Amphiphilic helix is essential for the activity of brain injury-derived neurotrophic peptide (BINP)

Tokiko Hama*, Miyuki Murayama, Rika Kato, Atsuko Ohtake, Kazuki Sato

Mitsubishi Kasei Institute of Life Sciences, 11, Minamiooya, Machida-shi, Tokyo 194, Japan

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Abstract To study the structure-activity relationships of brain injury-derived neurotrophic peptide (BINP), 12 analogs were synthesized by replacing each amino acid residue with Gly. BINP showed CD spectra typical of an α-helical conformation in TFE solution which mimics the membrane environment. In the α-helical conformation, BINP showed an amphiphilic profile. Neurotrophic activities of BINP and its analogs were estimated from the effects on supporting septal cholinergic neurons and on rescuing hippocampal neurons from injury caused by glutamate. Both assays showed that the residues on the hydrophobic side of the amphiphilic helix were essential for the neurotrophic activity.

Key words: Neurotrophic peptide; Amphiphilic helix; Cholinergic neuron; Hippocampal neuron

1. Introduction

Neuronal activities and survival are supported by several kinds of proteins. Among them, neurotrophic factors have been reported to play important roles in various developmental stages. Even after injury to or disease of a mature central nervous system, parts of neurons are rescued and are able to survive. Treatment with neurotoxins or mechanical injury to the brain increases the synthesis of several kinds of factors in the brain [1,2]. These factors, which are up-regulated after injury, are thought to act on neurons to support their survival. The activities of these factors were mainly investigated in vitro and also sometimes in vivo, and it was shown that they supported neuronal survival directly or indirectly via glial cells.

In the course of investigating neurotrophic factors that appeared after injury to rat brains and rescued neurons from injury or excitotoxin, we purified a protein from gelforms implanted in cavities of neonatal rat brains [3]. During analysis of amino acid sequences of peptide fragments, we found that a 13-mer peptide named BINP, which was synthesized based on an amino acid sequence of one of the fragmentary peptides, has a neurotrophic activity. BINP supported neuronal survival of septal cholinergic neurons and mesencephalic dopaminergic neurons in primary cultures from neonatal rats [3]. Furthermore, BINP rescued neurons from injury caused by glutamate even when it was applied at 4.5 h after treatment with glutamate in a primary culture of hippocampal neurons. Since these effects were reproducible in cultures with or without astroglial feeder layer, presumably BINP acted on the neurons. Because BINP is a small 13-mer peptide and may be of clinical interest, it is worth investigating which amino acid residue is important in this activity.

In the present study, we synthesized 12 analogs of BINP in which each amino acid residue except Gly^8 was replaced with a Gly residue in order to examine the structure-activity relationships of the peptide (Table 1). The effects of the substitutions were compared using many criteria, such as theoretical hydrophobicity, retention time on reverse phase HPLC, CD spectra, neurotrophic activity supporting the survival of cultured septal cholinergic neurons, and rescuing neurons from injury caused by glutamate treatment of cultured hippocampal neurons.

2. Materials and methods

2.1. Peptide synthesis

Fmoc amino acids and other reagents used on the synthesizer were obtained from Applied Biosystems Japan (Chiba, Japan). Fmoc-NH-SAL-resin was obtained from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). Other reagents of peptide synthesis grade were obtained from the Peptide Institute (Osaka, Japan) or Kokusan Chemical Works Ltd. (Tokyo, Japan).

Solid phase peptide synthesis was conducted on an Applied Biosystems 431A peptide synthesizer. Amino acid analyses were performed on a Beckman System Gold amino acid analyzer after hydrolysis in 6 M hydrochloric acid at 110°C for 24 h and derivatization by 4-dimethylaminoazobenzene-4′-sulfonyl chloride. FAB-MS were measured on a JEOL HX-100 mass spectrometer. Analytical HPLC was carried out on a Shimadzu LC-6A system with an ODS column (4.6×250 mm). Preparative HPLC was performed with a Shimadzu LC-8A system with an ODS column (20×250 mm).

All the analogs were synthesized by solid phase methodology of Fmoc chemistry. After TFA cleavage, crude peptides were extracted with 2 M AcOH and lyophilized. The crude products were purified by successive chromatography with Sephadex G-25F and preparative HPLC with an ODS column. Structures and purity of synthetic peptides were confirmed by analytical HPLC, amino acid analysis, and FAB-MS measurement.

2.2. CD measurements

CD spectra were recorded on a JASCO J-600 spectropolarimeter in H_2O solution (0.01 M sodium phosphate, pH 7.0) and in TFE solution at 20°C using a cell of 1 mm path length. The sample concentration was 0.05 mM and corrected by quantitative amino acid analysis. The spectra are expressed as mean residual ellipticity [θ].

