A 13-Mer Peptide of a Brain Injury-derived Protein Supports Neuronal Survival and Rescues Neurons from Injury Caused by Glutamate

(Received for publication, August 22, 1995, and in revised form, October 5, 1995)

Tokiko Hamat, Akihiko Ogura, Akira Omori, Miyuki Murayama, Misae Kubota, Mariko Sekiguchi, Mariko Ishiguro, Mutsumi Maruyama, Hiroshi Hatanaka, and Kazuki Sato

From the Mitsubishi Kasai Institute of Life Sciences, Machida, Tokyo 194, Japan.

Neuronal survival is mediated by several kinds of proteins. Among these, neurotrophic factors play important roles in the nervous system by supporting neuronal activity and survival. It has been suggested recently that certain factors promote neuronal survival in the case of brain injury. To examine this possibility, we purified a novel neurotrophic factor from Gelfoam that was implanted at the site of injury caused in neonatal rats. During amino acid sequence analysis, we found that a fragmental peptide of this neurotrophic protein consisting of 13 amino acids showed neurotrophic activity. This 13-mer peptide promoted survival of septal cholinergic and mesencephalic dopaminergic neurons in culture and rescued hippocampal neurons from injury caused by glutamate in culture. This peptide rescued neurons from cell death caused by glutamate, even when added 4.5 h after glutamate exposure.

Neuronal survival is supported by several factors. Among these, neurotrophic factors are thought to play important roles in both the peripheral and the central nervous system. Many studies have indicated that a lack of neurotrophic factor(s) causes neuronal cell death and therefore that injury to the central nervous system of an adult animal would lead to massive death of neurons. Recently, however, it has been reported that the central nervous system has the potential to partially recover from injury. Several studies showed that such neurotrophic activity was high around the site of injury (1-5). Messenger RNA of nerve growth factor and brain-derived neurotrophic factor increased after the induction of limbic seizure in the central nervous system of an adult animal would lead to massive neuronal activities in the injured brain even after the central nervous system is fully developed. We are in the process of isolating a novel neurotrophic factor, which promotes neuronal survival from Gelfoam implanted at the site of injury caused in neonatal rats, using the primary cultures of septal neurons from rat neonates as an assay system. While pursuing this factor, we found that a fragment of this protein consisting of 13 amino acids promoted neuronal survival and rescued neurons from injury caused by glutamate. This finding might open the possibility of therapeutic application of neurotrophic peptide to the injured brain.

Materials and Methods

Synthesis of 13-Mer Peptide—A peptide corresponding to the sequence of the tryptic fragment of a neurotrophic protein was synthesized by solid phase methodology of common t-butoxycarbonyl chemistry starting from p-methylbenzhydrylamine using a Biosearch model 9500 peptide synthesizer. After hydrogen fluoride deaggregation, the crude peptide was purified by successive chromatographies with Sephadex G-25F, preparative HPLC with ODS columns, and Sephadex G-25F. The structure and purity of the peptide were confirmed by analytical HPLC, amino acid analysis, and fast atom bombardment mass spectrometry measurement. Cell Culture—Primary cell cultures were prepared following the method of Hatanaka et al. (10). Briefly, the septal area was removed out from Wistar rat neonates (9-10 days of age). Cells were dissociated with papain and plated on polyethyleneimine-coated plastic dishes at a density of about 3 x 10^6 cells/cm^2. Cultures were maintained for 6 days with a DF medium (1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 medium) and supplemented with 5% precolostrum newborn calf and 5% heat-inactivated horse, and 1% rat sera. BNP or NGF was added at the indicated concentration on the next day of plating. Determination of ChAT activity for the extract of cell culture was done according to the method of Fonnum (11). Induction of Neuronal Injury—Hippocampal neurons were isolated with papain from Wistar rat embryos of gestational day 18, plated on polyethyleneimine-coated glass coverslips at a density of 1.5-2.5 x 10^5 cells/cm^2 (dish diameter, 15 mm), and maintained for 7 days in DMEM supplemented with 5% precolostrum newborn calf and 5% heat-inactivated horse sera (12). On the day of examination, after arbitrary view fields were photographed with a 10x phase-contrast optic, the culture medium was exchanged sequentially with 1) serum-free DMEM (5 min), 2) serum-free DMEM supplemented with or without 1.2 μM CaCl_2 and 1 mM glutamate (30 min), 3) serum-free DMEM (5 min), and 4) sera-containing DMEM. All treatments were done at 37°C. Twenty-four h later, the identical fields were photographed (to facilitate identification, the coverslips had grating printed on the back). Cells possessing phase-bright somata and neurites (larger than soma diameter, without bead) on pairs of photographs before and after exposure of glutamate were counted, and the rate of remaining cells was calculated.

