FEBS Letters 470 (2000) 360–364 FEBS 23490

Isolation of Ich-1S (caspase-2S)-binding protein that partially inhibits caspase activity

Akihiro Ito, Takashi Uehara, Yasuyuki Nomura*

Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

Received 11 January 2000; received in revised form 3 March 2000

Edited by Jesus Avila

Abstract Members of the caspase family are essential executors of apoptosis. Caspase-2 has two messenger RNAs generated by alternative splicing, which encode caspase-2L and caspase-2S. Although caspase-2L induces apoptosis, caspase-2S also has the ability to antagonize cell death. Experiments in caspase-2-deficient mice showed that caspase-2 functions to delay cell death in some neuronal populations, suggesting that caspase-2S dominantly acts for cell survival in the brain. However, the mechanism of caspase-2S-mediated anti-apoptotic effect is still unclear. Here, we isolated a protein that interacts with caspase-2S, designated as Ich-1S (caspase-2S)-binding protein (ISBP), by yeast two-hybrid screening using full-length caspase-2S cDNA as a bait. ISBP is identical to the recently isolated calcium and integrin-binding protein, and a small molecule calcium-binding protein with two EF-hand motifs of its C-terminus. In vitro transcribed and translated ISBP interacts specifically with glutathione-*S*-transferase-fused caspase-2S. Moreover, the interaction between ISBP and caspase-2S was observed in cultured cells. Northern blot analysis indicated that ISBP may be a ubiquitous protein. Interestingly, ISBP can partially inhibit the processing of pro-caspase-2L induced by anti-Fas antibody-treated Jurkat cytosolic lysates. These results suggested that ISBP may be the mediator for the survival function of caspase-2S.

Key words: Apoptosis; Caspase-2; Caspase-2S; Ich-1S-binding protein

1. Introduction

Apoptosis or programmed cell death is an essential process for normal development and homeostasis of multicellular organisms. Genetic analyses in the nematode *Caenorhabditis elegans* characterized several genes, including ced-3, ced-4 and ced-9, which are involved in programmed cell death [1]. The ced-3 and ced-4 genes are required for cell death to occur, while ced-9 antagonizes the function of ced-3 and ced-4 by protecting cells from programmed cell death [2,3]. Interleukin-1β-converting enzyme (ICE) was the first identified mammalian homologue of CED-3, which is a cysteine protease that converts the inactive form of interleukin-1β to its biologically active form [4,5]. To date, 14 mammalian homologues of CED-3, the caspases, have been identified and classified into three groups based on their homology, structure and substrate specificity [6,7]. Caspases are synthesized as proenzymes and are proteolytically activated in response to various cell death signals or by other members of the caspase family. These results suggested that caspases constitute a protease cascade [8–11].

Nedd-2 was initially identified as a gene that is highly expressed during early embryonic brain development and is down-regulated in the adult brain, and was later shown to encode the rodent homologue of the human ICE family member Ich-1/caspase-2 [12–14]. Caspase-2 (Nedd-2/Ich-1) has been implicated in apoptosis. Overexpression of Nedd-2/Ich-1 causes apoptosis in various cell types [13,14] and decreases in Nedd-2 expression level caused by an antisense construct delayed onset of apoptosis induced by trophic factor withdrawal [15,16]. Caspase-2 is also activated in response to various apoptotic stimuli such as DNA damage, anti-Fas antibody, nitric oxide, hypoxia and ceramide [17–21]. Two different mRNA species derived from alternative splicing encode two proteins, caspase-2L (Ich-1L) and caspase-2S (Ich-1S). Caspase-2L has homology to both p20 and p10 subunits of ICE, but Caspase-2S is a truncated protein containing only the p20 subunit. Interestingly, overexpression of Caspase-2L induces apoptosis, while that of Caspase-2S can antagonize cell death [14]. Experiments using caspase-2-deficient mice showed that oocytes or B lymphoblasts in mutant mice are resistant to cell death following treatment with chemotherapeutic drugs or mediated by granzyme B and perforin, while cell death of some neuronal populations is accelerated in caspase-2-deficient mice [22]. These results suggested that caspase-2 may act as both a positive and negative regulator of programmed cell death, depending on cell lineage and stage of development, and caspase-2S may dominantly function as a cell survival effector in the brain.

