RAT GONADOTROPIN-RELEASING HORMONE-ASSOCIATED PEPTIDE (GAP): BIOCHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION

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ABSTRACT

The purpose of this report is to characterize the biochemical and immunochemical properties of rat gonadotropin-releasing hormone-associated peptide (GAP) immunoreactivity (IR) in rat brain. Rat GAP(1-56) and related fragments such as GAP(46-56), GAP(41-56), GAP(34-56), GAP(28-56), and GAP(15-56) were synthesized by solid phase methodology. A GAP radioimmunoassay (RIA) was developed using synthetic rat GAP(28-56) and an antiserum generated against rat GAP(28-56) in rabbit. This RIA was used to characterize GAP-IR in extracts of the rat brain. The rat hypothalamus was found to contain the highest concentration of GAP-IR (298.70 ± 72.65 fmol/mg wet weight). Two GAP-IR forms were detected using gel chromatography. A large variant eluted near the position of a marker protein with a molecular weight of 25 K, and a smaller form coeluted with synthetic rat GAP. The larger variant coeluted with synthetic GAP when it was treated with 100 mM dithiothreitol in 8 M urea. These data demonstrate that the highest level of GAP-IR is found in the rat hypothalamus and that rat GAP and pro-LHRH may exist as a dimer. A carrier protein for GAP may also exist in the rat brain.

The amino acid sequence of the precursor protein for rat gonadotropin-releasing hormone (GnRH) was deduced from the nucleotide sequence of the cloned cDNA encoding the precursor protein using libraries derived from rat hypothalamic mRNA (2). This precursor protein contains the decapeptide, GnRH at the N-terminus followed by a Gly-Lys-Arg cleavage sequence and then a 56-amino acid peptide at the C-terminus. The C-terminal cryptic peptide, which was termed GnRH-associated peptide (GAP), shows approximately a 30% difference in the primary structures between the rat and human sequences. GAP-immunoreactivity (IR) has been detected by radioimmunoassay (RIA) and immunohistochemical studies in the rat

hypothalamus (1, 3, 6, 7, 9). In some of these studies, GAP-IR was detected by a RIA which used antisera generated against human GAP or its fragments. In order to elucidate more precisely the concentration and molecular forms of GAP in the rat, we synthesized rat GAP and its fragments. Using a GAP carboxy-terminal fragment GAP(28-56), we developed a RIA for rat GAP and characterized the distribution and molecular forms of GAP-IR in the rat brain.

MATERIALS AND METHODS

Synthesis of Rat GAP

The synthesis of rat GAP was carried out by a solid-phase technique using an automated

