Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 2009–2013, April 1985
Biochemistry

Brain-specific polypeptide 1B236 exists in multiple molecular forms

(peptides/proteases/radioimmunoassay/post-translational processing)

BERNARD MALFROY*, CHARLES BAKHIT*, FLOYD E. BLOOM*, J. GREGOR SUTCLIFFE†, AND ROBERT J. MILNER*‡

Departments of *Basic and Clinical Research and of †Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037

Contributed by Floyd E. Bloom, December 3, 1984

ABSTRACT **The COOH-terminal amino acid sequence of the rat brain-specific polypeptide 1B236 was previously deduced from molecular cloning and nucleotide sequence determination of its mRNA and the 1B236 protein shown to exist in the rat brain. The amino acid sequence of 1B236 contained at least three peptide sequences demarcated by pairs of basic amino acids—a structure similar to known neuropeptide and hormone precursors—which suggested that the protein might be processed *in vivo* to generate peptides. We have developed radioimmunoassays specific for 1B236 with antibodies against three synthetic peptides corresponding to putative cleavage products of this protein and have used these assays to define the molecular forms of 1B236 in rat brain extracts. The most abundant form is of high molecular weight (*ca.* 100,000) and requires detergent for solubilization; hence, it is probably membrane-bound. However, a small fraction of the high molecular weight material is soluble in the absence of detergent. In addition, several low molecular weight species are detectable in brain extracts prepared under conditions preventing proteolysis. These molecules correspond in size to two of the possible products of proteolytic processing predicted from the amino acid sequence of 1B236. The multiplicity of 1B236 forms, together with other data, suggests that this protein undergoes extensive post-translational modification, including proteolytic processing to generate peptides that may be physiologically relevant.**

Our laboratories (1, 2) have described an experimental strategy that utilizes recombinant DNA techniques to identify and characterize brain-specific molecules. A cDNA library was generated from rat brain poly(A)$^+$ mRNA and screened for clones of mRNAs expressed in brain but not in nonneuronal organs such as liver or kidney. Individual brain-specific clones were analyzed by nucleotide sequencing, providing the sequence of the corresponding mRNA and the amino acid sequence of its encoded protein. The novelty of each amino acid sequence was established by computer comparison with a protein sequence data base. To identify the novel proteins corresponding to these clones, we made antibodies against synthetic peptides mimicking several selected regions of the putative protein sequences.

One such brain-specific cDNA clone (p1B236) was studied in detail by this approach (2). This clone corresponded to a mRNA present in brain with an abundance of ≈0.01% but not detectable in liver or kidney (1). The nucleotide sequence of p1B236 provided the novel 318-amino acid carboxyl-terminal sequence of the corresponding protein 1B236. The relationships between the 1B236 mRNA, the cDNA clone p1B236, and the putative open reading frame for the protein 1B236 are shown in Fig. 1*A*. One of the few notable features of the 1B236-amino acid sequence was the presence

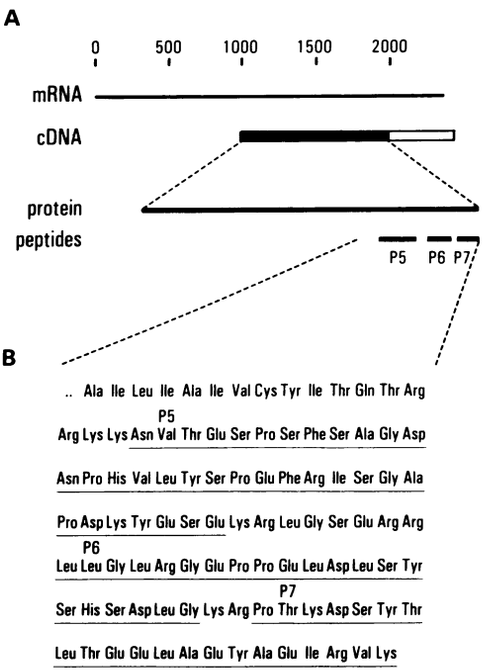

FIG. 1. Structure of the 1B236 protein. (*A*) The 1B236 mRNA, the p1B236 cDNA clone, and the 1B236 protein are schematically represented (2). The mRNA is ≈2500 nucleotides in length: the cDNA clone p1B236 corresponds to the 3′ 1500 nucleotides. The open reading frame in the cDNA sequence is shown by the filled bar; the 3′ noncoding region is shown by the open bar. Also indicated are the positions of peptides P5, P6, and P7. (*B*) The carboxyl-terminal sequence of the 1B236 protein is shown, with peptides P5, P6, and P7 underlined. Notice the pairs of basic amino acids flanking the underlined peptides.