Purification of Neurotrophic Factors—Neurotrophic activities were assayed to examine the promotion of the survival of septal cholinergic neurons by measuring ChAT activities with primary cultures from neonatal rats (10-14 days of age). Substance Gelfoam made of gelatin (3) was implanted in a cavity made in the frontal part of the cerebrum of a neonatal (4-5 days of age) rat brain and left for 7 days. Gelfoam (19 g) frozen at -80°C was homogenized with 200 ml of DF (minus serum).
medium and centrifuged at 100,000 × g for 60 min. The supernatant was concentrated by 30–60% ammonium sulfate and applied on a Superose 12 column equilibrated with 0.1% CHAPS in phosphate-buffered saline. The active fractions corresponding to molecular masses of about 20–14 kDa were applied on a Mono S ion-exchange column equilibrated with 0.1% CHAPS in 50 mM acetate buffer, pH 5.0. The unadsorbed fraction and fractions were applied several times on the same Mono S column. The unadsorbed fraction (protein concentration was 0.017 mg/ml) was concentrated about 6 times, and SDS-polyacrylamide gel electrophoresis was performed using Phast system (Pharmacia Biotech Inc.) with 8–25% acrylamide gradient gel. Proteins were stained by silver according to the method of Heukeshoven and Dernick (13).

Cell Staining—Staining for acetylcholinesterase (AChE) was performed according to the method of Hefti et al. (14). Following the fixation with 4% paraformaldehyde, the cultures were incubated for 5 days at 4 °C in 50 mM acetate buffer, pH 5.0, containing 4 mM acetylthiocholine iodide (substrate), 0.2 mM tetraisopropyl pyrophosphoramide (pseudocholinesterase inhibitor), and gelatin (prevention of diffusion of reaction product).

Measurement of Cellular Dopamine—Primary cultures were carried out by using the under part of the midbrain (containing mainly substantia nigra) from a postnatal 10-day-old rat in the same way as described in the cell culture (15). Six days after plating, cultures were sonicated in 0.1 M perchloric acid, and the contents of intracellular dopamine were measured by HPLC using an electrochemical detector.

**RESULTS**

Purification of Neurotrophic Factor—On the ion-exchange column chromatography, the unadsorbed fraction and fractions eluted with 0.25, 0.3, and 0.4 M NaCl (fractions I, II, and III, respectively, in Fig. 1B) showed significant neurotrophic activity. Fraction I contained β-NGF, which was detected by immunoblotting using anti-β-NGF. Fractions II and III have not yet been analyzed. The unadsorbed fraction contained a major band with a molecular mass of 15 kDa on SDS-polyacrylamide gel electrophoresis, which was not detected by anti-β-NGF (Fig. 1C). The protein with a molecular mass of 15 kDa was electrophoretically transferred to a polyvinylidene difluoride membrane and was digested with trypsin. The resulting fragmental peptides were separated on a C18 reverse-phase column with HPLC, and those amino acid sequences were analyzed. Based on the amino acid sequences of one or the fragmental peptides, a peptide consisting of 13 amino acids (EALELARGAIFQA) named BINP was authentically synthesized.

Effects of BINP on Septal Cholinergic Neurons—BINP was found to promote the survival of septal cholinergic neurons (Fig. 2). Various concentrations of BINP were added to the septal cell cultures, and 6 days later the activity of ChAT of the cultured cell extracts was measured as an index of the survival of cholinergic neurons. The ChAT activity of the cultures treated with BINP was remarkably higher than those of the control cultures (up to 5 times at the dose of 1.0 ng/ml) (Fig. 2A). The same effect was seen in the serum-free cultures of septum enriched in neurons (not shown).

The survival of septal cholinergic neurons was confirmed by staining for AChE. The number of AChE-positive neurons cultured on an astroglial feeder layer with a supplementation of BINP was greater than the number of AChE-positive cells in the control culture (without BINP), and with 1.0 ng/ml BINP, the number was almost 2.5 times greater than the number in the control culture (Fig. 2B). The AChE-positive neurons in the BINP-supplemented cultures had long and well arborized neurites (Fig. 2C).

Effects of BINP on Dopaminergic Neurons—Although BINP was identified as a survival-promoting factor for septal neurons, we thought it would be of interest to examine whether BINP has a similar effect on other classes of neurons. Therefore, we cultured the dopaminergic neurons in the substantia nigra from rat neonates with or without the addition of BINP. The amount of dopamine in the cell extract of the BINP-added (5.0 ng/ml) culture measured by HPLC with an electrochemical detector was about 2.5 times greater than that in the control culture (Fig. 3). This result coincides with the fact that the number of neurons immunostained by an antibody against tyrosine hydroxylase in the BINP-added culture was significantly greater than that in the control culture (not shown).

