was immunoprecipitated with anti-c-Myc IgG, followed by blotting with anti-Apaf-1 antibody. (B) Where indicated, recombinant GST-Hip-1 and GST protein were prepared. Western blot analysis was performed with anti-Hip1 antibodies or anti-GST antibodies to identify the proper expression of GST and Hip1. After cells were stimulated with staurosporine for 6 h, immunoprecipitation of cell lysates was followed using anti-Apaf-1 antibodies. The immunocomplexes were incubated with glutathione beads attached to either GST-Hip1 or GST for 24 h. After extensive washing, Western blot analyses of the final eluates were performed with anti-GST antibodies, as indicated. (C) After transfection with 6 μg of a plasmid encoding Hip1 for 24 h, the cytosolic fraction was immunoprecipitated with either anti-Apaf-1 or anti-caspase-9 IgGs, followed by blotting with anti-cytochrome *c* or Apaf-1 antibodies, as indicated.

to Apaf-1. In addition, the interaction between Hip1 and Apaf-1 was confirmed by using recombinant GST-Hip1 and in vitro pull-down assay. After cells were stimulated with staurosporine, cell lysates were prepared and immunoprecipitated with anti-Apaf-1 IgGs. As shown in Fig. 6B, anti-Apaf-1 immunocomplexes well bound to GST-Hip1, but not to GST alone, suggesting that Hip1 is functionally linked to neuronal cell death via binding to Apaf-1.

Next we examined whether released cytochrome *c* binds to Apaf-1. It is known that, as the formation of binding complex between cytochrome *c* and Apaf-1 generates the more wide-open conformation of Apaf-1, binding of deoxy-ATP or ATP to the CARD consequently allows for the multimerization of Apaf-1 and its association with procaspase-9. This results in generation of an apoptosome complex having about 1.0 MDa size, consisting of cytochrome *c*/Apaf-1/caspase-9 [24,25]. After the transient transfection of Hip1 plasmid, cells were fractionated to separate subcellular compartments. The cytosolic fraction was immunoprecipitated with anti-Apaf-1 IgG, followed by immunoblotting with anti-cytochrome *c* antibodies. As shown in Fig. 6C, Apaf-1 was bound to cytochrome *c*, indicating that overexpression of Hip1 induces the formation of cytosolic binding complex between cytochrome *c* and Apaf-1.

Furthermore, the possibility that Hip1 might play a role in making an apoptosome complex between caspase-9 and Apaf-1 was validated by assessing whether binding between caspase-9 and Apaf-1 occurs after transfection of Hip1 into H19-7 cells. As shown in Fig. 6C, transient overexpression of Hip1 induced the direct interaction between caspase-9 and Apaf-1. Taken together, these data suggest that the cell death signals of Hip1 are transmitted via the activation of the formation of apoptosome complex, which consists of cytochrome *c*, Apaf-1, and caspase-9. Formation of the apoptosome complex leads to the activation of caspase-3.

4. Discussion

Besides the role of Hip1 in the endocytic pathway and vesicle trafficking [18], there are conflicting data in the literature regarding the effect of Hip1 on cell viability. Originally, Hip1 was reported to function as a pro-apoptotic protein [19,26]. Although the mechanism of cell death was unclear, both

caspase 8-independent intrinsic and caspase 8-dependent extrinsic death receptor final pathways have been implicated. The latter has been shown to be mediated by a Hip1-protein binding partner, designated HIPPI (Hip1 protein interactor) [26]. The current study, along with our previous study, has shown the pro-apoptotic activity of Hip1 in hippocampal neuroprogenitor H19-7 cells [20]. By contrast, mutation of murine Hip1 in vivo leads to testicular degeneration due to apoptosis of post-meiotic spermatids, suggesting a primary role for full-length Hip1 in the survival of some cell types [27]. In addition, Hip1 was reported to transform fibroblasts and to be overexpressed in multiple cancers [28,29]. This discrepancy could be explained by the speculation: while the normal cellular function of Hip1, such as proper endocytosis and receptor trafficking, requires interaction with wild type huntingtin, increased polyglutamine tracts in HT and/or structural modification of Hip1 could disturb the normal interaction. This diminished interaction between Hip1 and HT could release Hip1 from the complex. The increase in the levels of unbound free Hip1, in turn, could lead to alteration of biochemical events at the membrane causing premature cell death and, ultimately, the clinical manifestations of HD.