We have isolated a protein, designated as Ich-1S-binding protein (ISBP), that interacts with caspase-2S (Ich-1S) using the yeast two-hybrid system. This protein is ubiquitously expressed and identical to the recently isolated calcium and integrin-binding protein (CIB) containing two EF-hand motifs in its C-terminus [23]. ISBP has the ability to partially suppress the processing of caspase-2L in vitro. Thus, ISBP may be involved in caspase-2S-mediated cell survival.

2. Materials and methods

2.1. Yeast two-hybrid screening

The full-length caspase-2S (Ich-1S) cDNA was amplified by polymerase chain reaction (PCR) from corresponding plasmid constructs, which were kindly provided by Dr. Miura (Osaka University) and cloned into the yeast GAL4 DNA-binding domain vector pAS2-1 (Clontech). The resulting construct was used as bait to analyze human

*Corresponding author. Fax: (81)-11-706 4987.
E-mail: nomura@pharm.hokudai.ac.jp

Abbreviations: CIB, calcium and integrin-binding protein; GST, glutathione-*S*-transferase; ICE, interleukin-1β-converting enzyme; Ich-1, ICE and CED-3 homolog; ISBP, Ich-1S-binding protein

PII: S0014-5793(00)01351-X

fetal cDNA libraries (Clontech) according to the Matchmaker two-hybrid system 2 protocol (Clontech).

2.2. Generation of glutathione-S-transferase (GST) fusion protein and in vitro translated protein

Full-length caspase-2S (Ich-1S) and ISBP cDNAs were cloned into the pGEX 6P vector (Pharmacia). The GST fusion protein was purified by glutathione-Sepharose affinity column chromatography (Pharmacia) according to the manufacturer's protocol. To cleave the ISBP protein from GST fusion protein, GST fusion ISBP protein was incubated with 20 U of PreScission protease (Pharmacia). [^{35}S]Methionine-labeled ISBP and caspase-2L protein were generated with a TNT T7 Coupled Reticulocyte Lysate system (Promega) according to the manufacturer's instructions.

2.3. In vitro binding assay

Aliquots of 4 μg of GST or GST fusion caspase-2S protein were incubated with ^{35}S-labeled ISBP in NETN buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.01% NP-40, 0.1% BSA) for 2 h and then incubated with 20 μl of 50% glutathione-Sepharose beads (Pharmacia) for 2 h. The beads were then washed five times with NETN buffer. Proteins on the beads were fractionated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized using a Fujix BAS 2000 (Fuji).

2.4. Interaction between ISBP and caspase-2S in vivo

The cDNAs encoding full-length ISBP and caspase-2S tagged with an HA and a FLAG epitope at the N-terminal end were constructed by PCR, respectively. They were subcloned into the mammalian expression vector pCR3.1, generating pCR3.1-HA-ISBP and pCR3.1-FLAG-caspase-2S. 2 μg of HA-tagged ISBP and FLAG-tagged caspase-2S were co-transfected into NIH-3T3 cells by using Effectene transfection reagent (Qiagen) [24,25]. 48 h after transfection, lysates from NIH-3T3 cells were immunoprecipitated with anti-HA antibody (Santa Cruz) or rabbit normal IgG. The immunoprecipitates were then separated by SDS–PAGE (15%) and immunoblotted with anti-FLAG antibody M2 as described [24,25].

2.5. Northern blot analysis

Northern blot analysis of human multiple tissue blots (Clontech) was performed according to the manufacturer's instructions, using the full-length ISBP cDNA or β-actin cDNA as a probe.