peptide synthesizer (Beckman, Model 990). H-Met-OCH₂-Pam-resin (Applied Biosystems, CA, U.S.A.) (3.4 g) was coupled with 0.8 mmol of Boc-Lys(Cl-Z)-OH in the presence of 0.8 mmol dicyclohexylcarbodiimide (DCC), and the unreacted free amino groups were blocked by acetic anhydride. The resulting protected dipeptide resin (5.0 mg) was hydrolyzed with 6 N HCl (0.5 ml) in propionic acid at 130°C for 3 h. Lysine content was quantitated by amino acid analysis and found to be 0.25 mmol/g of resin. Using this dipeptide-OCH₂-Pam-resin (3.38 g) as the starting material, appropriate Bocamino acids were coupled by the DCC method according to the program used for the synthesis of PHM (5). *N*^α-Bocamino acid derivatives used in this study were Asn, Gln, Gly, Ala, Val, Ile, Leu, Phe, Met, Trp, Asp(OcHex), Thr(Bzl), Glu(OBzl), Lys(Cl-Z), His(Tos), Arg(Tos) and Cys(4-CH₃-Bzl). A 4-fold excess of each *N*^α-Bocamino acid derivatives was used for the acylation in CH₂Cl₂ with the exception of Boc-Asn, Boc-Gln and Boc-Arg(Tos), which were coupled in DMF in the presence of 1-hydroxybenzotriazole. TFA-CH₂Cl₂ (50%) containing 0.2% 1,2-ethadithiol was used for removal of the *N*^α-Boc group of the peptide resin containing methionine and tryptophan residues. Throughout chain elongation, a portion of the peptide-resin (500 mg each) was removed from the reaction vessel for preparation of rat GAP(46-56), GAP(41-56), GAP(34-56), GAP(28-56) and GAP(15-56), after introduction of Boc-Leu (position 46), Boc-Gly (position 41), Boc-Asp(OcHex) (position 38), Boc-Ser(Bzl) (position 34), Boc-Thr(Bzl) (position 28), and Boc-Glu(OBzl) (position 15), respectively. After introduction of Boc-Asn, the amino terminal amino acid, a protected peptide-resin of rat GAP (3.67 g) was obtained. A portion of the protected peptide-resin (1.0 g) was treated with redistilled HF (10 ml) in the presence of anisole (1.0 ml) and L-methionine (1.0 g) at 0°C for 60 min. HF was removed at 0°C, and then the residue was retreated with 50% HF in anisole (v/v, 10 ml) at 0°C for 60 min to remove the 4-CH₃-Benzyl group used for the protection of the cysteine residue. After evaporation of HF, the residue was washed three times with ethyl acetate (30 ml each) and the crude peptide was extracted three times with nitrogen gas-

bubbled 3 M CH₃OH (30 ml each). Extracts were combined and lyophilized. The resultant powder was dissolved in 1 M CH₃OH (10 ml) and the solution was subjected to gel filtration on a Bio-Gel P-10 column (3.5×190 cm) using nitrogen gas-bubbled 1 M CH₃OH as an eluent. All fractions (10 g each) containing peptide were examined by analytical reverse-phase high performance liquid chromatography (HPLC) (column: YMC Pack R-ODS-5, 0.46×25 cm; eluent: 0.01 N HCl/CH₃CN, 75/25→50/50, v/v, 30 min; detection: 210 nm; flow rate: 1 ml/min). Fractions containing the desired product (fractions No. 55-74) were collected and lyophilized (yield, 186 mg). The crude peptide thus obtained was dissolved in 10 ml of 0.01 N HCl and the solution was subjected to HPLC using a column, YMC Pack D-ODS-5 (2×25 cm) and eluted with a solvent system of 0.01 N HCl/CH₃CN (75/25→50/50, v/v, 30 min). Eluates were monitored by absorbance at 210 nm. Fractions containing the desired material were collected and lyophilized. Further purification was achieved by reverse-phase HPLC using the same column as above with a solvent system of 0.01 N HCl/CH₃CN (75/25→60/40, v/v, 30 min) (yield, 12.6 mg).

Stability Test of Synthetic Rat GAP

The stability of synthetic rat GAP was examined by HPLC. Synthetic rat GAP (100 μg) was dissolved in nitrogen gas-bubbled 0.01 N HCl (500 μl), the solution was allowed to stand at 24°C for 1 and 24 h, respectively and then chromatographed on HPLC (column: YMC Pack R-ODS-5, 0.46×25 cm; eluent: 0.01 N HCl/CH₃CN, 75/25→60/40, v/v, 30 min; detection: 210 nm; flow rate: 1 ml/min). A part of the peak was used for amino acid analysis. The product thus obtained (50 μg) was treated with 1 mM dithiothreitol (DTT) (Sigma Chemical, St. Louis, MO, U.S.A.) (1.0 ml) for 48 h at 37°C to cleave the dimer product of the rat GAP.