2.3. Assay of neurotrophic activities

Primary cell cultures were prepared following the method of Hatanaka et al. [4,5]. Briefly, the septal area was dissected out from Wistar

*Corresponding author. Fax: (81) (427) 246314.
E-mail: hama@libra.ls.m-kagaku.co.jp

Abbreviations: BINP, brain injury-derived neurotrophic peptide; Boc, *t*-butoxycarbonyl; ChAT, choline acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high performance liquid chromatography; Fmoc, 9-fluorenylmethoxycarbonyl; NGF, nerve growth factor; ODS, octadecylsilane; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Analogs are designated by a letter and number indicating the identity and position of the replaced amino acid, followed by a letter indicating the identity of the replacement; for example, E1G indicates an analog in which Glu^1 is replaced with Gly

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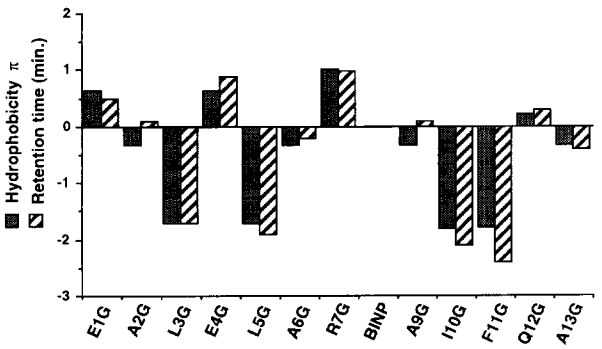

Fig. 1. Changes in the theoretical hydrophobicity and retention times on reversed-phase HPLC of BINP and its analogs. The solid line indicates the position of BINP as a standard. Analytical HPLC was carried out on a Shimadzu LC-6A system with a Shim-pack CLC-ODS column (4.6 × 250 mm) by applying a linear gradient of 5–65% CH_3CN in 0.1% TFA for 30 min at a flow rate of 1 ml/min with monitoring of the absorbance at 230 nm.

rat neonates (9–10 days of age). Cells were dissociated with papain and plated on polyethyleneimine-coated plastic dishes at a density of about 3×10^5 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) supplemented with 5% precolostrum newborn calf serum, 5% heat-inactivated horse serum and 1% rat serum. BINP or NGF was added at the indicated concentration on the next day of plating. Determination of ChAT (choline acetyltransferase) activity in the extract of cultured cells was done according to the method of Fonnum [6].

Hippocampal neurons were isolated with papain from Wistar rat embryos of gestational day 18, plated on polyethyleneimine-coated glass coverslips at a density of $1.5–2.5\times10^5$ cells/cm^2 (dish diameter 15 mm), and maintained for 7 days in DMEM supplemented with 5% precolostrum newborn calf serum and 5% heat-inactivated horse serum [3,7]. On the day of examination, after arbitrary view fields were photographed with a 10× phase-contrast optic, the culture medium was exchanged sequentially with (1) serum-free DMEM (10 min), (2) serum-free DMEM with or without 1.2 mM $CaCl_2$ and 1 mM glutamate (30 min), (3) serum-free DMEM (10 min) and (4) serum-free DMEM with or without peptide. All treatments were done at 37°C. 24 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 (>soma diameter, without beading) were counted for pairs of photographs and the rates of remaining cells were calculated [3,7].

3. Results

Most amino acids were assembled on the resin using standard Fmoc chemistry. However, to avoid spontaneous pyroglutamine formation of the N-terminal Glu residue during treatment with piperidine for the removal of the Fmoc group, an amino group of Glu^1 was protected with the Boc group. After that procedure, the Boc was removed by TFA treatment employed for the cleavage of peptide from resin support. The physicochemical properties of BINP and its analogs were evaluated in terms of the retention times on reversed-phase HPLC, which is more or less related to molecular hydrophobicity. As shown in Fig. 1, this property correlated well with theoretical hydrophobicity [8], showing that there were no anomalies in the molecular hydrophobicity.