2.6. Preparation of Jurkat cytosolic lysates

Jurkat cells (1×10^7/ml) were treated with 250 ng/ml of anti-Fas monoclonal antibody CH-11 (MBL) for 4 h, and cytosolic lysates were prepared as described [18]. Briefly, cells were washed twice with ice-cold RPMI 1640 medium and suspended in 400 μl of extraction buffer (10 mM HEPES–NaOH, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EDTA, 1 mM dithiothreitol (DTT), 40 μM β-glycerophosphate). After four cycles of freezing and thawing, cell lysates were centrifuged at $12\,000\times g$ for 15 min at 4°C and then further centrifuged at $100\,000\times g$ for 1 h at 4°C. The resulting supernatant was used as the cytosolic fraction.

2.7. In vitro cleavage assay

In vitro translated ^{35}S-labeled caspase-2L was incubated with anti-Fas antibody-treated Jurkat cytosolic lysates in reaction buffer (20 mM Tris–HCl, pH 7.4, 10 mM DTT, 0.1 mM EDTA) for the indicated times at 30°C in the presence or absence of 5 μg ISBP protein, and the reaction was terminated by addition of 5×SDS sample buffer. Samples were subjected to 15% SDS–PAGE and visualized using a Fujix BAS 2000 (Fuji).

3. Results

3.1. Isolation of cDNA clones encoding ISBP

We screened a human fetal brain cDNA library constructed in pACT2 plasmid encoding the GAL4 activation domain using caspase-2S full-length cDNA as bait, because caspase-2S is highly expressed in the embryonic brain [14]. From approximately 1×10^6 transformants, six positive clones were obtained as determined by activation of *his* and *lacZ* reporter genes. Sequence analysis revealed that these six clones were identical, and the clone was designated as ISBP. Database searches utilizing the BLAST program showed that ISBP is identical to the CIB recently isolated from a human fetal liver cDNA library by yeast two-hybrid system using the cytoplasmic domain of α_{IIb} as bait [23]. CIB consists of 191 amino acids with a molecular mass of about 25 kDa and is a small molecule calcium-binding protein with two EF-hand motifs of its C-terminus. To confirm that full-length ISBP interacts specifically with caspase-2S, full-length ISBP cDNA was amplified by reverse transcriptase-PCR from total RNA of Jurkat cells, cloned into the pACT2 vector and re-transformed into the yeast Y187 strain with an empty pAS2-1 vector, a pAS2-1 hybrid with a caspase-2S insert, or a pAS2-1 hybrid with a caspase-2L (Fig. 1A). ISBP interacted only with the pAS2-1 hybrid with caspase-2S, but neither pAS2-1 empty vector

