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Biochemical and Biophysical Research Communications 352 (2007) 1–5
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Homer1c interacts with Hippi and protects striatal neurons from apoptosis

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Received 14 September 2006
Available online 7 November 2006

Abstract

By the two hybrid screening of mouse brain cDNA library, we identified Hppi, a cell death-promoting protein, as a binding partner of postsynaptic scaffold protein Homer1c. Hippi interacted specifically with Homer1c but not with its homologue Homer2. It was reported that Hippi, when complexed with Hip1, induces the apoptosis in striatal neurons and may cause Huntington's disease. We found that this apoptotic effect of Hippi was specific to the striatum and was not observed in hippocampal neurons. Furthermore, the apoptotic effect of Hippi was prevented when Homer1c was co-expressed in cultured striatal neurons. The protective effect of Homer1c was diminished when Hippi binding domain was deleted. These results suggest that Homer1c may play an important role in the mechanisms of neuronal death in the striatum.

Keywords: Homer; Huntingtin; Huntington's disease; Striatum; Apoptosis; Caspase

Homer family proteins are postsynaptic scaffold proteins and are encoded by three genes, *homer1*, *2*, and *3* [1–3]. All of the family members have a common property with which they interact with group I metabotropic glutamate receptors, IP3 receptor, ryanodine receptor, and shank through highly conserved EVH-1 domain in their N-terminal regions [1–3]. The amino acid sequences of their N-terminal regions are highly homologous to each other, and are thought to be the basis of the common property of the members.

The C-terminal regions of Homer proteins, on the other hand, contain coiled-coil domains and form homo- and hetero-multimers. However, the amino acid sequences of the C-terminal regions are diverse from each other, and are thought to be the source of the specific properties of different members [2,3]. Although much is not known about possible binding partners of the C-terminal region, they are expected to be specific to different members of the family. In fact, we recently identified a protein which specifically interacts with the C-terminal domain of Homer2 but not with that of Homer1c [4]. Rho family small GTPases were also reported as Homer2-specific binding partners [5].

Huntington's disease is an autosomal dominant progressive neurodegenative disorder characterized by neuronal cell loss primarily in striatum and cerebral cortex [6]. Cell death in Huntington's disease is suggested to be apoptosis, caused by expansion of polyglutamine tract of Huntingtin. Caspases are known to be central executers of apoptosis. Pro-caspases are produced as inactive form, and oligomerization of pro-caspases are essential for conversion to active form by proteolytic cleavage. Gervais et al. reported that Hip1 (Huntingtin interacting protein 1), Hippi (Hip1 protein interactor) and caspase-8 formed multimeric complex, which lead to activation of caspase-8 and mammalian cell death [7]. They proposed that expanded polyglutamine tract of Huntingtin favors the release of Hip1, which normally is bound to Huntingtin. Free Hip1 interacts with

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doi:10.1016/j.bbrc.2006.10.167

Hippi, and promotes oligomerization of caspase-8, leading to apoptosis of striatal neurons.

In the present study, we tried to identify proteins that specifically interact with the C-terminal region of Homer1c by yeast two-hybrid screening. In this report, we demonstrate that Hippi specifically interacts with Homer1c. We also report that exogenous Homer1c expression prevented the Hippi–Hip1-mediated cell death of striatal neurons, and propose new roles of Homer1c in the apoptotic pathway of striatal neurons.

Materials and methods

Plasmids. The FLAG (DYKDDDDK)-tagged Homer constructs FLAG-Homer1c and FLAG-Homer2b have been described elsewhere [2]. Myc (EQKLISEEDL)-tagged Homer2, LB5, Hippi, and Hip1 constructs were generated by PCR using specific primers and subcloned into a mammalian expression vector pcDNA3 (Invitrogen). Fusion proteins of Homer1c or Hippi with fluorescent proteins CFP or YFP were prepared as described [4,8]. GST-fused Homer1c and MBP-fused Hippi were prepared according to the procedures described previously [2,4].

Antibodies. Antibodies used were: anti-synapsin antibody (Chemicon, affinity-purified rabbit polyclonal IgG), Cy5-conjugated anti-rabbit goat IgG (Molecular Probes), mouse anti-FLAG antibody (Sigma), or rabbit anti-Myc or anti-GST antibodies (Sigma), anti-rabbit or anti-guinea pig antibodies conjugated with alkaline phosphatase (Sigma).