Production of Anti-Rat GAP(28-56) Antiserum

Rat GAP(28-56) (10 mg) was conjugated to bovine serum albumin (BSA; 25 mg) by the method described previously (9). The result-

ing conjugate (3 mg) was emulsified with complete Freund's adjuvant (1.5 ml; Carbiochem-Behring, La Jolla, CA, U.S.A.) and then the emulsion was given intradermally into three mixed bred female rabbits. Each rabbit received approximately 1 mg of the conjugate for the primary immunization. Booster injections were given at two week intervals using half the dose of the conjugate used for the primary immunization. After the fourth immunization, the rabbit was bled from the marginal ear vein 10 days following each booster injection. Serum was then subjected to a titer check.

Rat GAP RIA

Since tryptosine and histidine residues are not present in rat GAP(28-56), 3-(4-hydroxyphenyl)-propionylated rat GAP(28-56) [HPP-rat GAP(28-56)] was synthesized for radioiodination. HPP-rat GAP(28-56) was iodinated by the chloramine T method (4) and the iodinated compound was purified on a Sephadex G10 column (1.0×30 cm) with 1 M acetic acid as the eluent. The RIA buffer was 0.01 M phosphate-buffered saline (pH 7.4) containing 0.025 M EDTA and 0.5% BSA. To assay tubes, standard rat GAP(28-56) or specimen (0.1 ml) and appropriately diluted anti-rat GAP(28-56) serum (R-0246) (0.1 ml; final dilution 1:70,000) were added. The mixture was incubated at 4°C for 48 h. ¹²⁵I-HPP rat GAP(28-56) was then added, and the mixture was further incubated at 4°C for 24 h. Separation of bound and free iodinated ligands was accomplished by the second antibody-polyethylene glycol (PEG) method (9). Briefly, bound and free tracer ligands were separated by the addition of 0.1 ml of diluted normal rabbit serum (1:150, Daiichi Radioisotope Labs., Tokyo), 0.1 ml of diluted goat anti-rabbit IgG serum (1:30, Daiichi Radioisotope Lab.) and 0.5 ml of 10% (w/v) PEG. The mixture was incubated at 4°C for 3 h, and then centrifuged at 3,000 rpm at 4°C for 30 min. The free ligand in the supernatant was aspirated and the precipitate was counted in a gamma counter. Standard curves were constructed and unknown values interpolated with a computer program which utilized a log-logit transformation to produce linear dose-response curves.

Extraction of Rat Brain Tissues

Fresh rat brains (Male Wistar rats, approximately 250 g) were removed and dissected. The dissected tissues were homogenized by a polytron homogenizer in a 5-fold volume excess of 0.1 M acetic acid in an ice bath. Homogenates were heated at 100°C for 10 min, adjusted to 1 M concentration with glacial acetic acid and then centrifuged at 15,000 g for 30 min at 4°C. Supernatants were saved and the pellets were washed with 1 M acetic acid and recentrifuged. Supernatants were pooled and lyophilized. Lyophilized extracts were constituted in the RIA buffer for assay.

Sephadex Chromatography

Sephadex G-50 superfine chromatography was done on a column (1.6×100 cm) eluted with 1 M acetic acid. Fractions (2 ml each) were collected, lyophilized, and reconstituted in assay buffer for RIA of rat GAP-IR.

Chemical Treatment of Rat Brain Extracts

The 25 K GAP-IR peak detected upon Sephadex chromatography was collected and lyophilized. The resultant powder was dissolved in 100 mM DTT (1.5 ml) containing 8 M urea. After incubation for 24 h at 24°C, the mixture was acidified with glacial acetic acid to 1 M concentration and then subjected to Sephadex G-50 column chromatography (1.6×100 cm). Fractions were assayed by RIA for rat GAP to monitor the elution position of GAP-IR.