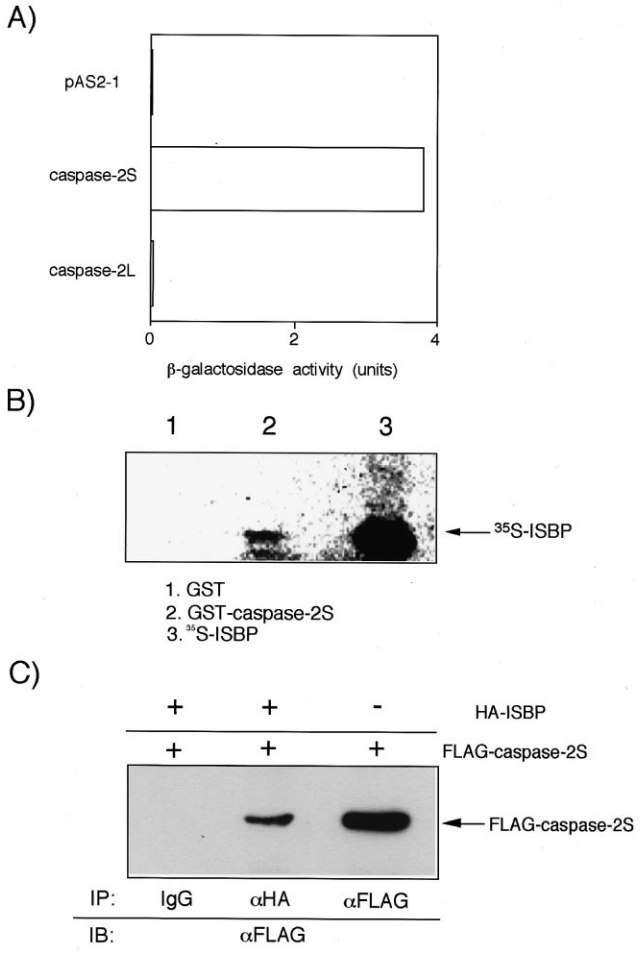

Fig. 1. Interaction between ISBP and caspase-2S in vitro and in vivo. (A) The expression vector encoding ISBP fused to the GAL4 DNA activation domain was co-transformed with empty vector, expression vector encoding caspase-2S or caspase-2L fused with the GAL4 DNA-binding domain in yeast Y187 cells. Each transformant was assayed for β-galactosidase activity. (B) In vitro interaction of ISBP with caspase-2S. In vitro translated [^{35}S]ISBP was incubated with GST alone or purified GST fusion caspase-2S protein and processed as described in Section 2. (C) In vivo interaction of ISBP with caspase-2S. NIH-3T3 cells were transiently transfected with HA-ISBP and FLAG-caspase-2S. Cell lysates were prepared and immunoprecipitated as described in Section 2. Then, the immunoprecipitates were analyzed by Western blotting using anti-FLAG antibody M2.

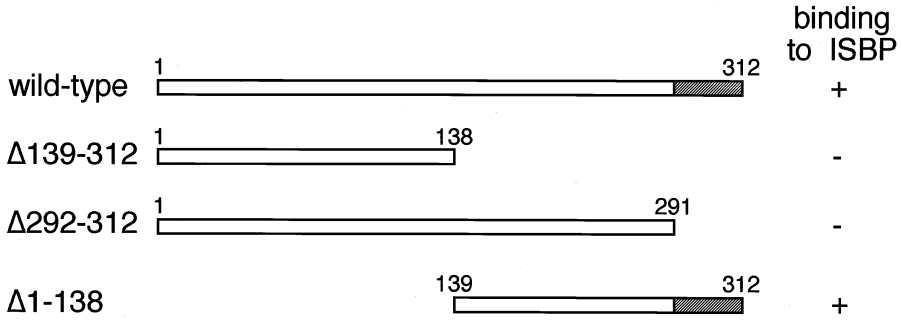

Fig. 2. Interaction of ISBP with caspase-2S deletion mutants. Yeast Y187 cells were co-transformed with expression vector encoding various caspase-2S deletion mutants fused to the GAL4 DNA-binding domain and ISBP fused to the GAL4 DNA activation domain. Each transformant was analyzed by the β-galactosidase filter assay. The shaded boxes indicate caspase-2S specific sequences.

alone nor pAS2-1 hybrid with caspase-2L, indicating a true positive interaction of ISBP with caspase-2S. CIB shares homology with calcineurin B, the regulatory subunit of protein phosphatase 2B (28% identity, 58% homology) and calmodulin (27% identity, 55% homology) [23]. To investigate the possibility that caspase-2S can also interact with calcineurin B, calcineurin B cDNA was cloned into the pACT2 vector and transformed into the yeast Y187 strain with a pAS2-1 hybrid with a caspase-2S insert. Calcineurin B failed to interact with caspase-2S (data not shown).