This prediction was supported by the finding that intact Hip1 can be processed into fragment after either Hip1 overexpression or stimulation with apoptosis-inducing drugs. These events results in the inhibition of normal Hip1-mediated protein transport and, subsequently, induction of cell death. In addition, co-expression of Hip1 with a normal huntingtin fragment capable of binding Hip1 was shown significantly to reduce Hip1-induced cell death [19]. Furthermore, transfection of Hip1 deletion mutants leads to the induction of apoptosis in multiple cell types, including HEK293T cells [28,29].

Although the regulation of Hip1 expression and the mechanism by which it is increased in tumors is largely unknown, the levels of Hip1 were unaffected by the expression of polyglutamine-expanded huntingtin in transgenic mice and in HD patient brain samples [7]. The present study also demonstrates that while the total amount of endogenous Hip1 was not changed at all, overepxression of Hip1 alone or with stimulation by toxic stimuli caused the digestion of intact Hip1 into its partial fragment. In addition, increase in the levels of wild type huntingtin also blocked the occurrence of cell death as well as the generation of cleaved Hip1 proteins. This suggests that wild type huntingtin might bind to Hip1 in the cells, which decreases Hip1 toxicity. Fragmentation of Hip1 appears to contribute the Hip1-induced neuronal cell death.

Additionally, there are several lines of evidence to suggest that overexpression of normal HT provides resistance to cell death, as would be predicted if wild-type huntingtin sequesters Hip1 and prevents it from inducing the apoptotic pathway. Rigamonti et al. demonstrated that expression of wild-type HT protected clonal striatal derived cells from several apoptotic stimuli [30]. Furthermore, increasing the expression of wild-type HT in transgenic mice protects against the toxic effects of mutant huntingtin, potentially by binding to Hip1 [31].

Additional elucidation of the signaling pathways revealed that apoptotic signals of Hip1 transmit through the intrinsic mitochondrial pathways via the activation of caspase-9 and the formation of the apoptosome complex. The involvement of the intrinsic apoptotic pathway in Hip1-induced cell death was implicated by a previous report that apoptosis mediated by the deletion mutant of Hip1 is inhibited by a dominant-negative caspase-9 [28]. Further studies can search for the unknown extra-binding partners of Hip1 in CNS neuronal cells. For example, an interaction between the death effector domain-containing proteins Hip1 and Hip1-protein interactor (Hippi) induces the oligomerization and activation of caspase-8. This subsequently leads to mammalian cell death [26]. Based on that report, it will be also interesting to test whether the Hip1/Hippi interaction occurs under the current conditions of neuronal cell death in H19-7 cells. These studies will help us to understand the functional role of Hip1 and its links to the pathogenesis of HD and other neurodegenerative diseases.

Acknowledgements: The authors thank M.R. Hayden for plasmids and antiserum, and S.R. Paik, J. Kim, and M.R. Rosner for their helpful discussions and technical assistance. This study was supported by a grant from the Brain Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology of Republic of Korea (M103KV010002-03K2201-00600 to K.C.C.), by Basic Science Research Grant from the Korea Research Foundation (KRF2003-015-C00527 to K.C.C.), by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A050181 to K.C.C.), and by Basic Research Grant from the Korea Science and Engineering Foundation (R01-2004-000-10673-0 to K.C.C.). It was also partly supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2004-005-E00017 to K.C.C).