of several pairs of basic amino acids (Arg-Arg-Lys-Lys, Lys-Arg, Arg-Arg, Lys-Arg) in its COOH-terminal region (Fig. 1*B*). Such sequences, particularly the dipeptide Lys-Arg, previously have been found to demarcate neuropeptides or other peptide hormones in their precursor proteins and have been shown to be the sites of proteolytic processing to generate bioactive peptides (3, 4). In this respect, 1B236—a wholly novel protein—appeared to resemble neuropeptide or peptide hormone precursors.

Based on this structural similarity, we postulated that the 1B236 protein might be proteolytically processed *in vivo* to generate physiologically relevant neuropeptides and suggested that the most likely cleavage products would be the peptides P5, P6, and P7 (Fig. 1*B*) (2). To detect and characterize the 1B236 protein and at the same time to test this hypothesis, we selected these peptides for synthesis and antibody

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: NP-40, Nonidet P-40.

‡To whom reprint requests should be addressed.

production. These antibodies were used for immunocytochemical localization of 1B236 and detected immunoreactive material in neuronal cells and fibers distributed throughout the rat central nervous system. Immunoreactivity was prominent in hindbrain, particularly spinal cord and cerebellum, midbrain structures such as hippocampus, and cingulate and somatosensory cortex. The patterns were distinct from any other previously characterized neuronal system (2, 5).

In this report we describe the detection of the 1B236 protein in brain extracts, using specific RIAs developed with the antibodies against the synthetic peptide fragments of 1B236. Our data provide evidence for the existence of both high molecular weight and peptide forms of 1B236, suggesting that this protein is indeed subjected to extensive proteolytic processing *in vivo* and providing further support for the hypothesis that the 1B236 molecule is the precursor for a previously undescribed family of neuropeptides.

METHODS

RIA of 1B236 Peptides. Rabbit antibodies directed against synthetic peptides P5, P6, and P7 from the 1B236 protein were obtained as described (2). Synthetic peptides P5, P6, and P7 were radiolabeled by the chloramine-T procedure (6) and purified by chromatography through Sep-Pak cartridges (Waters). The iodinated peptides were eluted with 5 mM trifluoroacetic acid containing 50% acetonitrile and were stored at −20°C. Each RIA tube contained 100 μl of antibody diluted in buffer A [150 mM sodium phosphate buffer (pH 7.5) containing 0.1% bovine serum albumin (Sigma) and 0.1% Triton X-100 (Bio-Rad)], 100 μl of iodinated peptide (≈6000 cpm) in buffer A, and 300 μl of peptide standard or brain extract in buffer B (one part of buffer A plus one part of 0.1 M HCl containing 1% Triton X-100). Antisera used were: P5, rabbit 5862 at 1:25,000 final dilution; P6, rabbit 5751 at 1:5000; and P7 rabbit 5864 at 1:2500. After 14–20 hr at 4°C, 1 ml of ice-cold 150 mM sodium phosphate buffer (pH 7.5) containing 5% activated charcoal (Sigma), 0.3% dextran (Sigma), and 15% heat-deactivated horse serum (M. A. Bioproducts, Walkersville, MD) was added, and each tube was mixed in a Vortex mixer and centrifuged at 3000 × *g* for 30 min (7). The radioactivity in each supernatant (containing the antibody–antigen complexes) was measured in a Micromedic 10/600 multichannel gamma counter directly interfaced with an Apple IIe microcomputer. The standard curves (serial dilutions of cold synthetic peptide, ranging from 7.5 fmol to 1 pmol per tube) were analyzed, and the antigenic equivalents in each tube were calculated by using the Four-Parameter Logistic Method (8) adapted for the Apple II microcomputer (M. L. Jaffe, P.O. Box 846, Silver Spring, MD 20901). Each peptide was detected specifically by its appropriate antibody, with no crossreactivity from the other two peptides. The detection limit in each assay was about 20 fmol per tube.