Fig. 2 shows the CD spectra of BINP and its analogs in H_2O and in TFE solution. In H_2O solution, CD spectra of all the analogs as well as BINP showed a simple profile with large negative Cotton effects of approximately 195 nm, which is typical of a random conformation. In TFE solution, which mimics the membrane environment, all peptides showed CD spectra with a double minimum of approximately 205 nm and 222 nm, typical of a helical conformation. Fig. 3 shows the helical contents of the peptides calculated from mean residual ellipticity at 222 nm [9]. Since Gly residue is a 'helix breaker' [10], helical conformation is destabilized by this substitution, especially at the central part of the molecule. It is interesting

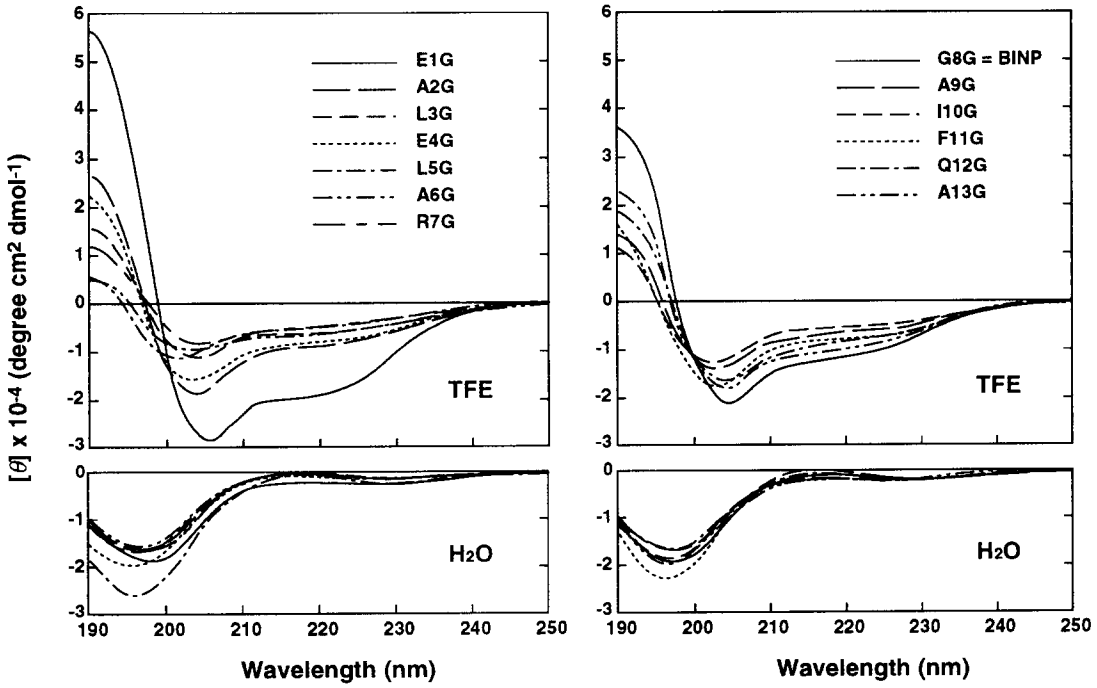

Fig. 2. CD spectra of BINP and its analogs in TFE solution (upper) and in H_2O (0.01 M sodium phosphate, pH 7.0) solution (lower) at 20°C. The spectra are expressed as mean residual ellipticity [θ].

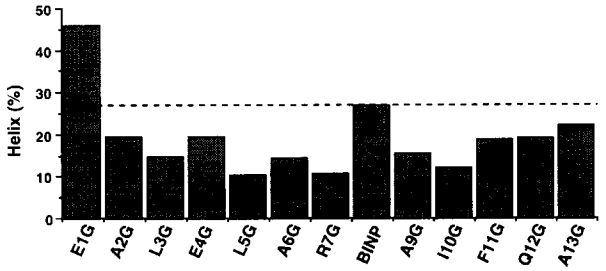

Fig. 3. Helix contents of BINP and its analogs calculated from CD spectra in TFE solution. The broken line indicates the helix content of BINP as a standard.