Anti-Homer1c antibody was prepared as described [9]. Anti-Hippi antisera were obtained by immunizing guinea-pigs with full length LB5 (Hippi). Hippi was expressed in *Escherichia coli* as a fusion protein with maltose-binding protein (MBP), and purified and separated from MBP using amylose-resin and factor-Xa protease (New England Biolabs) according to the manufacturer's instruction.

Cell cultures, immunochemical procedures, and two-hybrid screening. COS7 cells were cultured as described [10]. Hippocampal or striatal neurons were isolated from E18 rat embryos and cultured on cover slips precoated with poly-D-lysine as described [9]. Immunocytochemistry and immunoblotting were performed essentially as described [4,10,11]. Cells were observed with a Zeiss LSM-510 confocal microscope. Puncta were defined as dots with a diameter of 0.2–1.5 μm.

Yeast two-hybrid screening was performed as described previously [4].

Transfection procedures. COS7 cells were transfected with DNA constructs by electroporation as described [10]. Hippocampal or striatal neurons were transfected by lipofectamine method as follows. Neurons (11–16 days *in vitro*) were transferred to 12-well dishes with Neurobasal-B27 medium (500 μl/well) and incubated at 37 °C before the addition of DNA/lipofectamine complexes. Fifty microliters of Neurobasal medium per well was combined with 1.25 μl of Lipofectamine 2000 (1 mg/ml, Invitrogen) and DNA (0.8 μg), and allowed to form DNA/lipofectamine complexes for 30 min at room temperature. The mixture (50 μl/well) was then added to the culture. After 90 min, the medium was exchanged to conditioned medium.

Cell toxicity assays. pCDNA3-Myc-Hippi, pCDNA3-Myc-Hip1, or empty pCDNA3-Myc were transfected alone or in combination, and cell toxicity effects of these plasmids were assayed essentially as described by Gervais et al. [7]. In some cases, pCDNA3-FLAG-Homer1c or pCDNA-FLAG-Homer1cΔC-terminal (176–366) was also co-transfected, as the reporter, pEGFP was co-transfected (at one-fifth amount of the constructs indicated above). After 48 h, EGFP-positive healthy neurons were counted under the confocal microscope.

In vitro binding assays. *Escherichia coli* cells expressing GST-Homer1c, MBP-Hippi, or MBP were sonicated in sonication buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1 mM DTT). The homogenate was centrifuged at 5000 rpm for 10 min at 4 °C. Protein extracts from *E. coli*, expressing MBP-Hippi or MBP was incubated for 1 h at 4 °C with amylose resins (New England Biolabs) and washed three times with sonication buffer. GST-Homer1c was mixed with MBP-Hippi- or MBP-binding amylose resins and incubated for 1 hr at 4 °C. The resins were washed three times with sonication buffer and precipitated. Precipitated proteins were subjected to SDS–PAGE and detected by the anti-GST antibody and alkaline phoshphatase-conjugated secondary antibody as described [4,10,11].

Immunoprecipitation assays. COS-7 cells transfected for 72 h with DNA constructs for FLAG-tagged or Myc-tagged proteins were collected with Hepes-buffer saline (25 mM Hepes (pH 7.4), 150 mM NaCl) and were lysed in lysis buffer (25 mM Hepes (pH 7.4), 150 mM NaCl, 1% Nonidet-P40, 1 mM PMSF, 5 μg /ml aprotinin, 10 μg /ml leupeptin, and 5 mM EDTA) at 4 °C for 30 min. The lysate was centrifuged for 15 min at 1000*g* to remove cell debris. The supernatants were precleared with protein A/G–agarose beads (Santa Cruz Biotechnology) at 4 °C for 30 min. One micrograms of anti-FLAG antibody and 20 μl of protein A/G–agarose beads were added to each of the precleared cell lysates, and the lysates were incubated overnight. After a brief centrifugation, the precipitates were washed three times with lysis buffer and subjected to SDS–PAGE. Immunoblot was performed using anti-Myc antibody and alkaline phoshphatase-conjugated secondary antibody.