Extraction Procedures. Male 180- to 200-g Sprague–Dawley rats (Charles River Breeding Laboratories), housed under standard conditions, were used in all experiments. After decapitation and dissection, the whole brain was extracted by one of the following two procedures: (*i*) homogenization (Polytron, Brinkmann) in 8 ml of ice-cold 10 mM Tris·HCl buffer, pH 7.5/150 mM NaCl (Tris/NaCl), followed by centrifugation for 60 min at 25,000 × *g*; and (*ii*) homogenization in the same buffer containing 1% Nonidet P-40 (NP-40) (BDH) (Tris/NaCl/NP-40), followed after a 30-min incubation on ice by centrifugation (60 min at 25,000 × *g*). In each case, the supernatants were taken for further study. In some experiments, immediately after dissection, the brains were sealed in 50-ml polypropylene tubes (Corning) and exposed to microwave irradiation for 2 min at maximal power in a microwave oven and then extracted by one of the above procedures.

Gel Filtration Chromatography. Aliquots (2 ml) of brain extracts were loaded on a 1 × 50 cm column of Sephadex G-75 (coarse) equilibrated and run at 4°C under gravity in buffer B. Fifty fractions (≈900 μl each) were collected and directly assayed by RIA. Brain extracts also were fractionated on HPLC protein analysis columns. Aliquots (500 μl) of brain extracts were loaded on two Protein Pak 300 SW protein analysis columns (Waters) connected in series and were eluted at 0.5 ml/min with 100 mM sodium phosphate buffer, pH 7.4/30% acetonitrile. One hundred fractions (≈350 μl each) were collected and diluted with an equal volume of buffer B before RIA.

Stability and Recovery Studies. Brains were homogenized by the procedures described above (Tris/NaCl, with or without prior microwave irradiation) or in 8 ml of 2 M acetic acid/1% Triton X-100. Two hundred microliters of each homogenate were incubated with 300 μl of the corresponding extraction buffer containing 100 pmol of synthetic peptides P5, P6, or P7. The incubations were stopped immediately or after 2, 15, 30, and 60 min at 25°C by heat denaturation in boiling water for 10 min. The incubation media were diluted 200 times in buffer B and assayed for P5, P6, and P7 by RIA.

RESULTS

To define the molecular forms of 1B236, we extracted rat brains under a variety of different conditions and centrifuged the extracts to remove insoluble material. Aliquots of each

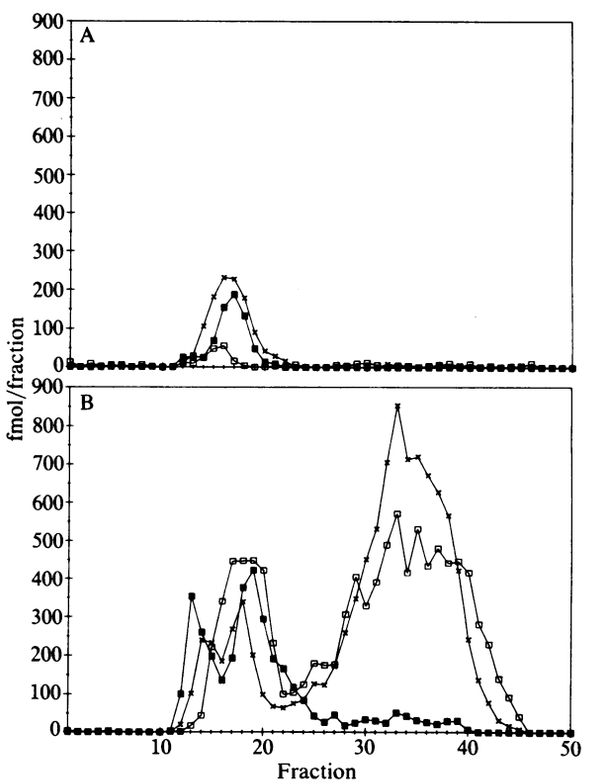

FIG. 2. Molecular forms of 1B236 in neutral extracts of fresh rat whole brain. Whole rat brains were extracted in 8 ml of Tris/NaCl (*A*) or Tris/NaCl/NP-40 (*B*), and a 2-ml aliquot of each extract was subjected to Sephadex G-75 chromatography. Eluted fractions were directly assayed for P5 (■), P6 (×), and P7 (□) immunoreactivity. The volume assayed in each fraction corresponds approximately to an extract of 1/12th of a brain. Values represent means of three to six independent experiments.