3.2. Association of ISBP with caspase-2S in vitro and in vivo

We performed an in vitro binding assay to confirm the specific interaction of caspase-2S with ISBP that was observed in the two-hybrid system. GST and GST fusion caspase-2S protein were tested for interaction with ^{35}S-labeled ISBP protein generated by in vitro transcription and translation. As shown in Fig. 1B, GST fusion caspase-2S protein but not GST associated with ISBP. In addition to binding ISBP in vitro, we next investigated the possibility that ISBP also interacts with caspase-2S in vivo. NIH-3T3 cells were transiently transfected with HA-tagged ISBP and FLAG-tagged caspase-2S. The ability of caspase-2S to associate with ISBP in mammalian cells was assessed by determining whether the two proteins co-immunoprecipitate. Transfection of NIH-3T3 cells with caspase-2S and ISBP led to the interaction as demonstrated by the co-immunoprecipitation of ISBP with caspase-2S using anti-HA antibody (Fig. 1C).

3.3. ISBP interacts with the C-terminus of caspase-2S

Caspase-2S is a truncated form of caspase-2 generated by insertion of a 61 bp intron, resulting in a stop codon 21 amino acids downstream of the insertion [14]. Hence, the sequence from 21 amino acids downstream of the active domain QACRG in caspase-2S is different from that of caspase-2L. To investigate the region of caspase-2S that interacts with ISBP, several pAS2-1 hybrids with mutants of caspase-2S were co-transformed with pACT2 hybrid with ISBP into the yeast Y187 strain. The pACT2 hybrid with ISBP interacted with an N-terminus-lacking mutant (Δ1–138), but not a mutant lacking the C-terminus (Δ139–312) or a mutant lacking 21 amino acids from the C-terminus (Δ292–312) (Fig. 2).

3.4. Detection of ISBP mRNA in human tissues

Although the expression of CIB mRNA has been detected in some cell lines and platelets [23], it remains unclear whether CIB is expressed in other tissues. To examine the expression of ISBP in different tissues, we performed Northern blot analysis with a radioactive probe corresponding to the ISBP cDNA (Fig. 3). ISBP mRNA was found to be expressed in all adult and embryonic human tissues examined, indicating that ISBP is probably a ubiquitous protein.

3.5. ISBP partially inhibits caspase activity

To investigate the effects of ISBP on caspase activity, we used a cell-free system using anti-Fas antibody-induced apoptotic Jurkat cytosolic lysates. Jurkat cells were induced to

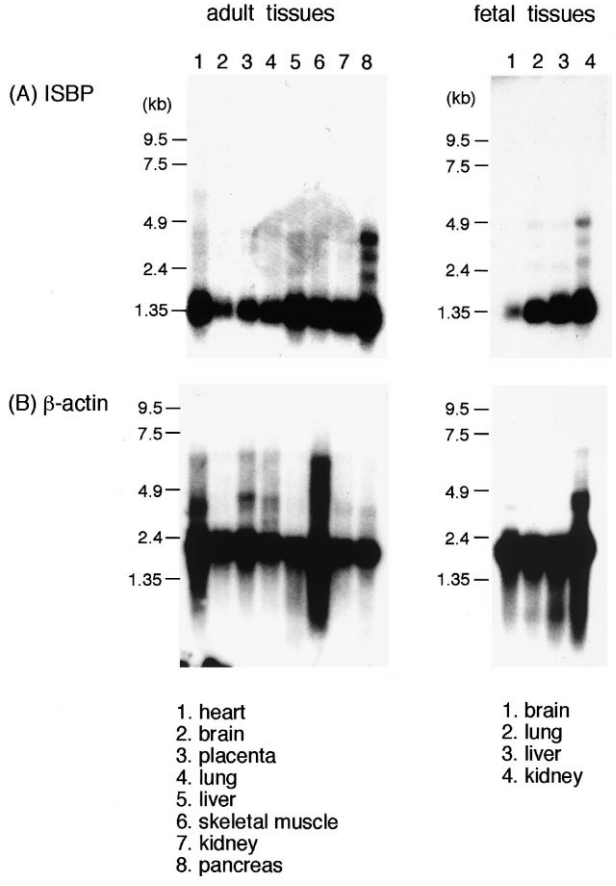

Fig. 3. Northern blotting analysis of ISBP mRNA in multiple human tissues. Northern blotting analysis of ISBP mRNA was performed on multiple human adult and embryonic tissues. The same filters were reprobed with β-actin as a control.