RESULTS

Synthesis of Rat GAP

The purified peptide showed a sharp single peak on analytical HPLC (Fig. 1). The primary structure of the purified material was confirmed by sequence analysis of each tryptic fragment. The amino acid ratio in an acid hydrolysate (6 N HCl, 110°C, 24 h) was: Asp(5)4.91, Thr(2)1.89, Ser(2)1.86, Glu(14)-14.35, Gly(3)3.05, Ala(3)3.03, Val(2)1.98, Met(3)2.86, Ile(1)0.95, Leu(5)5.21, Phe(2)-1.89, His(2)1.89, Lys(3)3.04, Arg(4)3.95 and Pro(3)2.96. The retention time on HPLC was 17.0 min (column: YMC Pack R-ODS-5,

Fig. 1 Analytical HPLC of synthetic rat GAP. Column: YMC Pack R-ODS-5, 0.46×25 cm. Eluent: 0.01 N HCl/CH₃CN, 75/25→60/40, v/v, 30 min. Detection: 210 nm. Flow rate: 1 ml/min

0.46×25 cm; eluent: 0.01 N HCl/CH₃CN, 75/25→60/40, v/v, 30 min; detection: 210 nm; flow rate: 1 ml/min). R_f^I 0.01 (1-C₄H₉OH:CH₃OH:H₂O=4:1:5, upper phase). R_f^{II} 0.11 (1-C₄H₉OH:pyridine:CH₃OH:H₂O=30:20:6:24). $[\alpha]_D^{25} -209^\circ$ (c 0.1 1 M CH₃OH).

Chemical Stability Test

As shown in Fig. 2a, after a 1 h incubation of synthetic rat GAP(I-56) in nitrogen gas-bubbled 0.01 N HCl, a small peak, which was undetected previously, appeared at 21 min upon HPLC. After incubation of rat GAP in 0.01 N HCl for 24 h, a main peak eluted at 21 min (Fig. 2b). The amino acid ratio of the product which eluted at 21 min was found to be identical to that of rat GAP(I-56). After treatment of the fraction with a retention time of 21 min with DTT, a major peak appeared at 17 min, which coincides with the retention time of rat GAP(I-56) (Fig. 2c).

Development of a Rat GAP C-Terminal Specific RIA

One of the immunized rabbits produced an anti-rat GAP(28-56) serum (R-0246) which bound approximately 30% of the tracer, ¹²⁵I-HPP-rat GAP(28-56), at a final dilution of 1:70,000. Fig. 3 shows a characterization of

Fig. 2 Analytical HPLC of synthetic rat GAP. Rat GAP was rechromatographed after incubation with 0.01 N HCl for 1 h (a) and 24 h (b). A portion of the main peak in (b) was treated with DTT for 48 h at 37°C and rechromatographed (c). Column: YMC Pack R-ODS-5, 0.46×25 cm. Eluent: 0.01 N HCl/CH₃CN, 75/25→60/40, v/v, 30 min. Detection: 210 nm. Flow rate: 1 ml/min

this antiserum (R-0246). This antiserum did not recognize LHRH and cross-reacted very weakly with human GAP(25-56) (0.002% on a molar basis). Based on the cross-reactivities of synthetic rat GAP(38-56), rat GAP(34-56), rat GAP(28-56) and rat GAP, the immunogenic determinant of this antiserum seems to be localized within the 38-56 sequence of rat GAP. The sensitivity and ID₅₀ (50% inhibition of radiolabeled ligand) of the present rat GAP RIA are 8 and 128 fmol/tube, respectively.

Fig. 3 Characterization of anti-rat GAP(28-56) serum (R-0246). R-0246 was used at a final dilution of 1:70,000, and ^{125}I -HPP-rat GAP(28-56) was used as tracer. Rat GAP(38-56) (▲), rat GAP(34-56) (■), rat GAP(28-56) (●), rat GAP (★), human GAP(25-56) (Δ) and LHRH (○)

Table 1 Distribution of GAP Immunoreactivity in Rat Brain

Region	Immunoreactivity (fmol/mg wet weight)
Cerebral cortex	0.72 ± 0.02
Corpus striatum	0.78 ± 0.07
Hippocampus	0.62 ± 0.06
Thalamus	0.95 ± 0.06
Midbrain	0.76 ± 0.09
Pons	0.66 ± 0.03
Cerebellum	0.58 ± 0.07
Medulla oblongata	0.67 ± 0.13
Hypothalamus	298.70 ± 72.65
Pituitary	4.16 ± 1.28