Table 1. Molecular forms of 1B236 in rat brain extracts

Extraction condition*		1B236-peptide immunoreactivity†					
		P5		P6		P7	
NP-40	μwave	HMW	LMW	HMW	LMW	HMW	LMW
Without	Without	10 ± 2 (4)	ND	13 ± 2 (5)	ND	1.6 ± 0.5 (3)	ND
With	Without	41 ± 10 (6)	ND	27 ± 9 (6)	121 ± 31 (6)	20 ± 9 (3)	61 ± 1 (3)
Without	With	ND	13 ± 3 (5)	ND	ND	2 ± 1 (4)	8 ± 2 (4)
With	With	4 ± 2 (3)	18 ± 6 (3)	3 ± 2 (3)	2 ± 1 (3)	5 ± 3 (3)	13 ± 3 (3)

*Brains were extracted in Tris/NaCl with and without 1% NP-40 and with and without prior microwave (μwave) irradiation.

†Amounts of high molecular weight (HMW) and low molecular weight (LMW) P5, P6, and P7 immunoreactive material were calculated from Sephadex G 75 column profiles and were expressed as pmol of antigenic equivalents per brain. Numbers of independent experiments are shown in parentheses. ND = not detectable (<1 pmol per brain).

supernatant were fractionated by gel filtration on Sephadex G-75, and the column fractions were assayed for 1B236 with each of the three RIAs directed against P5, P6, and P7. We observed that 1B236 immunoreactive material could be divided into distinct high and low molecular weight forms (Fig. 2). High molecular weight material was eluted in the void volume of the Sephadex G-75, with a mass of >13 kDa (cytochrome *c*), whereas low molecular weight material was eluted in the volume of the column, with a mass of <13 kDa. The results from the different extraction conditions are summarized in Table 1.

When brains were extracted with neutral saline (Tris/NaCl), only high molecular weight material could be detected with each of the three RIAs (Fig. 2*A*). Addition of a nonionic detergent (NP-40) to the extraction buffer resulted both in an increase in the amounts of P5, P6, and P7 high molecular weight immunoreactive material extracted and in the appearance of large amounts of immunoreactive P6 and P7 low molecular weight material (Fig. 2*B*). The Sephadex G-75 profile of detergent extracts seems to suggest the existence of two peaks of high molecular weight materials. We observed, however, that the two peaks were not apparent when the fractions were diluted prior to RIA (Fig. 3), suggesting that the apparent valley separating these peaks resulted from the interference of high protein concentration in each RIA. Moreover, an immunoblot analysis with anti-P5 antibody of fractions eluted from Sephadex G-75 revealed a single high molecular weight band of ≈100 kDa (Fig. 3).

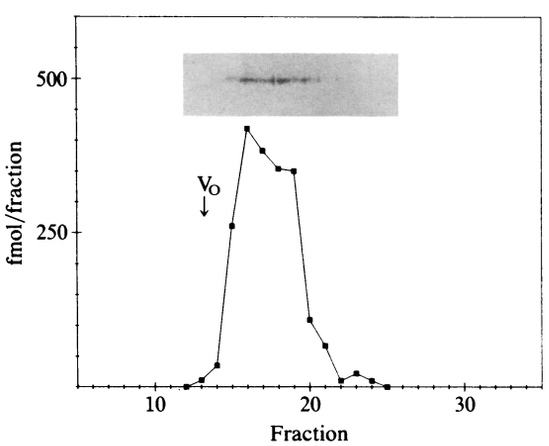

FIG. 3. RIA and immunoblot analysis of high molecular weight 1B236. An extract of brain in Tris/NaCl/NP-40 was fractionated on Sephadex G-75, and fractions were assayed for 1B236 by RIA for P5. In parallel, aliquots of each fraction were assayed for 1B236 by immunoblotting (9) with anti-P5 antibodies: the photograph of the blot showing the immunoreactive band of molecular weight 100,000 is aligned so that each gel slot corresponds to its appropriate fraction.