Rats were sacrificed by decapitation, and the hippocampus was dissected out immediately. Hippocampus was homogenized and sonicated in TNE buffer (50 mM Tris–HCl, 150 mM NaCl, and 1 mM EDTA [pH 7.5]) with 1% CHAPS, 1 mM PMSF, and 1 mM DTT (0.15 g tissue/ml). The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C. One milliliters of the hippocampus extract was preincubated for 1 h at 4 °C with 50 μl of protein A–Sepharose beads to remove non-specific binding proteins, and incubated with anti-Hippi antibody for 1 h at 4 °C. Fifty microliters of protein A–Sepharose beads was added and incubated for an additional 2 h at 4 °C. After washing three times with TNE buffer for 10 min each at 4 °C, bound proteins were eluted from the beads with 25 μl of SDS-loading buffer. Proteins were separated by SDS–PAGE and Homer1c was detected by Western blot with an anti-Homer1c antibody [9].

Isolation of hip1 cDNA. Total RNA was isolated from rat brains and RT-PCR was performed to obtain the full-length cDNA of rat *hip1*. Primers were designed based on the conserved regions of human and mouse *hip1* (5′-*Eco*RI site +ATG AAG CAG GTA TCC AAC CCG CTG-3′, 5′-*Not*I site +CTA CTC TTT GTC CGG TAT TGC TTC TTGG-3′). The 3.1-kb cDNA fragment amplified by RT-PCR was then cloned into the *Eco*RI and *Not*I site of the pBluescript SK II vector and the DNA sequence was determined.

Results and discussion

Identification of Hippi as a Homer1c-interacting protein

To identify neural proteins that interact with Homer1c, we performed yeast two-hybrid screening of mouse brain cDNA library, using the C-terminal 190 amino acid residues of rat Homer1c as the bait. As a result, we obtained 18 candidate clones. One of them, termed LB5, was further examined for its specific binding. LB5 was found to bind specifically to Homer1c, but not Homer2 in heterologously expressed cells (Fig. 1a).

After isolation of the full-length clone and partial characterization of LB5, Gervais et al. [7] reported a protein, Hippi, the protein interactor of Huntingtin interacting protein 1 (Hip1). The amino acid sequence of full length LB5 was found to be identical to that of Hippi protein reported by them. The originally isolated LB5 corresponded to the C-terminal 200 amino acid residues of Hippi protein which consists of 426 amino acid residues.

It has been shown that both Hip1 and Hippi contain a death effector domain-like motif, forming a complex which

