MYELIN BASIC PROTEIN: A SUBSTANCE THAT RELEASES IMMUNOREACTIVE GROWTH HORMONE IN VITRO

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A protein has been isolated from ovine hypothalamus on the basis of its ability to stimulate release of growth hormone by in vitro cultures of dispersed pituitary cells. This protein has been identified as being myelin basic protein. With no similar biological activity in vivo, myelin basic protein is thus to be recognized as a potentially interfering substance in any search for the physiological growth hormone releasing factor using in vitro assay systems.

Several types of hypothalamic extracts have been reported to release immunoreactive growth hormone in vivo in rats (1-2) and dogs (3) and in vitro in pituitary cell cultures (3-6). A hypothalamic, physiologically meaningful, growth hormone releasing factor (GRF) has been postulated to explain the observed biological activities. So far, such a GRF has not been isolated or identified. In recent attempts at characterizing GRF we have isolated from ovine hypothalamic extract a substance that is a powerful stimulant of the release of immunoreactive growth hormone in vitro, but has no similar activity in vivo. The substance was identified as myelin basic protein (MBP), a well-known structural component of myelin. We wish to report these observations in some detail as they show MBP to be a potential artifact in current or contemplated programs to isolate GRF using in vitro bioassays.

MATERIALS AND METHODS

The residue from a previous extraction (7) of 490,000 ovine hypothalami with 90% C_2H_5OH-$CHCl_3$-HOAc (900:100:5) was homogenized with 2N HOAc at 25°C in a 1-gallon Waring blender for 30 min. Homogenates were filtered in a Buchner funnel through Whatman No. 1 paper and filtrates, clarified further by centrifugation, were lyophilized. A 15 x 40 cm column of Whatman CM32 was used for ion-exchange chromatography; elution was carried out with a linear gradient of water and 1M NH_4OAc at pH 7. Gel filtration was per-

TABLE I. Purification Sequence

	Stage	Fragments	Weight
1.	Lyophilized hypothalami	4.9×10^5	28 kg
2.	90% C_2H_5OH-$CHCl_3$-HOAc extraction	4.9×10^5	25 kg
3.	2N HOAc extraction	4.9×10^5	2 kg
4.	Ion-exchange chromatography (CMC)	4.2×10^5	275 g
5.	Gel filtration, G-25	4.1×10^5	182 g

formed on a 10 x 200 cm column of Sephadex G-25 in 0.5M HOAc. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was done according to previously published methods (8). Digestion with trypsin was done in 0.2M triethylamine-bicarbonate buffer, pH 8.2, at 37° for 16 hr with a 20:1 ratio of protein to enzyme. Two dimensional maps were done as previously described (9). Chromatograms were visualized by fluorescence under UV light after dipping in 0.003% fluorescamine in acetone-pyridine (50:1). Amino acid analyses and dansylation were done as previously described (7). Maleylation was done according to published methods (10). Digestion with pepsin was done in 0.1M acetic acid-ammonium acetate buffer, pH 3, at 25°C for 30 min with a 500:1 ratio of protein to enzyme. Samples of characterized bovine and porcine MBP and of the tryptophan containing peptide of MBP, residues 113-121, were generous gifts of Dr. Fred Westall (Salk Institute).

Release of growth hormone *in vitro* was followed with cultures of enzymatically dispersed rat anterior pituitary cells as used routinely in this laboratory (11). Male Sprague Dawley rats (100g) housed in temperature and humidity controlled quarters with 14 hours of light and 12 hours of dark were used in the *in vivo* bioassay. All animals were fed purina rat chow and tap water ad libitum. To ensure uniform basal levels of growth hormone, rats were etherized twice at periods ten minutes apart. Administration of test substances was via the external jugular vein. Blood samples were obtained by decapitation five minutes after each injection.

Plasma growth hormone levels were determined utilizing a double antibody method with the following reagents: NIAMDD rat GH standard (GH-RP-1); NIAMDD monkey anti-rat GH (GH-Serum-3); highly purified rat GH for iodination obtained from Dr. Standley Ellis, Ames Research Center, Moffett Field, California.