The view that high molecular weight P5, P6, and P7 immunoreactive material in Tris/NaCl/NP-40 brain extracts consists mainly of a single species is further supported by HPLC analysis using a protein analysis column, which provides separation by molecular size (Fig. 4). As shown with assays for P5 and P7 (P6, not shown), the high molecular weight immunoreactive material was eluted between myosin and bovine serum albumin, consistent with a molecular weight of 100,000. The low molecular weight P7 immunoreactive species migrated as a broad peak between the elution positions of synthetic P5 (34 residues) and [Leu^5]enkephalin (five residues). In addition, the HPLC analysis of the Tris/NaCl/NP-40 extracts also revealed a small amount of a low molecular weight P5 immunoreactive form with an elution position similar to enkephalin (Fig. 4).

A different pattern emerged when rat brains were heated rapidly by microwave irradiation immediately after dissection and before Tris/NaCl or Tris/NaCl/NP-40 extraction (Fig. 5). The most noticeable difference was that low molecular weight P5 immunoreactive material could be consistently detected: similar amounts were found with or without detergent (Fig. 5; Table 1). Furthermore, low molecular weight P7 immunoreactive material could now be detected in ex-

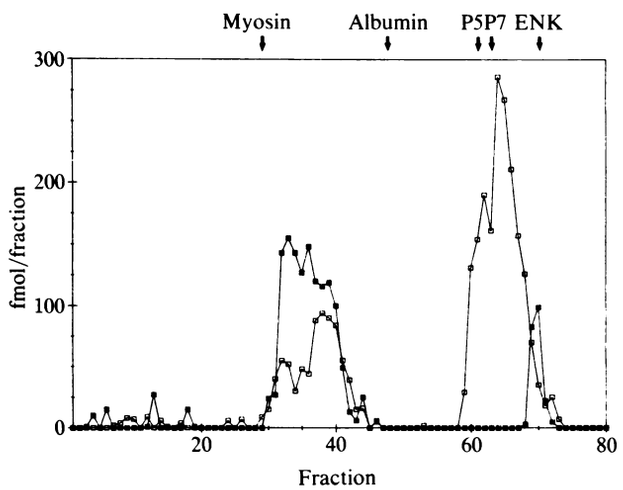

FIG. 4. HPLC analysis of 1B236 molecular forms in a extract of fresh rat whole brain. A 500-μl aliquot of a Tris/NaCl/NP-40 extract from rat whole brain was subjected to HPLC analysis on two protein analysis columns mounted in series. Eluted fractions (350 μl) were diluted with an equal volume of buffer B and assayed by RIA for P5 (■) and P7 (□). The volume assayed in each fraction corresponds to ≈1/32th of a brain. This experiment was repeated three times with similar results. The elution positions of standard proteins and peptides are indicated by arrows: myosin, bovine serum albumin, B chain of insulin, and [Leu^5]enkephalin (ENK) (5 μg each) were detected by absorbance at 210 nm; synthetic peptides P5 and P7 (5 pmol) also were injected and detected by RIA.