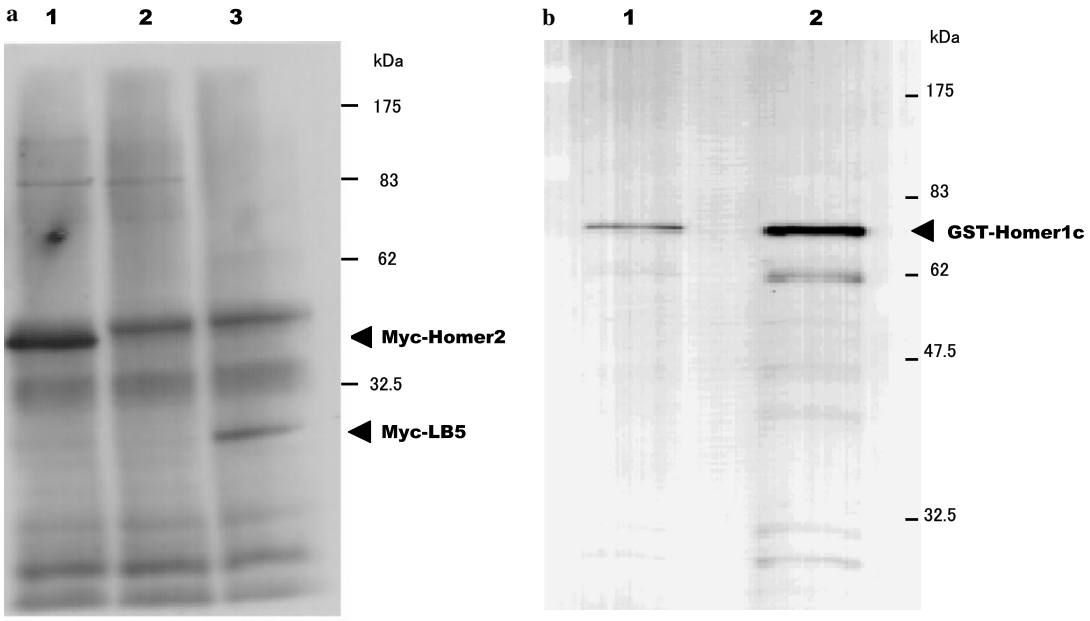

Fig. 1. Specific binding of LB5 or Hippi to Homer-1c. (a) The lysates from COS-7 cells co-expressing FLAG-tagged and Myc-tagged proteins were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-Myc antibody. The lysates analyzed were from COS-7 cells expressing FLAG-Homer1c and Myc-Homer2 (lane 1), FLAG-Homer2 and Myc-LB5 (lane 2), or FLAG-Homer1c and Myc-LB5 (lane 3). LB5 corresponds to the C-terminal 200 amino acid residues of Hippi (b) Extracts of *E. coli* expressing GST-Homer1c were mixed with amylose resins binding MBP (lane 1) or MBP-Hippi (lane 2). After incubation, the beads were precipitated. The precipitates were analyzed by SDS–PAGE and immunoblotted with anti-GST antibody and alkaline phoshphatase-conjugated secondary antibody.

can induce apoptosis through activation of caspase-8 [7,12]. This Hip1–Hippi-mediated apoptosis pathway may play an important role in neuronal death of Huntington's disease.

Hippi interacts with Homer1c in vitro and in vivo

To confirm the interaction of Hippi (full length LB5) and Homer1c, an *in vitro* binding assay was performed. The bacterial lysate containing GST-Homer1c was incubated with MBP-Hippi fixed on amylose resins and the bound Homer1c was detected by Western blot with the anti-GST antibody. The signal of Homer1c was detected in the lane with MBP-Hippi but not in MBP alone (Fig. 1b). This result indicates that GST-Homer1c specifically binds to Hippi.

We then examined the interaction *in vivo*. CHAPS (1%) extracts of rat hippocampi were incubated with anti-Hippi antisera, and then with protein A–Sepharose. The eluate of the protein A–Sepharose was analyzed by Western blot using anti-Homer1c antibodies. These results indicate that the endogenous Homer1c interacts with Hippi *in vivo* (Fig. 2).

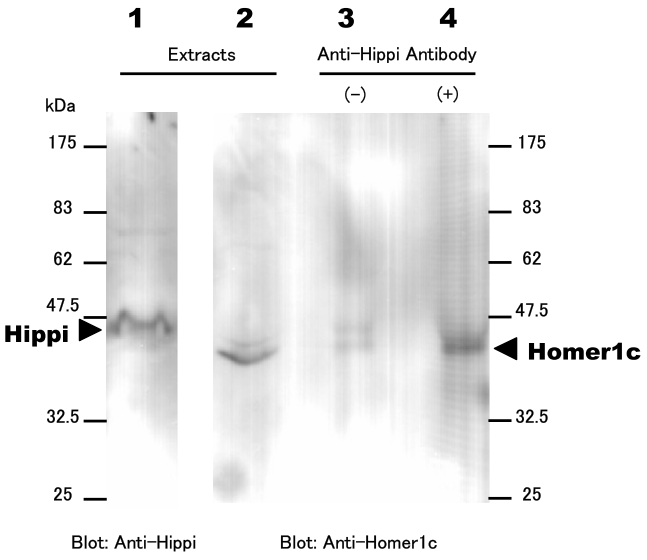

Fig. 2. Immunoprecipitation analyses of rat hippocampus extracts. Hippocampus extracts were incubated with or without anti-Hippi antibody and precipitated with protein A–Sepharose beads. The extracts (lanes 1 and 2) or precipitated proteins (lanes 3 and 4) were analyzed by SDS–PAGE. Immunoblotting was performed using anti-Hippi (lane 1) or anti-Homer1c (lanes 2–4) and alkaline phoshphatase-conjugated secondary antibody.

Exogenously expressed Homer1c and Hippi colocalize at postsynaptic region of hippocampal neurons

To examine the subcellular localization of Homer1c and Hippi in neurons, YFP–Hippi and CFP–Homer1c were co-expressed in primary cultures of hippocampal neurons. Both Homer1c and Hippi were found at dendrites and formed punctate structures (Fig. 3a). Most of Homer1c puncta (94%) colocalized with Hippi puncta. Similarly, 90% of Hippi puncta colocalized with Homer1c puncta (Fig. 3). This result indicates that Homer1c and Hippi colocalize in hippocampal neurons.