Mean $\pm$ SEM, $n=5$

GAP-IR in Rat Brain Extracts

Table 1 shows the concentrations of rat GAP-IR in rat brain tissue extracts assayed by the present rat GAP C-terminal specific RIA. The hypothalamus contains the highest level of GAP-IR (298.70 ± 72.65 fmol/mg wet weight) when compared to extracts of other brain regions. For example, the pituitary and thalamus contain 4.16 ± 1.28 and 0.95 ± 0.06 fmol/mg wet weight, respectively.

In a crude extract of the rat hypothalamus, two GAP-IR peaks are detected upon gel chromatography. One elutes near the position of a 25 K marker protein, and another coelutes with synthetic rat GAP (Fig. 4a). When the

Fig. 4 Gel chromatography of rat hypothalamus extracts. The samples were chromatographed on a Sephadex G-50 superfine column (1.6×100 cm). Crude extract of rat hypothalamus (a). Rechromatography of the component collected from the large peak of the crude extract with (c) and without (b) treatment of 100 mM DTT in 8 M urea.

larger molecular form was incubated with distilled water at room temperature for 24 h and then rechromatographed, only one GAP-IR peak was observed. This peak eluted with same K_{ad} as the larger peak of the original extract (Fig. 4b). However, when the larger variant form was treated with 100 mM DTT in 8 M urea, the majority of the immunoreactivity coeluted with synthetic GAP (Fig. 2c).

DISCUSSION

In the present study, rat GAP and its related fragments, such as rat GAP(15-56), GAP(28-56), GAP(34-56), GAP(41-56), and GAP(46-56) were synthesized. Rat GAP, a 56-amino

acid peptide, was synthesized with high yield, and careful assessment of the product showed that this synthetic peptide and its related fragments were highly homogeneous. In addition, during the purification of crude synthetic rat GAP, a HPLC peak was observed with a retention time of 21 min. Interestingly, this product has an amino acid composition identical to that of synthetic rat GAP (retention time, 17 min). Treatment of this material with DTT converted the retention time to 17 min, using the HPLC conditions described in the present study. These results suggest that dimerization of rat GAP occurs easily. According to a hypothetical three-dimensional model of human GAP, a loop encompassing residues 23–34 exposes a hydrophilic region which can result in dimerization of GAP via a Cys residue at position 27 (8).

Characterization of our antiserum (R-0246) showed that the antiserum did not cross-react with LHRH. In addition, it showed negligible cross-reactivity with synthetic human GAP(25–56) (0.002%), while it cross-reacted similarly with synthetic rat GAP and examined fragments. These results indicate that the antiserum (R-0246) is specific for the C-terminal of rat GAP.

Using the presently described rat GAP RIA, distribution of GAP-IR in the rat brain was examined. The results show that the highest level of GAP-IR occurs in the hypothalamus (298.70 ± 72.65 fmol/mg wet weight). This level is much higher than the level reported previously (3.87 ± 0.09 fmol/mg wet weight) using an antiserum (R-0242) that was generated against human GAP(34–56) (9). This difference may reflect the different affinities of the anti-rat GAP(28–56) serum (R-0246) and anti-human GAP(34–56) serum (R-0242) for the rat GAP molecule.

Gel chromatography of a rat hypothalamic extract showed two GAP-IR forms: one eluted in the position of a 25 K marker protein and another coeluted with synthetic rat GAP. Interestingly, treatment of the larger molecular weight peak (25 K) with 100 mM DTT in 8 M urea converted it to a gel chromatography peak that coeluted with synthetic rat GAP. The present results strongly suggest the presence of GAP and a dimer of GAP with a car-

rier protein in rat hypothalamic extracts.

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