References

[1] DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P. and Aronin, N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277, 1990–1993.
[2] van Dellen, A. and Hannan, A.J. (2004) Genetic and environmental factors in the pathogenesis of Huntington's disease. Neurogenetics 5, 9–17.
[3] Landles, C. and Bates, G.P. (2004) Huntingtin and the molecular pathogenesis of Huntington's disease. EMBO Rep. 5, 958–963.
[4] DiFiglia, M., Sapp, E., Chase, K., Schwarz, C., Meloni, A., Young, C., Martin, E., Vonsattel, J.P., Carraway, R. and Reeves, S.A. (1995) Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. Neuron 14, 1075–1081.
[5] Harjes, P. and Wanker, E.E. (2003) The hunt for huntingtin function: interaction partners tell many different stories. Trends Biochem. Sci. 28, 425–433.
[6] Li, S.H. and Li, X.J. (2004) Huntingtin-protein interactions and the pathogenesis of Huntington's disease. Trends Genet. 20, 146–154.
[7] Kalchman, M.A., Koide, H.B., McCutcheon, K., Graham, R.K., Nichol, K., Nishiyama, K., Kazemi-Esfarjani, P., Lynn, F.C., Wellington, C., Metzler, M., Goldberg, Y.P., Kanazawa, I., Gietz, R.D. and Hayden, M.R. (1997) HIP1, a human homologue of S. cerevisiae Sla2p, interacts with membrane-associated huntingtin in the brain. Nat. Genet. 16, 44–53.
[8] Wanker, E.E., Rovira, C., Scherzinger, E., Hasenbank, R., Walter, S., Tait, D., Colicelli, J. and Lehrach, H. (1997) HIP-I: a huntingtin interacting protein isolated by the yeast two-hybrid system. Hum. Mol. Genet. 6, 487–495.
[9] Metzler, M., Legendre-Guillemin, V., Gan, L., Chopra, V., Kwok, A., McPherson, P.S. and Hayden, M.R. (2001) HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. J. Biol. Chem. 276, 39271–39276.
[10] Mishra, S.K., Agostinelli, N.R., Brett, T.J., Mizukami, I., Ross, T.S. and Traub, L.M. (2001) Clathrin- and AP-2-binding sites in HIP1 uncover a general assembly role for endocytic accessory proteins. J. Biol. Chem. 276, 46230–46236.
[11] Waelter, S., Scherzinger, E., Hasenbank, R., Nordhoff, E., Lurz, R., Goehler, H., Gauss, C., Sathasivam, K., Bates, G.P., Lehrach, H. and Wanker, E.E. (2001) The huntingtin interacting protein HIP1 is a clathrin and a-adaptin-binding protein involved in receptor-mediated endocytosis. Hum. Mol. Genet. 10, 1807–1817.

[12] Metzler, M., Li, B., Gan, L., Georgiou, J., Gutekunst, C.A., Wang, Y., Torre, E., Devon, R.S., Oh, R., Legendre-Guillemin, V., Rich, M., Alvarez, C., Gertsenstein, M., McPherson, P.S., Nagy, A., Wang, Y.T., Roder, J.C., Raymond, L.A. and Hayden, M.R. (2003) Disruption of the endocytic protein HIP1 results in neurological deficits and decreased AMPA receptor trafficking. EMBO J. 22, 3254–3266.

[13] Dragunow, M., Faull, R.L., Lawlor, P., Beilharz, E.J., Singleton, K., Walker, E.B. and Mee, E. (1995) In situ evidence for DNA fragmentation in Huntington's disease striatum and Alzheimer's disease temporal lobes. Neuroreport 6, 1053–1057.

[14] Green, D.R. (1998) Apoptotic pathways: the roads to ruin. Cell 94, 695–698.

[15] Bredesen, D.E. (2000) Apoptosis: overview and signal transduction pathways. J. Neurotrauma 17, 801–810.

[16] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479–489.

[17] Rodriguez, J. and Lazebnik, Y. (1999) Caspase-9 and APAF-1 form an active holoenzyme. Genes Dev. 13, 3179–3184.

[18] Hyun, T.S. and Ross, T.S. (2004) HIP1: trafficking roles and regulation of tumorigenesis. Trends Mol. Med. 10, 194–199.

[19] Hackam, A.S., Yassa, A.S., Singaraja, R., Metzler, M., Gutekunst, C.A., Gan, L., Warby, S., Wellington, C.L., Vaillancourt, J., Chen, N., Gervais, F.G., Raymond, L., Nicholson, D.W. and Hayden, M.R. (2000) Huntingtin interacting protein 1 induces apoptosis via a novel caspase-dependent death effector domain. J. Biol. Chem. 275, 41299–41308.