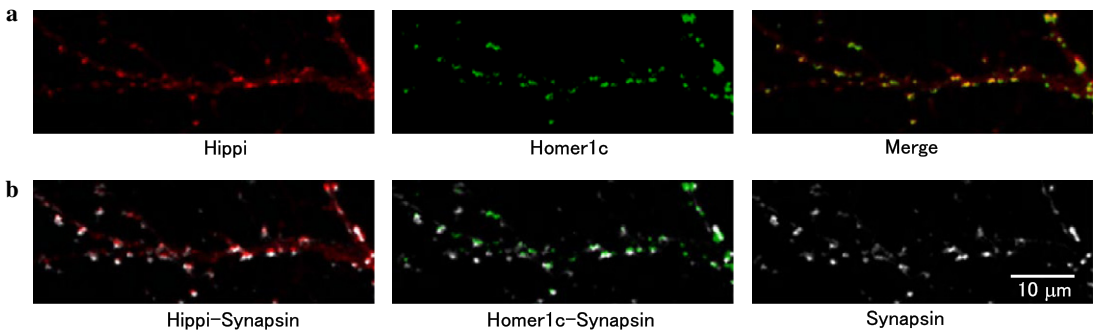

Fig. 3. Distribution of Homer1c, Hippi, and synapsin in hippocampal neurons. Cultured neurons (14 days *in vitro*) were transiently transfected with YFP–Hippi (red) and CFP–Homer1c (green), and then immunostained for synapsin (white) with anti-synapsin antibody and Cy5-conjugated secondary antibody. (a) Hippi and Homer1c form clusters which colocalize with each other: 93.5% of Homer1c clusters and 90.2% of Hippi clusters were positive for both Homer1c and Hippi. (b) Close association of Homer1c or Hippi with synapsin: 87.3% of Homer1c clusters and 90.6% of Hippi clusters were associated with synapsin.

Neurons expressing YFP–Hippi and CFP–Homer1c were counterstained for synapsin, a presynaptic vesicle protein. Most of the Homer1c and Hippi puncta in dendrites were associated with synapsin puncta (Fig. 3b). Eighty-seven percent of Homer1c puncta and 91% of Hippi puncta were associated with synapsin puncta (Fig. 3). This result suggests that Homer1c and Hippi localize at postsynaptic regions. This pattern of distribution is essentially the same as that of Homer1c itself in the absence of Hippi expression. In other words, Hippi does not appear to affect or modify the distribution of Homer1c, but rather seems to be attracted by Homer1c.

Hippi–hip1-mediated cell death occurs in striatal neurons but not in hippocampal neurons

It is suggested that the interaction of Hippi and Hip1 activates caspase-8 and induces cell death in striatal neurons [7]. Therefore, we first attempted to confirm the Hippi–Hip1-mediated cell death. Hippi and Hip1 along with a reporter EGFP were expressed in primary hippocampal or striatal neurons and the EGFP-positive viable neurons were counted (Fig. 4). In striatal neurons, the cell viability was significantly decreased (to 35.9%; Fig. 4a), confirming the results of Gervais et al. [7]. In hippocampal neurons,

