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Development of a monoclonal antibody against recombinant neuroendocrine 7B2 protein

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Mouse monoclonal antibody MON-100 was raised against the neuroendocrine protein 7B2 using bacterially produced hybrid proteins. In Western blot analysis, MON-100 reacted with 3 different 7B2 hybrid proteins and not with the respective carrier proteins. Furthermore, MON-100 was reactive with recombinant 7B2 cleaved from a hybrid protein. In an immunohistochemical study, MON-100 exhibited strong reactivity with the intermediate lobe of the *Xenopus* pituitary gland, a tissue previously shown to contain 7B2 mRNA. Using MON-100, immunoprecipitation analysis of newly synthesized proteins produced by in vitro incubated *Xenopus* neurointermediate lobes revealed the biosynthesis of a single protein of M_r 24 kDa, the expected size of the 7B2 protein. It appears, therefore, that the anti-7B2 monoclonal antibody MON-100 can be successfully used for Western blot analysis and immunohistochemical analysis as well as for immunoprecipitation experiments.

Protein, 7B2; Monoclonal antibody; Immunochemistry; Protein, neuroendocrine; Protein, prokaryotic hybrid

1. INTRODUCTION

7B2 is a nonglycosylated neuroendocrine polypeptide consisting of approximately 180 amino acids [1] and intracellularly compartmentalized within secretory granules [2]. This 19–24 kDa protein [3–5], originally isolated from porcine anterior pituitary glands [1], is widely distributed within the central nervous system [5] as well as in endocrine tissues and tumors [6–8]; 7B2 may be a plasma marker for endocrine tumors [6]. Recently we showed that the 7B2 protein is highly conserved during 350 million years of vertebrate evolution [9]. The physiological function of the 7B2 protein is yet unknown. It has been proposed that 7B2 could be a growth factor [10], a hormone, a neurotransmitter/neuromodulator [11] or it could be a factor regulating some intracellular function [12]. The fact that 7B2 is an unstable protein [13] and that it is present in neural and endocrine tissues in only minute amounts [6,10] hampers functional studies of this interesting neuroendocrine protein. We applied recombinant DNA technology and bacterial expression vectors to produce relatively large amounts of recombinant human 7B2 protein. Using the recombinant 7B2 protein it was possible to develop anti-7B2 monoclonal antibodies. The availability of the recombinant protein and the monoclonal antibody will allow us to perform studies aimed at elucidating the function of 7B2.

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2. MATERIALS AND METHODS

2.1. *Preparation of hybrid proteins*

Using standard recombinant DNA techniques [14], 3 hybrid proteins were obtained consisting of human 7B2 [15] linked to

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β-galactosidase (β-gal), to anthranilate-synthetase (AS) or to worm glutamylcysteinylglycine-binding protein (GBP). The GBP-7B2 hybrid protein was purified with affinity chromatography using glutamylcysteinylglycine-agarose (Sigma). The purified hybrid protein GBP-7B2 was treated with the site-specific serine protease thrombin, resulting in the production of intact GBP and 7B2; the consensus sequence for this cleavage is: P4-P3-Pro-Arg-P1-P2, where P3 and P4 are hydrophobic amino acids and P1 and P2 are nonacidic amino acids [16].

2.2. *Development of monoclonal antibodies directed against 7B2*

Female Balb/c mice, 2–3 months old, were used for intraperitoneal (i.p.) immunization with 50 μg of recombinant hybrid protein in complete Freund's adjuvant. After 4 weeks, the mice were rechallenged i.p. using incomplete Freund's adjuvant. Ten days after the last injection, a final dose of 25 μg of protein was given intravenously (i.v.) and 3 days later the spleen was removed.

The first fusion experiment was performed after 4 rechallenges using complete bacterial lysate containing AS-7B2. For the second fusion experiment, a mouse was immunized with partly purified GBP-7B2 and one rechallenge was sufficient to raise an immune response against 7B2. Fusions were performed according to the polyethylene glycol (PEG) procedure of Lane [17], using Sp_2/O-Ag14 myeloma cells [18] as a fusion partner, except that PEG (Kodak) was diluted in RPMI 1640 instead of dimethylsulfoxide and phosphate-buffered saline. No feeder macrophages were used. By this procedure, up to 20000 clones can be generated in each fusion.