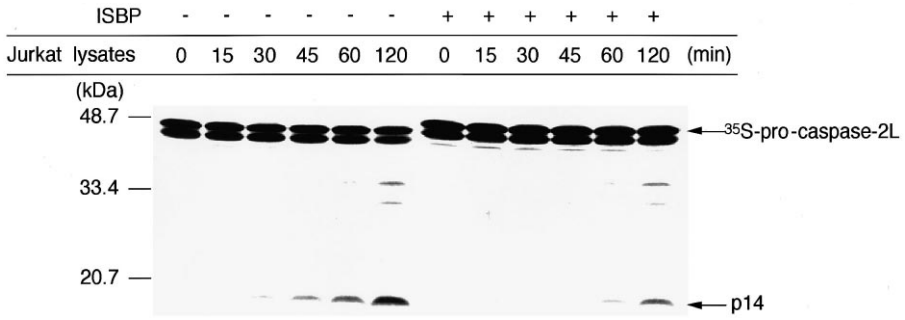

Fig. 4. The suppressive effect of ISBP on in vitro cleavage of pro-caspase-2L. 1×10^7/ml Jurkat cells were treated with 250 ng/ml anti-Fas monoclonal antibody CH-11 for 4 h and cytosolic lysates were prepared as described in Section 2. In vitro translated ^{35}S-labeled pro-caspase-2L was incubated with cytosolic lysates for the indicated times at 30°C in the presence or absence of recombinant ISBP protein.

undergo apoptosis in the presence of 250 ng/ml of anti-Fas antibody for 4 h. Cytosolic extracts treated with anti-Fas antibody were isolated and incubated for the indicated times with ^{35}S-labeled pro-caspase-2L translated in vitro in the presence or absence of recombinant ISBP protein. Cleavage of pro-caspase-2L into the 14 kDa fragment was induced by apoptotic Jurkat cytosolic lysate in a time-dependent manner and this cleavage was significantly delayed in the presence of ISBP (Fig. 4).

4. Discussion

The aim of this study was to isolate a protein that may interact with caspase-2S, which is a truncated form of caspase-2 generated by alternative splicing and antagonizes cell death, using the yeast two-hybrid system. The isolated cDNA encoded a polypeptide of 191 amino acids that has recently been isolated as a protein which binds to the integrin α_{IIb} cytoplasmic domain, designated as CIB [23]. The integrins are adhesion molecules and transmembrane glycoproteins composed of α and β subunits, which mediate the adherence of cells to the extracellular matrix important for various physiological processes such as homeostasis, angiogenesis, cell proliferation and differentiation [26]. Integrin-mediated adhesion to the extracellular matrix also plays an important role in anchorage-dependent cell survival [27,28]. In hematopoietic cell lines, the induction of apoptosis was caused by fibronectin via its interaction with very late antigen that belongs to the integrin β_1 subfamily [29]. Since it has been reported that CIB only binds to the cytoplasmic domain of integrin α_{IIb}, but not other integrin cytoplasmic domains such as α_v, α_2, α_5, β_1 and β_3 [23], it has been speculated that CIB/ISBP is not mainly involved in the regulation of fibronectin-induced cell death. Furthermore, integrin $\alpha_{IIb}\beta_2$ functions as the platelet fibrinogen receptor and is specifically expressed in the megakaryocytic lineage. On the other hand, CIB is expressed in platelets. As platelets do not have nuclei, therefore, it is unlikely that ISBP is involved in the regulation of cell survival by integrins. Northern blotting analysis revealed that ISBP is expressed in all human adult and embryonic tissues examined to date where the expression of caspase-2S was detected (Fig. 3). These results suggested that ISBP is a ubiquitous protein and functions as not only the regulatory molecule for platelet integrin $\alpha_{IIb}\beta_2$, but also as a co-factor for the anti-apoptotic action of caspase-2S.