RESULTS AND DISCUSSION

The sequence of isolation of the active material from ovine hypothalamus, shown in Table I, was accomplished by following the zone of growth hormone release *in vitro*. We judged the material from stage 5 (Table I) to be nearly pure myelin basic protein on the basis of several

TABLE II. Amino Acid Ratios

	Bovine MBP		Ovine MBP[1]	Peptide P1[2]	Peptide P2[2]	Bovine MBP Residues 1-88[3]	Bovine MBP Residues 89-169[3]
Lys	13[3]	13.1[4]	12.8	6.1	7.0	5	8
His	10	9.6	9.2	4.4	2.4	8	2
Arg	18	16.2	17.1	7.7	8.5	10	8
Asp	11	11.7	11.3	5.2	4.3	7	4
Thr	7	7.0	6.8	3.4	2.8	4	3
Ser	18	15.9	15.2	7.6	8.8	8	10
Glu	10	10.6	10.5	5.1	4.0	6	4
Pro	12	13.2	12.8	6.2	6.0	6	6
Gly	25	25.0	25.0	11.9	14.8	10	15
Ala	14	14.2	14.2	6.8	5.5	9	5
Val	3	2.0	1.8	0.8	0.6	2	1
Met	2	1.7	1.8	0.8	0.9	1	1
Ileu	3	2.4	2.3	1.2	1.4	1	2
Leu[5]	10	10.0	10.0	5.0	5.0	5	5
Tyr	4	4.0	4.0	2.1	2.0	2	2
Phe	8	8.2	8.0	3.8	3.9	4	4
Trp	1					0	1

1. Material from stage 5 (Table I).
2. Obtained from pepsin digest of ovine MBP. Peptide P1 contained minor impurities as judged by polyacrylamide gel electrophoresis; peptide P2 appeared nearly pure.
3. Ref. 9-10.
4. Experimental values.
5. Ratios normalized to leucine.

criteria. SDS-polyacrylamide gel electrophoresis revealed essentially one band corresponding to a molecular weight of 20,000. The amino acid ratios (Table II) for this material were very similar to those of bovine MBP (9,12). In addition, peaks corresponding to the unusual amino acids, N^G,N^G-dimethyl-L-arginine and N^G-monomethyl-L-arginine, which are known to occur in MBP (9), were present. No free N-terminus was detectable by dansylation in agreement with the occurrence of an acetyl blocked N-terminus in bovine MBP (9). The two dimensional peptide maps of the tryptic digests of stage 5 material and of bovine MBP were very similar but not identical.

TABLE III. Effect of MBP on Growth Hormone Release *in vitro*.

Treatment	Dose (µg/ml)	rGH (ng/dish)	p[1]
Control		176 ± 22	
Ovine MBP	20	330 ± 26	<.05
	60	527 ± 12	<.01
	200	1730 ± 447	<.01
Bovine MBP	60	445 ± 31	<.01
	200	1052 ± 282	<.01
Porcine MBP	60	390 ± 28	<.01
	200	1500 ± 50	<.01

1. P value for difference between control and other treatments determined by Dunnett's test on log transformed values.

Digestion with pepsin and subsequent ion-exchange chromatography on CMC of the active material yielded two peptide fragments, P1 and P2 (Table II), with amino acid compositions (Table II) similar in the case of P1 and nearly identical in the case of P2 to residues 1-88 and 89-169, respectively, of bovine MBP (9,12).

The data in Table III shows that ovine, bovine, and porcine MBP have similar capacities for stimulating growth hormone secretion from pituitary cell cultures. We recognized early that these proteins are active at doses (10^{-6}M) well above the minimum doses (10^{-10}M) at which the well characterized hypothalamic factors, thyrotropin releasing factor (TRF), luteinizing hormone releasing factor (LRF), and somatostatin, are active (13,14). Furthermore, MBP lacked activity *in vivo* (Table IV) in the rat, even at doses (0.1 µmol) 500 to 10,000 times greater than minimally active doses of TRF (13), LRF (13) and somatostatin (14). In the same experiments in the rat, prostaglandin E_2 (PGE_2) resulted in a significant elevation of growth hormone levels, an observation we have previously reported (15). Similarly negative results were obtained when either our highly purified material or

TABLE IV. Effect of MBP on Release of Growth Hormone in vivo.