Antibodies produced by hybridomas generated in the first fusion were analyzed using a rapid immunoblot screening technique [19]. As a first screening assay for the second fusion experiment, an ELISA was developed. Culture supernatant of each clone was tested for reactivity with complete bacterial lysates containing AS-7B2, GBP or GBP-7B2, coated to microtiter dishes (Costar). Positive hybridoma cultures were subsequently cloned three times by the limiting dilution method. Cultures of hybridomas were maintained in RPMI 1640 supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), 2 mM L-glutamine and 10% fetal bovine serum (High Clone).

2.3. *Protein analysis*

Protein samples were analyzed using 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) [20], electrotransferred onto nitrocellulose [21] and tested for immunoreactivity with antisera essentially as described by Mulders et al. [22]. Neurointermediate lobe cells of South African clawed toads, *Xenopus laevis*, were labeled according to Martens et al. [23] for 15 min with [^{3}H]lysine and [^{3}H]proline, and subsequent immunoprecipitation was performed according to Deppert and Haug [24]. Toads were kept in black buckets under constant illumination for at least 3 weeks.

2.4. *Immunocytochemistry*

Xenopus brain and pituitary were fixed in Bouin-Hollande, Paraplast-embedded, and 5 μm sections were immunostained using the peroxidase anti-peroxidase method according to Sternberger [25] using diaminobenzidine as oxygen acceptor and ammonium nickel sulphate enhancement. As a control, non-immune mouse serum was applied.

3. RESULTS AND DISCUSSION

The major objective of this study was to isolate relatively large amounts of the 7B2 protein in order to develop 7B2-specific monoclonal antibodies. The availability of the recombinant protein and the antibody might enable us to study the neuroendocrine protein 7B2 more extensively and these products might be instrumental to elucidate the unknown function of 7B2. Since only a limited amount of 7B2 is present in tissues [10], bacterial expression of 7B2 might constitute a valuable alternative approach to obtain relatively large amounts of this small non-glycosylated protein. We here report that 7B2 can indeed be produced in large quantities as part of stable hybrid proteins (fig.1A, lanes 2, 4 and 6); we have been unsuccessful to bacterially produce the 7B2 protein itself (G.J.M. Martens and P.J.T.A. Groenen, unpublished). Injection of rabbits and mice with hybrid protein raised a strong immune response. Failure to produce polyclonal antisera in rabbits with small amounts of the native porcine 7B2 has been reported by others [10]. This may be due to immunological tolerance because of the highly conserved nature of 7B2. This problem was apparently not encountered when 7B2 hybrid proteins were used for immunization, possibly because conjugation of 7B2 to a carrier protein (AS or GBP) might have made the recombinant protein more immunogenic.

Fusion experiments resulted in the isolation of 3 hybridomas producing monoclonal antibodies with reactivity towards 7B2 on immunoblots and in ELISA analysis (data not shown). Of these monoclonal antibodies, MON-100 showed the strongest reactivity with the 7B2 protein moiety of each of the hybrid proteins. As shown in fig.1B, the MON-100 antibody recognizes β-gal-7B2 (136 kDa) (lane 2), AS-7B2 (57 kDa) (lane 4) and GBP-7B2 (46 kDa) (lane 6), and does not react with β-gal (116 kDa) (lane 1), AS (37 kDa) (lane 3) or GBP (29 kDa) (lane 5). Hence, this monoclonal antibody clearly has specificity for 7B2. To further establish that MON-100 recognizes an epitope within the 7B2 structure, the hybrid protein GBP-7B2 was cleaved with the site specific enzyme