ISBP is similar to calcineurin B and calmodulin. Calcineurin B is a small regulatory subunit of calcineurin or phosphoprotein phosphatase 2B, and has four EF motifs. Calcineurin is a hetero dimer that consists of calcineurin A (a large catalytic subunit) and calcineurin B. Calcineurin A binds to calmodulin, while calcineurin B binds to four atoms of calcium [30]. The activity of calcineurin is regulated by calmodulin and calcium. Calcium signaling is believed to be involved in apoptosis in certain pathways such as neuronal death induced by glutamate, inducers of calcium influx and calcineurin activation, and cell death in T cells [31–33]. Sustained increases in cytosolic calcium result in the activation of calcineurin and subsequent apoptosis in susceptible cells [34] and overexpression of constitutively active calcineurin induced apoptosis in mammalian cells [35]. These observations and the similarity between ISBP and calcineurin B raise the possibility that ISBP is involved in calcium-induced apoptosis as a regulatory subunit for an unknown enzyme or caspase-2S.

Interestingly, in vitro cleavage experiments showed that recombinant ISBP has the ability to partially attenuate the processing of pro-caspase-2L induced by treatment with anti-Fas antibody in a cell-free system. This suggested the attenuation of caspase activation in response to anti-Fas antibody by ISBP in vitro. However, the mechanism of ISBP-mediated attenuation of caspases activity is still unclear. Since the C-terminal 21 amino acids of caspase-2S (a region specific to caspase-2S, but not caspase-2L) are critical for the interaction with ISBP, ISBP is unlikely to bind caspase-2L directly. Hence, the interaction of ISBP with pro-caspase-2L was not implicated in this inhibition. Moreover, we found that ISBP is not a substrate for caspases in this cell-free system (data not shown), indicating that ISBP-mediated suppression of caspase activity is not due to competition with pro-caspase-2L and -2S. It has been shown that caspase-2S mRNA is expressed in Jurkat cells [14]. Therefore, ISBP may attenuate the caspase activity through its interaction with caspase-2S in the quiescent state.

ISBP is the first candidate molecule to mediate and/or regulate the cell survival function of caspase-2S. The cell death of motor neurons during development was increased in caspase-2-deficient mice, suggesting that caspase-2S predominantly acts as a cell survival factor in some neuronal populations during development [22]. These results suggested that the interaction of caspase-2S with ISBP may have some role(s) in apoptosis. Thus, it will be of interest to elucidate the main machinery of caspase-2S-mediated anti-apoptotic function to understand embryonic neuronal death.