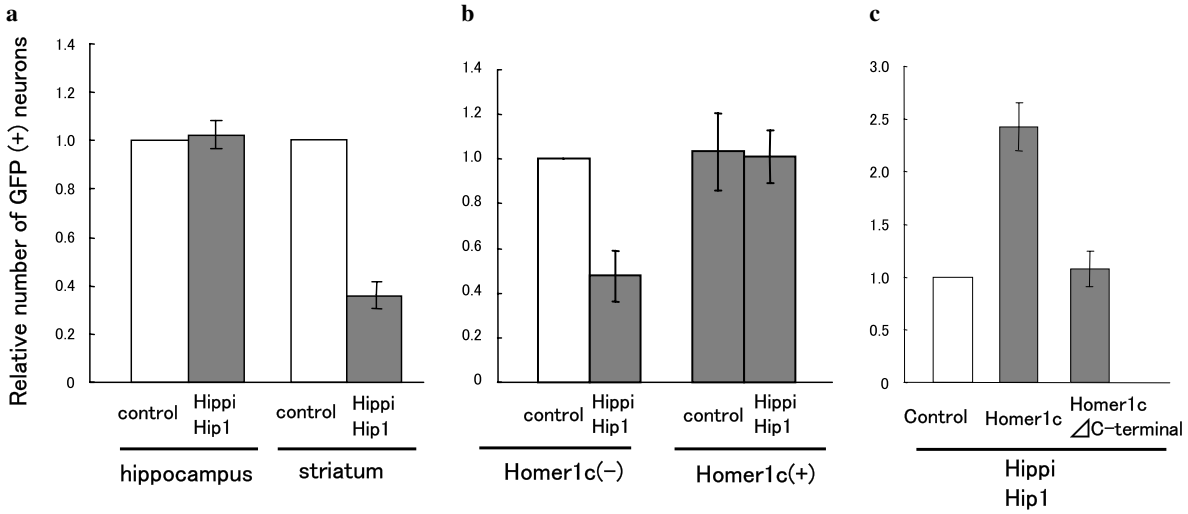

Fig. 4. Hippi and Hip1 induce cell death in the striatum but not in the hippocampus, and Homer1c protects striatal neurons from the Hippi–Hip1-mediated cell death. (a) Cultured neurons (14–21 days *in vitro*) were transfected with DNAs coding Hippi and Hip1 or control vector DNAs with a reporter EGFP, and 24–48 h later the numbers of healthy neurons expressing EGFP were counted. Left, hippocampus; right, striatum. The average cell numbers under control conditions were 61.6 (hippocampus) and 53.4 (striatum). Mean ± SEM, $n = 3$ independent experiments. (b) Cultured striatal neurons (14–21 days *in vitro*) were transfected as in (a) with DNAs coding Homer1c or control vector, and 24–48 h later the numbers of healthy neurons expressing EGFP were counted. (c) Cultured striatal neurons were transfected as in (a) with DNAs coding Homer1c, Homer1c ΔC-terminal, or control vector, and 24–48 h later the numbers of healthy neurons expressing EGFP were counted. The protective effects of Homer1c were lost in its mutant lacking its C-terminal domain (from 166 to 366 amino acid residues). The average cell numbers under control conditions were 80.8 (b) and 69.2 (c). Mean ± SEM, $n = 3$ independent experiments.

however, we found that the cell viability was not affected by the expression of Hippi and Hip1 (Fig. 4a). This is a sharp contrast with the striatum, and appears to correspond to the fact that Huntington's disease results from the specific loss of striatal neurons. Thus, our results may provide a clue to investigate the mechanisms of specific loss of neurons in the striatum but not in the hippocampus in the Huntington's disease.

Homer1c protects neurons from Hippi–Hip1-mediated cell death in striatal neurons

Since Homer1c interacts with Hippi, it is likely that Homer1c may affect the function of Hippi. Therefore we asked whether the co-expression of Homer1c might influence the Hippi–Hip1-mediated neuronal toxicity. Homer1c was co-expressed with Hippi and Hip1, together with EGFP as a reporter in striatal neurons, and the number of EGFP-positive viable neurons was counted. We found that the Hippi–Hip1-mediated toxicity was almost completely suppressed by the co-expression of Homer1c (Fig. 4b). Homer1c alone did not affect the viability of control cells in the absence of Hippi and Hip1 (Fig. 4b). These results indicate that Homer1c protects striatal neurons from Hippi–Hip1-mediated toxicity.

This result raised a possibility that Hippi–Hip1 may cause cell toxicity in striatal neurons but not in hippocampal neurons because the amount of Homer1c may be larger in the hippocampus than in the striatum. We therefore measured the amount of Homer1c protein in the hippocampus and the striatum, but we did not find the significant difference (Yano, Udo, and Sugiyama, unpublished results). At present, the reason why Hippi–Hip1 did not cause cell toxicity in hippocampal neurons is not clear. The difference is not explained simply by the difference in the amount of Homer1c. The difference in the localization, or the status of the interactions with other proteins in the cells might be important. Further investigations may be needed.

The C-terminal region of Homer1c is required for the protection of neuronal cell death

To further investigate the role of Homer1c in the protection of neuronal death, a mutant of Homer1c was co-expressed along with Hip1 and Hippi in striatal neurons. When Homer1c ΔC-terminal, a mutant lacking the C-terminal region (from 176 to 366), were co-expressed, it failed to protect striatal neurons from cell death (Fig. 4c). The mutant Homer1c does not bind to Hippi. This result shows that Homer1c mediated protection from cell death may require the direct interaction between Homer1c and Hippi through the C terminal region of Homer1c.

In summary, we found that Homer1c specifically interacts with Hippi which, together with Hip1, causes neuronal death specifically in the striatum but not in the hippocampus, and that Homer1c could prevent this Hippi–Hip1-mediated neuronal death. Although the detailed mechanisms are not clear at present, Homer1c–Hippi interaction may be involved and play important roles in the regulation of neuronal death in Huntington's disease.

Acknowledgments

We thank S. Ogawa and Y. Inoue for their help in hippocampal cell cultures.

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