Treatment	GH ng/ml plasma	p[1]
Control	41.8 ± 11.0	
Ovine MBP (1.6 mg)	35.3 ± 4.0	
Bovine MBP (1.6 mg)	30.6 ± 2.6	
PGE_2 (10 µg)	246.6 ± 110.6	<.01

1. P Value for difference between control and other treatments by Dunnett's test.

characterized MBP of bovine origin were tested in the dog by Dr. M. Grumbach and collaborators (University of California, San Francisco).

Because the possibility exists that a small fragment of MBP may be responsible for its activity in vitro, we generated peptides from our purified material in several ways. The trypsin digest was inactive in vitro. Maleylation, i.e., blocking of the ε-amino groups of the lysine residues by reaction with maleic anhydride, followed by digestion with trypsin and removal of the blocking groups at pH 2.4, gave a mixture of peptides that was inactive in vitro. Fragments P1 and P2 (Table II), generated by pepsin digestion, were also inactive in vitro, as was the tryptophan containing peptide of MBP, residues 113-121. The complete lack of activity of these peptides, especially of the large peptic fragments P1 and P2, suggests that the entire sequence of MBP is necessary for activity in vitro.

The highly basic nature (ca. 18% lysine plus arginine) of MBP is possibly responsible for its activity in vitro, in view of the inactivity of maleylated MBP, in which the basic side chains of the lysine residues have been converted to acidic ones. Two other highly basic substances, calf thymus histone and poly-L-lysine, showed toxicity effects in vitro when tested at the same level as MBP. In addition, our material appeared to

release other pituitary hormones (TSH, LH, FSH) *in vitro*, but at the same time it was realized that MBP interfered with the radioimmunoassays for these hormones.

Of the previously reported (3-6) *in vitro* growth hormone releasing activities, all have been extracted with acidic solvents which are capable of extracting MBP. One (6) was tested at a dose level (3140 µg/ml) which is toxic *in vitro* in our hands. The other activities (3-5) were tested at levels (8-300 µg) nearly equal to or above the minimum active dose of MBP *in vitro* (Table III). Of the two activities reported by Currie *et al.* (4) one, called "A-GHRH", reportedly (4) excluded on Bio Gel P2, has properties which are consistent with those of MBP. "A-GHRH" appears to be spread (4) on partition chromatography in the system n-butanol- acetic acid-water (4:1:5), exhibiting a wide range of R_f values. Our purified material behaves similarly in the same system, its R_f varying as a function of load. The second activity described by Currie *et al.* (4), "B-GHRH", does not appear similar to MBP on the basis of its behavior on ion exchange chromatography (16). The "GRF" reported by Sawano *et al.* (3) is apparently retarded on Sephadex G-25 and is adsorbed to CM-Sephadex. Our material behaved anomalously on Sephadex G-25, the bulk of the active material eluting as one band centered at *ca.* 1.8 Vo. Rechromatography of aliquots of this material at a 10-fold higher dilution gave a single band at *ca.* 1.1 Vo. Because the chromatography of the "GRF" reported by Sawano *et al.* (3) was not sufficiently detailed, we are unable to distinguish it from MBP. The "GRF" reported by Krulich *et al.* (5) does appear to be of small molecular size, being retarded on Sephadex G-15 and passing through Diaflo UM 2 (cutoff of 1000). It is also soluble in 90% ethanol. Our material is excluded on Sephadex G-15, retarded by both UM 2 and UM 10 (cutoff of 10,000), and insoluble in 90% ethanol.

Using an antiserum against bovine MBP, kindly furnished by Dr. V. Lennon (Salk Institute), Dr. M. Dubois (INRA, Nouzilly, France) was un-

able to observe by immunohistofluorescence any evidence of MBP-like material in the perikarya or the axoplasms of hypothalamic neurons while clearly observing the expected outer axonal location of MBP in myelinated fibers. These observations not only demonstrate the presence of myelinated nerve fibers in hypothalamic tissue (17) but also reaffirm the location of MBP in the myelin sheath. In consequence, the presence of MBP in acid extracts is not surprising. In fact, a protein was previously isolated (without regard to biological activity) from porcine hypothalami (18) and later recognized by another group (19) to be porcine MBP on the basis of its similarity to bovine MBP.

Myelin basic protein thus appears to be a powerful artifact, which must be recognized by others using methods of chemical purification and bioassays closely related to those reported here.

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