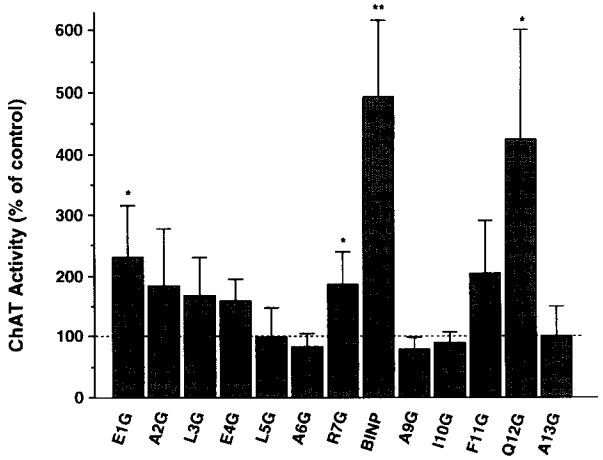

Fig. 4. Activity of BINP analogs on survival of septal cholinergic neurons in cultures. Cells from septal area of P10 rat brain were cultured as described in Section 2. On the next day of plating cells on culture dishes, each peptide or BINP was added in culture medium to a final concentration of 50 ng/ml or 5 ng/ml, respectively. Cells were cultured for 6 days and homogenized with 10 mM Tris-HCl, pH 7.5 containing 0.1% Triton X-100. ChAT activities in cell extracts were measured. ChAT activities of cells treated with peptide were compared with those of control culture (no addition). Each value is the mean of 4–5 samples. The ChAT activity of cells treated with NGF (100 ng/ml) was 473% of control (mean of 4 samples). $^{*}P < 0.05$, $^{**}P < 0.001$ by Student's t-test.

that the removal of a side chain of Glu[1] residue resulted in stabilization of helical conformation.

Neurotrophic activities of BINP and its analogs were measured using two different bioassays. The effects on supporting septal cholinergic neurons are summarized in Fig. 4. The analogs L5G, A6G, A9G, I10G, and A13G showed almost no activity at the concentration of 50 ng/ml. At higher concentration (10 μg/ml), the analogs E1G, A2G, L3G, E4G and R7G showed higher activities of 310, 226, 305, 457 and 469% of control (mean of 4–5 samples), respectively. These values were quite significant ($P < 0.01$) by Student's t-test, indicating that these analogs retained an intrinsic activity. On the other hand, the analogs L5G, A6G, A9G, I10G, and A13G showed no significant increase of activity even at this high concentration, suggesting that they do not interact with BINP receptor.

In another assay for rescuing neurons from injury caused by glutamate, the analogs A2G, L5G, A6G, A9G and I10G showed very low activity (Fig. 5). Although the results of the two assays were not completely in parallel as shown in the case of A2G and A13G, four residues at the central part, Leu[5], Ala[6], Ala[9] and Ile[10], were commonly essential for both activities.

4. Discussion

In many biologically active peptides, amino acid residues critical for the activity are the characteristic ones such as basic Arg or Lys, acidic Asp or Glu, or aromatic Tyr or Phe. It is interesting that Glu[1], Arg[7], Phe[11], and Gln[12] in BINP are not important for neurotrophic activity and all the essential residues are simple hydrophobic aliphatic amino acids. This may suggest that the hydrophobic interaction is critical for the binding of BINP to its target molecules.

Secondary structure prediction using the Chou and Fasman method [11] suggests that BINP tends to take on a helical conformation. This prediction was supported by CD spectrum analysis (Figs. 2 and 3). Interestingly, hydrophobic residues are clustered on one side of this helical conformation, and the hydrophilic ones on the other side (Fig. 6). It is also noteworthy that four Ala residues align almost straightforward in the hydrophobic side. According to the method of Eisenberg et al. [12], BINP has an average hydrophobicity value of 0.21 and a hydrophobic moment of 0.33, which are typical of the amphiphilic helix of a surface-seeking peptide.

Although the Gly substitution affected the conformation of the analogs (Fig. 3), the decrease of activity cannot simply be explained by the conformational changes. For example, R7G, which is one of the analogs with lowest helicity, was potent in both assays. Interestingly, all the residues essential for the activity are located on the hydrophobic side of the helix. It

Table 1
Amino acid sequences of BINP and its analogs[a]

Amino acid sequence	Peptide
Glu-Ala-Leu-Glu-Leu-Ala-Arg-Gly-Ala-Ile-Phe-Gln-Ala-NH_2	BINP
Gly-Ala-Leu-Glu-Leu-Ala-Arg-Gly-Ala-Ile-Phe-Gln-Ala-NH_2	E1G
Glu-**Gly**-Leu-Glu-Leu-Ala-Arg-Gly-Ala-Ile-Phe-Gln-Ala-NH_2	A2G
Glu-Ala-**Gly**-Glu-Leu-Ala-Arg-Gly-Ala-Ile-Phe-Gln-Ala-NH_2	L3G
Glu-Ala-Leu-**Gly**-Leu-Ala-Arg-Gly-Ala-Ile-Phe-Gln-Ala-NH_2	E4G
Glu-Ala-Leu-Glu-**Gly**-Ala-Arg-Gly-Ala-Ile-Phe-Gln-Ala-NH_2	L5G
Glu-Ala-Leu-Glu-Leu-**Gly**-Arg-Gly-Ala-Ile-Phe-Gln-Ala-NH_2	A6G
Glu-Ala-Leu-Glu-Leu-Ala-**Gly**-Gly-Ala-Ile-Phe-Gln-Ala-NH_2	R7G
Glu-Ala-Leu-Glu-Leu-Ala-Arg-Gly-**Gly**-Ile-Phe-Gln-Ala-NH_2	A9G
Glu-Ala-Leu-Glu-Leu-Ala-Arg-Gly-Ala-**Gly**-Phe-Gln-Ala-NH_2	I10G
Glu-Ala-Leu-Glu-Leu-Ala-Arg-Gly-Ala-Ile-**Gly**-Gln-Ala-NH_2	F11G
Glu-Ala-Leu-Glu-Leu-Ala-Arg-Gly-Ala-Ile-Phe-**Gly**-Ala-NH_2	Q12G
Glu-Ala-Leu-Glu-Leu-Ala-Arg-Gly-Ala-Ile-Phe-Gln-**Gly**-NH_2	A13G