**BINP Rescues Neurons from Injury Caused by Glutamate**—Next we examined whether BINP rescues the hippocampal neurons injured by excessive excitation by glutamate. The hippocampal neurons exposed to glutamate usually undergo disintegration with the passage of time, and this phenomenon has been widely used as a model system for the analysis of ischemic neuronal death (16–18). As shown in Fig. 4, BINP was effective in rescuing the neurons from glutamate excitotoxicity. As shown in the dose-effect relationship in Fig. 4A, not all hippocampal neurons were rescued, but we have not determined whether resuculable and unresuculable cells belong to distinct populations of neurons. For the resuculable cells, the half-effective dose of BINP was around 1.0 ng/ml. It is noteworthy that BINP exerted its effect even after the exposure to glutamate (Fig. 4B), though the effect gradually diminished with the delay of the timing of application.
DISCUSSION

BINP not only promotes neuronal survival but also rescues neurons from injury caused by glutamate. Since these effects were reproducible in cultures with or without astroglial feeder layers, BINP presumably acted directly on the neurons. Most of the known substances reducing the glutamate excitotoxicity (including glutamate receptor antagonists (16, 19), calcium channel blockers (16, 20), calcium chelators (21), etc.) were effective only when applied prior to exposure to glutamate. From this viewpoint, BINP may be of clinical interest. Basic fibroblast growth factor (22, 23), NGF (23), insulin-like growth factors (24), and TNFs (25) can protect neurons against metabolic excitotoxicity caused by glucose deprivation in culture.

![Image](https://i.imgur.com/123456.png)

**FIG. 2.** Survival-promoting effect of BINP to septal cholinergic neurons cultured with or without the addition of BINP or NGF. A, ChAT activities. Means ± S.D. of five determinations are shown. Asterisks indicate statistical significance in Student’s t test: *, p < 0.05; **, p < 0.01; ***, p < 0.001. B, the numbers of AChE-positive neurons. Means ± S.D. of five countings are shown. Statistical significance levels in Student’s t test are: *, p < 0.05; **, p < 0.01. C, septal cells stained for AChE. Cells were sampled from the cultures without BINP (1), with BINP (2, 0.5 ng/ml), and with NGF (3, 100 ng/ml).

![Image](https://i.imgur.com/789012.png)

**FIG. 3.** Survival-promoting effect of BINP on cultured nigral dopaminergic neurons. Cultures were prepared as explained under "Materials and Methods." Six days after plating, cultures were sonicated in 0.1 M perchloric acid, and the contents of intracellular dopamine were measured by HPLC using an electrochemical detector. Means ± S.D. of five determinations are shown. Statistical significance levels in Student’s t test are: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

![Image](https://i.imgur.com/345678.png)

**FIG. 4.** Rescuing effect of BINP from glutamate excitotoxicity in cultured hippocampal neurons. A, dose-effect relationship. BINP (10 ng/ml) was added in cultures before glutamate exposure or after glutamate exposure at each time indicated. Asterisks indicate statistical significance in Student’s t test; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

TNFs and interleukin 6 (26) were also effective in protecting neurons from glutamate treatment; however, pretreatment with TNFs was required for protection of neurons from glutata-
mate toxicity (25). Reduction of glutamate excitotoxicity by BINP is not due to competition with glutamate, since BINP was effective even when applied after the exposure to glutamate. BINP per se did not lower the cytoplasmic Ca\(^{2+}\) concentration nor did it suppress the magnitude of glutamate-evoked cytoplasmic Ca\(^{2+}\) elevation.\(^2\) BINP may interfere with a signal cascade leading to cell death in the downstream of Ca\(^{2+}\) and act by using a mechanism different from those factors.

When the amino acid sequence of a fragmental 17-mer peptide of the 15-kDa protein containing BINP was compared with the protein data bank, there was no homology with any neurotrophic factors or cytokines. Interestingly, the 17-mer fragmental peptide showed the highest homology with the chicken proteasome C1 subunit (70% residue identity); however, the rat proteasome C1 subunit showed less similarity with the 17-mer fragmental peptide (41% residue identity) than that of chicken proteasome C1. We have obtained one more peptide and sequenced it. The amino acid sequence of this peptide has also been compared with the Protein Data Bank and GenBank. This peptide showed no similarity with either neurotrophic factors or cytokines, and moreover, it had no similarity with the proteasome C1 subunit. The activity of this peptide has not been examined because it was small. Therefore, the neurotrophic factor containing BINP must be a novel one, and the relationship between proteasome and BINP needs further investigation. From the data of immunoblotting using polyclonal anti-BINP antibody, this novel neurotrophic factor is synthesized from astrocyte.\(^3\)

The complete amino acid sequence of the protein will soon be published elsewhere. Not only is the structure of the protein of interest, but the fact that its small fragment (M, = 1385.59) is sufficient in exerting the neurotrophic and neuroprotective effects suggests the possibility of its clinical and research applications.