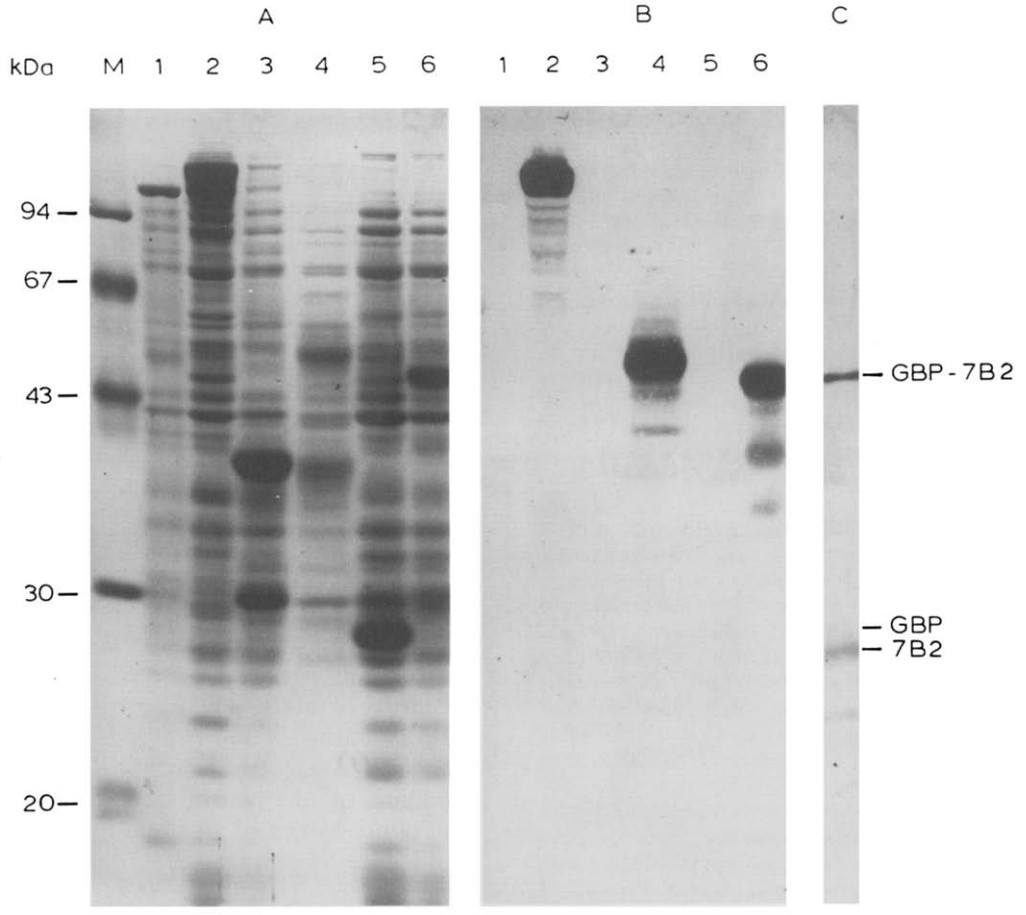

Fig.1. Analysis of 7B2 hybrid proteins. (A) Lysates of *E. coli* expressing relatively large amounts of β-gal (116 kDa) (lane 1), β-gal-7B2 (136 kDa) (lane 2), AS (37 kDa) (lane 3), AS-7B2 (57 kDa) (lane 4), GBP (29 kDa) (lane 5) and GBP-7B2 (46 kDa) (lane 6) were analyzed on SDS-PAGE. (B) Proteins, as in A, were electroblotted onto nitrocellulose and this immunoblot was analyzed with the anti-7B2 monoclonal antibody MON-100. (C) Western blot showing the reactivity of MON-100 with partially thrombin-digested GBP-7B2.