References

[1] Shaham, S. and Horvitz, H.R. (1996) Genes and Dev. 10, 578–591.
[2] Ellis, H.M. and Horvitz, H.R. (1986) Cell 44, 817–829.
[3] Hengartner, M.O., Ellis, H.M. and Horvitz, H.R. (1992) Nature 356, 494–499.
[4] Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and Horvitz, H.R. (1993) Cell 75, 641–652.
[5] Miura, M., Zhu, H., Rotello, R., Hartwieg, E.A. and Yuan, J. (1993) Cell 75, 653–660.
[6] Nicholson, D.W. and Thornberry, N.A. (1997) Trends. Biochem. Sci. 22, 299–306.
[7] Wolf, B.B. and Green, D.R. (1999) J. Biol. Chem. 274, 20049–20052.
[8] Martin, S.J. and Green, D.R. (1995) Cell 82, 349–352.
[9] Enari, M., Talanian, R.V., Wong, W.W. and Nagata, S. (1996) Nature 380, 723–726.
[10] Shimizu, S., Eguchi, Y., Kamiike, W., Matsuda, H. and Tsujimoto, Y. (1996) Oncogene 12, 2251–2257.
[11] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cell 91, 479–489.
[12] Kumar, S., Tomooka, Y. and Noda, M. (1992) Biochem. Biophys. Res. Commun. 85, 1155–1161.
[13] Kumar, S., Kinoshita, M., Noda, M., Copeland, N.G. and Jenkis, N.A. (1994) Gene and Dev. 8, 1613–1626.
[14] Wang, L., Miura, M., Bergeron, L., Zhu, H. and Yuan, J. (1994) Cell 78, 739–750.
[15] Kumar, S. (1995) FEBS Lett. 368, 69–72.
[16] Troy, C.M., Stefanis, L., Greene, L.A. and Shelanski, M.L. (1997) J. Neurosci. 17, 1911–1918.
[17] Harvey, N.L., Butt, A.J. and Kumar, S. (1998) J. Biol. Chem. 272, 13134–13139.
[18] Li, H., Bergeron, L., Cryns, V., Pasternack, M.S., Zhu, H., Shi, L., Greenberg, A. and Yuan, J. (1998) J. Biol. Chem. 272, 21010–21017.
[19] Uehara, T., Kikuchi, Y. and Nomura, Y. (1998) J. Neurochem. 72, 196–205.
[20] Araya, R., Uehara, T. and Nomura, Y. (1998) FEBS Lett. 13, 168–172.
[21] Ito, A., Uehara, T., Tokumitsu, A., Okuma, Y. and Nomura, Y. (1999) Biochim. Biophys. Acta 1452, 263–274.
[22] Bergeron, L., Petez, G.I., Macdonald, G., Shi, L., Sun, Y., Jurisicova, A., Varmuza, S., Latham, K.E., Flaws, J.A., Salter, J.C.M., Hara, H., Moskowitz, M.A., Li, E., Greenberg, A., Tilly, J.L. and Yuan, J. (1998) Genes and Dev. 12, 1304–1314.
[23] Naik, U.P., Patel, P.M. and Parise, L.V. (1997) J. Biol. Chem. 272, 4651–4654.
[24] Uehara, T., Matsuno, J., Kaneko, M., Nishiya, T., Fujimuro, M., Yokosawa, H. and Nomura, Y. (1999) J. Biol. Chem. 274, 15875–15882.
[25] Tanaka, S., Uehara, T., and Nomura, Y. (2000) J. Biol. Chem. in press.
[26] Hynes, R.O. (1992) Cell 69, 11–25.
[27] Ruoslahti, E. and Reed, J.C. (1994) Cell 77, 477–478.
[28] Frisch, S.M. and Francis, H. (1994) J. Cell Biol. 124, 619–626.
[29] Sugahara, H., Kanakura, Y., Furitsu, T., Ishihara, K., Oritani, K., Ikeda, H., Kitayama, H., Ishikawa, J., Hashimoto, K., Kanayama, Y. and Matsuzawa, Y. (1994) J. Exp. Med. 179, 1757–1766.
[30] Crabtree, G.R. and Clipstone, N.A. (1994) Annu. Rev. Biochem. 63, 1045–1083.
[31] Choi, D.W. (1992) J. Neurobiol. 23, 1261–1276.
[32] Reed, J.C. (1994) J. Cell. Biol. 124, 1–6.
[33] Wang, G.W., Pathan, N., Ethell, I.M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., Mckeon, F., Bobo, T., Franke, T.F. and Reed, J.C. (1999) Science 284, 339–343.
[34] Rao, A., Luo, C. and Hogan, P. (1997) Annu. Rev. Immunol. 15, 707–747.
[35] Shibasaki, F. and McKeon, F. (1995) J. Cell Biol. 131, 735–743.