Acknowledgments—We are grateful to S. C. Fujita, Y. Kudo, I. Nihonmatsu, and Y. Takagaki for discussions and support.

REFERENCES

1. Niet-Sampedro, M., Lewis, E. R., Cotman, C. W., Manthorpe, M., Skaper, S. D., Barbin, G., Longo, F. M. & Varon, S. (1982) Science 217, 860–861.
2. Niet-Sampedro, M., Manthorpe, M., Barbin, G., Varon, S. & Cotman, C. W. (1983) J. Neurosci. 3, 2219–2229.
3. Manthorpe, M., Nieto-Sampedro, M., Skaper, S. D., Lewis, E. R., Barbin, G., Longo, F. M., Cotman, C. W. & Varon, S. (1983) Brain Res. 267, 47–86.
4. Mattson, M. P., Cheng, B., Culwell, A. R., Esch, F. S., Lieberburg, I. & Rydel, R. E. (1993) Neuron 10, 243–254.
5. Mattson, M. P., Cheng, B. & Smith-Swintosky, V. L. (1990) Semin. Neurosci. 5, 295–303.
6. Gall, C. M. & Isackson, P. J. (1989) Science 245, 758–761.
7. Isackson, P. J., Huntsman, M. M., Murray, K. D. & Gall, C. M. (1991) Neuron 6, 937–948.
8. Ip, N. Y., Wiegand, S. J., Morse, J. & Rudge, J. S. (1993) Eur. J. Neurosci. 5, 25–33.
9. Tchelingenan, J.-L., Quinonero, J., Boose, J. Q. & Jacque, C. (1993) Neuron 10, 213–224.
10. Hatanaka, H., Tsukui, H. & Nihonmatsu, I. (1987) Neurosci. Lett. 79, 85–90.
11. Fonnum, F. A. (1975) J. Neurochem. 24, 407–409.
12. Ogura, A., Akita, K. & Kudo, Y. (1990) Neurosci. Res. 9, 103–113.
13. Heukeshoven, J. & Dernick, R. (1988) Electrophoresis 9, 28–33.
14. Hefni, F., Hartikka, J., Eckerstein, F., Gnahn, H., Heumann, R. & Schwab, M. (1985) Neurosciences 14, 55–68.
15. Hama, T., Kushima, Y., Miyamoto, M., Kubota, M., Takei, N. & Hatanaka, H. (1991) Neuroscience 40, 445–452.
16. Ogura, A., Miyamoto, M. & Kudo, Y. (1988) Exp. Brain Res. 73, 447–458.
17. Choi, D. (1987) J. Neurosci. 7, 369–379.
18. Rothman, S., Thurston, J. H. & Hauhart, R. E. (1987) Neuroscience 22, 471–480.
19. Frandsen, A., Drejer, J. & Schousboe, A. (1989) J. Neurochem. 53, 297–299.
20. Rich, K. M. & Hoflöwel, J. P. (1990) Science 248, 1419–1421.
21. Kudo, Y., Takeda, K. & Yamazaki, K. (1990) Brain Res. 528, 48–54.
22. Mattson, M. P., Murraing, M., Guthrie, P. B. & Kater, S. B. (1989) J. Neurosci. 9, 3728–3740.
23. Cheng, B. & Mattson, M. P. (1991) Neuron 7, 1031–1041.
24. Cheng, B. & Mattson, M. P. (1992) J. Neurosci. 12, 1558–1566.
25. Cheng, B., Christakos, S. & Mattson, M. P. (1994) Neuron 12, 139–153.
26. Yamada, M. & Hatanaka, H. (1994) Brain Res. 643, 173–180.

---

\(^2\) A. Ogura and T. Hama, unpublished observation.

\(^3\) T. Hama, M. Ishiguro, and M. Maruyama, unpublished data.
A 13-Mer Peptide of a Brain Injury-derived Protein Supports Neuronal Survival and Rescues Neurons from Injury Caused by Glutamate

Tokiko Hama, Akihiko Ogura, Akira Omori, Miyuki Murayama, Misae Kubota, Mariko Sekiguchi, Mariko Ishiguro, Mutsumi Maruyama, Hiroshi Hatanaka and Kazuki Sato

*J. Biol. Chem.* 1995, 270:29067-29070.
doi: 10.1074/jbc.270.49.29067

Access the most updated version of this article at [http://www.jbc.org/content/270/49/29067](http://www.jbc.org/content/270/49/29067)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 7 of which can be accessed free at [http://www.jbc.org/content/270/49/29067.full.html#ref-list-1](http://www.jbc.org/content/270/49/29067.full.html#ref-list-1)