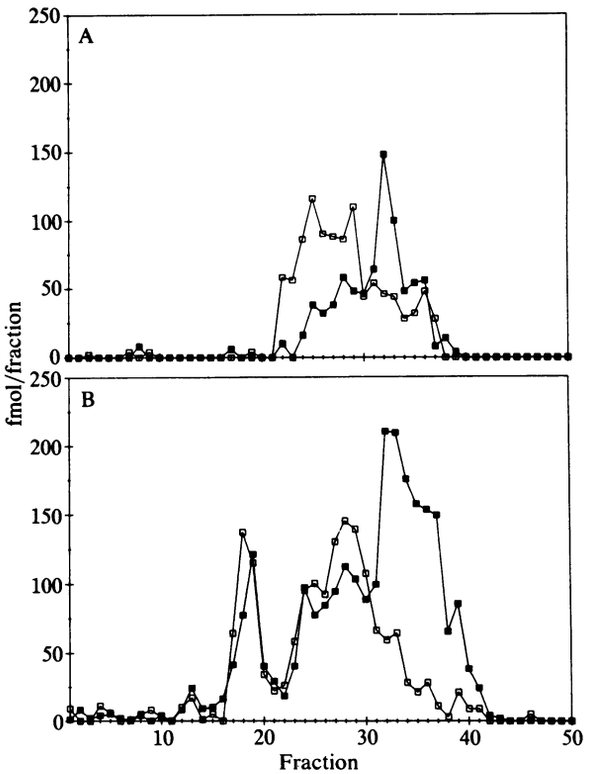

FIG. 5. Molecular forms of 1B236 in neutral extracts of a rat whole brain after microwave irradiation. Brains were subjected to microwave irradiation immediately after dissection and extracted in Tris/NaCl (*A*) or Tris/NaCl/NP-40 (*B*). A 2-ml aliquot of each extract was subjected to Sephadex G-75 chromatography, and eluted fractions were assayed directly by RIA for P5 (■) and P7 (□) as described for Fig. 2. The data shown are the means of three to five experiments.

tracts in the absence of detergent. An approximately equal amount of low molecular weight P7 was also recovered in the presence of detergent; however, the amount detected was considerably reduced compared to detergent extracts of fresh brains (Table 1). Similar results were obtained when brains were extracted in 2 M acetic acid (not shown). Microwave irradiation also resulted in a large decrease in the recoverable amounts of high molecular weight 1B236, soluble and detergent-extracted (compare Fig. 5*A* versus Fig. 2*A* and Fig. 5*B* versus Fig. 2*B*; see Table 1). In addition, using either of these extraction conditions, we consistently have been unable to detect any P6 immunoreactivity after microwave treatment (Table 1); we have no simple explanation for this loss of antigenic material. HPLC analysis on a protein analysis column of Tris/NaCl extracts of brains subjected to microwave irradiation revealed that the low molecular weight P7 and P5 immunoreactive species migrated in the same positions as the corresponding synthetic peptides. A smaller P5 peak also was observed at the position of enkephalin (Fig. 6).

To assay for the effects of endogenous peptidases on the stability and recovery of 1B236-derived peptides, small, known amounts of unlabeled synthetic peptides were incubated with brain homogenates prepared under various conditions, and their recovery was evaluated by RIA after different times. As shown for P5 (Fig. 7; similar results were obtained for P6 and P7 but are not shown), the peptides were rapidly degraded in Tris/NaCl homogenates of fresh brains but were completely stable either in an acid homogenate or in a Tris/NaCl homogenate from a microwave-irradiated brain for at least 1 hr at room temperature.

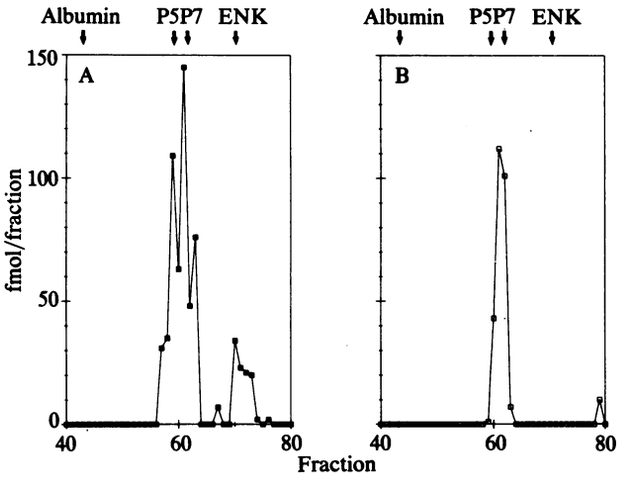

FIG. 6. HPLC analysis of low molecular weight forms of 1B236 in extracts of rat whole brain after microwave irradiation. A rat whole brain was subjected to microwave irradiation immediately after dissection, extracted in Tris/NaCl, and a 500-μl aliquot was injected on two HPLC protein analysis columns mounted in series. The eluted fractions were assayed by RIA for P5 (*A*) and P7 (*B*) as described for Fig. 4. This experiment was repeated four times with similar results.