[20] Kang, J.E., Choi, S.A., Park, J.B. and Chung, K.C. (2005) Regulation of the pro-apoptotic activity of huntingtin interacting protein 1 by Dyrk1 and caspase-3 in hippocampal neuroprogenitor cells. J. Neurosci. Res. 81, 62–72.

[21] Eves, E.M., Tucker, M.S., Roback, J.D., Downen, M., Rosner, M.R. and Wainer, B.H. (1992) Immortal rat hippocampal cell lines exhibit neuronal and glial lineages and neurotrophin gene expression. Proc. Natl. Acad. Sci. USA 89, 4373–4377.

[22] Min, Y.K., Park, J.H., Chong, S.A., Kim, Y.S., Ahn, Y.S., Seo, J.T., Bae, Y.S. and Chung, K.C. (2003) Pyrrolidine dithiocarbamate-induced neuronal cell death is mediated by Akt, casein kinase 2, c-Jun N-terminal kinase, and IκB kinase in embryonic hippocampal progenitor cells. J. Neurosci. Res. 71, 689–700.

[23] Gross, A., Jockel, J., Wei, M.C. and Korsmeyer, S.J. (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J. 17, 3878–3885.

[24] Cain, K., Bratton, S.B. and Cohen, G.M. (2002) The Apaf-1 apoptosome: a large caspase-activating complex. Biochimie 84, 203–214.

[25] Zou, H., Yang, R., Hao, J., Wang, J., Sun, C., Fesik, S.W., Wu, J.C., Tomaselli, K.J. and Armstrong, R.C. (2003) Regulation of the Apaf-1/caspase-9 apoptosome by caspase-3 and XIAP. J. Biol. Chem. 278, 8091–8098.

[26] Gervais, F.G., Singaraja, R., Xanthoudakis, S., Gutekunst, C.A., Leavitt, B.R., Metzler, M., Hackam, A.S., Tam, J., Vaillancourt, J.P., Houtzager, V., Rasper, D.M., Roy, S., Hayden, M.R. and Nicholson, D.W. (2002) Recruitment and activation of caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hippi. Nat. Cell Biol. 4, 95–105.

[27] Rao, D.S., Chang, J.C., Kumar, P.D., Mizukami, I., Smithson, G.M., Bradley, S.V., Parlow, A.F. and Ross, T.S. (2001) Huntingtin interacting protein 1 Is a clathrin coat binding protein required for differentiation of late spermatogenic progenitors. Mol. Cell. Biol. 21, 7796–7806.

[28] Rao, D.S., Hyun, T.S., Kumar, P.D., Mizukami, I.F., Rubin, M.A., Lucas, P.C., Sanda, M.G. and Ross, T.S. (2002) Huntingtin-interacting protein 1 is overexpressed in prostate and colon cancer and is critical for cellular survival. J. Clin. Invest. 110, 351–360.

[29] Rao, D.S., Paulson, H.L., Pendse, S.J., Saionz, S.J., Bonini, N.M. and Pittman, R.N. (2003) Altered receptor trafficking in huntingtin interacting protein 1-transformed cells. Cancer Cell 3, 471–482.

[30] Rigamonti, D., Bauer, J.H., De-Fraja, C., Conti, L., Sipione, S., Sciorati, C., Clementi, E., Hackam, A., Hayden, M.R., Li, Y., Cooper, J.K., Ross, C.A., Govoni, S., Vincenz, C. and Cattaneo, E. (2000) Wild-type huntingtin protects from apoptosis upstream of caspase-3. J. Neurosci. 20, 3705–3713.

[31] Hyun, T.S., Li, L., Oravecz-Wilson, K.I., Bradley, S.V., Provot, M.M., Munaco, A.J., Mizukami, I.F., Sun, H. and Ross, T.S. (2004) Hip1-related mutant mice grow and develop normally but have accelerated spinal abnormalities and dwarfism in the absence of HIP1. Mol. Cell. Biol. 24, 4329–4340.