[a]The substituted Gly residue is shown in bold.

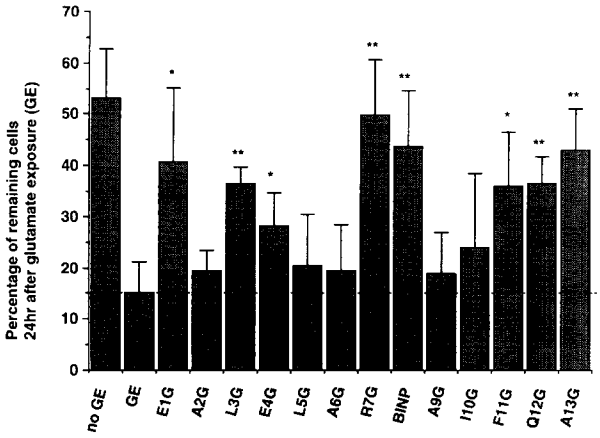

Fig. 5. Activity of BINP analogs on rescuing hippocampal neurons from injury caused by glutamate. Cells from hippocampus of E18 rat brain were cultured as described in Section 2. After being cultured for 5 days in DMEM containing serum, cells were washed with DMEM (−serum) and exposed to 1 mM glutamate for 30 min. After glutamate was removed from culture medium and washed with DMEM (−serum), cells were cultured for 24 h with or without peptide (10 nM). Numbers of neurons were counted and compared as described in Section 2. Each value is the mean of 8 samples. $^{*}P < 0.01$, $^{**}P < 0.001$ by Student's t-test.

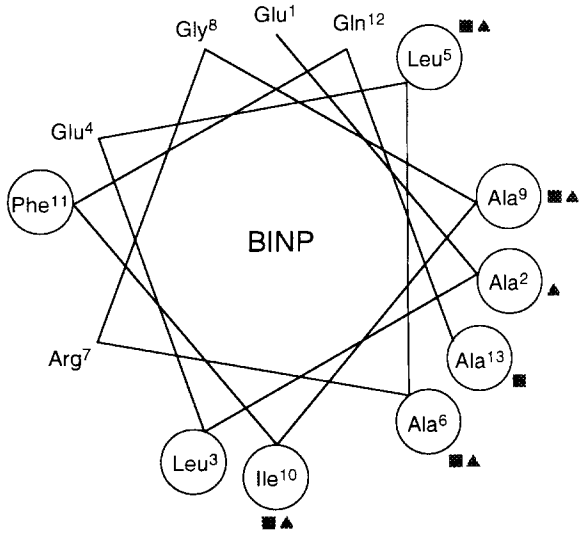

Fig. 6. Helical projection of BINP. Hydrophobic residues are indicated by circles. Squares and triangles indicate the residues important for the activity in the assays on cholinergic and hippocampal neurons, respectively.

is well known that some linear peptides take on a helical conformation when they bind to their receptors on the cell surface membrane, irrespective of their taking on a random conformation in aqueous solution [13]. Therefore, BINP may interact with its receptor on the membrane on the hydrophobic side of its amphiphilic helix. And differences between the two assays may indicate the differences of two kinds of receptors on two different neurons. Another explanation for differences of the activity may be the susceptibility of each peptide to secreted peptidases from each area of the brain in an assay medium. We are currently working on identifying the receptor for BINP and its parent molecule.

From the viewpoint of future clinical application, it is noteworthy that the N-terminal Glu residue is not essential for neurotrophic activity, because this N-terminal Glu residue tends to cyclize to a pyroglutamic residue and the resulting peptide has low solubility in aqueous solution. Synthesis and biological evaluation of the truncated analogs are now in progress in order to search for a smaller peptide having neurotrophic activity, which will be essential for future drug design.

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