DISCUSSION

In this paper we report the existence of high and low molecular weight forms of the brain-specific polypeptide 1B236, detected using RIAs against three nonoverlapping peptide regions of this protein. The high molecular weight material is found predominantly in a form requiring detergent for solubilization, but a fraction also exists as a soluble protein. Low molecular weight 1B236 species of peptide size also can be reproducibly detected under conditions where endogenous protease activity is abolished. This material probably corresponds to peptides present *in vivo*.

The experimental approach we chose was to prepare rat brain extracts under conditions suitable for the extraction of protein or peptide-size material and to analyze these extracts after gel filtration by RIAs for 1B236. Our hope was to find an extraction condition that would allow direct detection and quantitation of all molecular forms of 1B236 in the same ex-

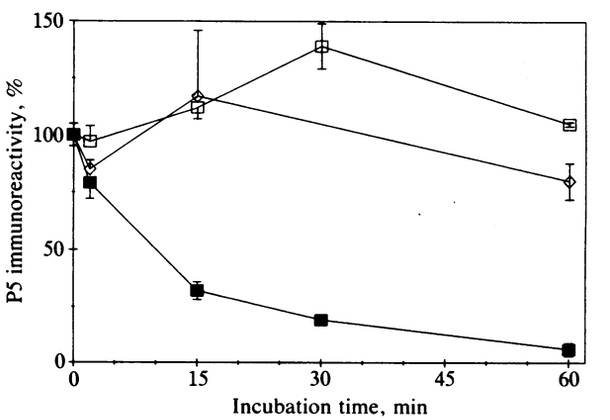

FIG. 7. Stability of synthetic 1B236 peptides in brain homogenates. Known amounts of synthetic P5 were incubated at 25°C with brain homogenates prepared in Tris/NaCl (■), Tris/NaCl with prior microwave irradiation (□), or 2 M acetic acid containing 1% Triton X-100 (◇). Data (means from four experiments) show the amount of synthetic P5 remaining after incubation for various times expressed as a percentage of the initial concentration. Similar data were obtained for P6 and P7 (not shown).

tract. However, because of the multiplicity of 1B236 molecular forms, this was not feasible: detection of high molecular weight material required a neutral extraction of fresh brains, while detection of low molecular weight material required either acidic extraction or neutral extraction of brains treated by microwave irradiation to inhibit endogenous proteolytic activity. These conditions decreased the recovery of high molecular weight material.

We previously had detected high molecular weight 1B236 in $NaDodSO_4$-solubilized rat brain extracts using immunoblotting (10). This protein has an apparent molecular weight of 100,000 and subsequently has been found to be a membrane-bound glycoprotein (unpublished data). The coelution of the 1B236 protein detected by immunoblotting and by RIA (Fig. 3) indicates that this species corresponds to the high molecular weight form extracted by detergent and reactive with anti-P5, -P6, and -P7 antibodies (Fig. 2*B*). There also may be a second high molecular weight 1B236-like protein that appears to be soluble in the absence of detergent (Fig. 2*A*).

In addition to solubilizing the high molecular weight form of 1B236, the detergent NP-40 apparently solubilized large amounts of low molecular weight material, detected by anti-P6 and -P7 antibodies (Fig. 2*B*), which were not present in the absence of detergent (Fig. 2*A*). A possible explanation for this phenomenon might be that these low molecular weight materials are contained in vesicles or similar compartments that remain intact when brains are extracted in isotonic neutral buffer but are disrupted by the detergent NP-40, releasing the immunoreactive peptides. However, since attempts to release such peptides by lysing vesicles by sonication or hypo- or hypertonic extractions have not been successful (not shown), this explanation is unlikely. A more likely explanation is that these low molecular weight immunoreactive materials are released from the membrane-bound 1B236 protein by the action of proteases during the 30-min incubation necessary for protein solubilization.

The method of subjecting a brain to microwave irradiation prior to homogenization, which enables a rat brain to be heated in a few seconds, has already been successfully used in the extraction of enkephalins and substance P (11–13), which are very sensitive to proteolysis. The stability of synthetic P5, P6, and P7 peptides in homogenates from microwave-irradiated brains or in homogenates made in acetic acid suggests that any similar peptides present *in vivo* would not be degraded during extraction under these conditions. Furthermore, since it is probable that these procedures inactivate all protease activities, any peptides detected in these extracts are unlikely to have been generated by protein degradation at the moment of extraction. Therefore, we conclude that the low molecular weight P5 and P7 immunoreactive materials extracted under these conditions (Figs. 5 and 6; Table 1) are endogenous peptides.

Thus, these results show that peptidase activities may influence the pattern of peptides recovered in a brain homogenate in two opposite ways. As already demonstrated in a number of cases (12), endogenous peptidases are primarily responsible for the degradation of peptides at the moment of their extraction. This probably accounts for the fact that low molecular weight P5 immunoreactivity can only be demonstrated in extracts from microwave-irradiated brains (Table 1). On the other hand, the increase in the amounts of low molecular weight P6 and P7 material recovered after homogenization of fresh brains in NP-40, compared to extraction after microwave irradiation, suggests that P6- and P7-like peptides can be released from the 1B236 protein by protease activities. Thus, the 1B236 protein and its peptide products appear to be particularly sensitive to proteolysis.

We have used antibodies directed against P5, P6, and P7 to visualize 1B236 immunoreactivity in brain sections (2), to map completely its distribution in brain (5), and to provide a preliminary characterization of the protein itself (10). We now have extended those observations. The same antibodies against synthetic peptides have been used to develop RIAs for 1B236: these assays can detect both high and low molecular weight forms of 1B236 and will allow the eventual purification of these species. Although predominantly of high molecular weight, 1B236 is also detected in substantial amounts in forms similar to the peptides P5 and P7, which we had postulated as proteolytically derived products of 1B236 based on its primary structure (2). The multiplicity of 1B236 molecular forms demonstrated here, together with evidence that 1B236 is glycosylated (10), indicates that this molecule undergoes extensive post-translational modification, including proteolytic processing to generate a previously undisclosed family of brain-specific peptides. Isolation of these peptides, determination of their exact relationship to the other forms of 1B236, and the demonstration that they are physiologically relevant will be necessary for further understanding of this system.

We acknowledge the expert technical assistance of Derf Lewis, Lynne Randolph, Eric Johnson, and Mary Kiel. We thank Dr. Richard Houghten (Department of Molecular Biology, Scripps Clinic) for helpful discussions and peptides. This work was supported in part by National Institutes of Health Grants NS 20728 and GM 32355 and by McNeil Laboratories. B.M. acknowledges a fellowship from the Fogarty International Program. This is publication 3734-BCR from the Research Institute of Scripps Clinic.

1. Milner, R. J. & Sutcliffe, J. G. (1983) *Nucleic Acids Res.* **11,** 5497–5520.
2. Sutcliffe, J. G., Milner, R. J., Shinnick, T. M. & Bloom, F. E. (1983) *Cell* **33,** 671–682.
3. Douglass, J., Civelli, O. & Herbert, E. (1984) *Annu. Rev. Biochem.* **53,** 665–715.
4. Loh, Y. P., Brownstein, M. J. & Gainer, H. (1984) *Annu. Rev. Neurosci.* **7,** 189–222.
5. Bloom, F. E., Battenberg, E. L. F., Milner, R. J. & Sutcliffe, J. G. (1985) *J. Neurosci.*, in press.
6. Greenwood, F. D., Hunter, W. H. & Glover, J. S. (1963) *Biochem. J.* **89,** 114–123.
7. Ghazarossian, V. E., Chavkin, C. & Goldstein, A. (1980) *Life Sci.* **27,** 75–86.
8. Munson, P. J. & Rodbard, D. (1978) *Anal. Biochem.* **88,** 1–19.
9. Towbin, H., Staehelin, T. & Gordon, J. (1982) *Proc. Natl. Acad Sci. USA* **79,** 4540–4544.
10. Sutcliffe, J. G., Milner, R. J. & Bloom, F. E. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **48,** 477–484.
11. Yang, H.-Y., Hong, J. S. & Costa, E. (1977) *Neuropharmacology* **16,** 303–307.
12. Bayon, A., Shoemaker, W. J., McGinty, J. F. & Bloom, F. E. (1983) *Int. Rev. Neurobiol.* **24,** 51–92.
13. Kanazawa, I. & Jessel, T. (1976) *Brain Res.* **117,** 362–367.