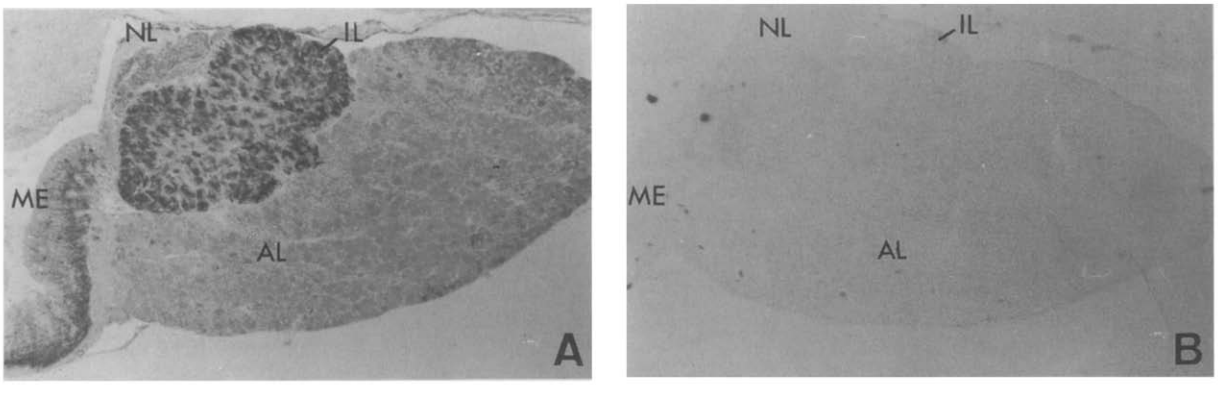

Fig.2. Immunocytochemical analysis of *Xenopus* pituitary using anti-7B2 monoclonal antibody MON-100 (A) or normal mouse serum (B). AL, anterior lobe; IL, intermediate lobe; NL, neural lobe; ME, median eminence.

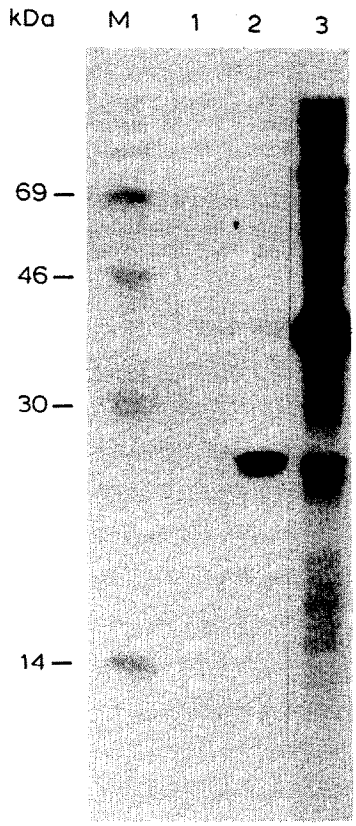

Fig.3. SDS-PAGE of immunoprecipitation analysis of ^{3}H-labeled proteins produced by *Xenopus* neurointermediate lobes (4 animals per lane) using normal mouse serum (lane 1) or anti-7B2 monoclonal antibody MON-100 (lane 2). Lane 3: total extract of labeled proteins (equivalent to 0.2 lobe), M, molecular weight markers.

thrombin and the cleavage products were subjected to Western blot analysis. As shown in fig.1C, MON-100 is reactive with the hybrid protein GBP-7B2 and intact 7B2; since GBP has no internal thrombin cleavage sites, the minor immunoreactive proteins of lower molecular weight represent 7B2 cleavage products. From fig.1 it can be concluded that the epitope recognized by MON-100 resides within the 7B2 protein structure.

To further evaluate the usefulness of MON-100, an immunocytochemical analysis of the pituitary gland of the toad *Xenopus*, a tissue previously shown to contain 7B2 mRNA [9], was performed. Immunoreactivity for 7B2 was found in the three lobes of the *Xenopus* pituitary (fig.2), in line with the previously reported distribution of 7B2 in mouse and rat pituitary [5].

As a final test for a possible application of MON-100, immunoprecipitation analysis of in vitro pulse labeled proteins produced by *Xenopus* neurointermediate lobes was performed. This analysis revealed a single protein of approximately 24 kDa, while the normal mouse serum did not precipitate any protein, suggesting that this newly synthesized 24 kDa protein is 7B2.

We conclude that MON-100 recognizes 7B2 specifically in all immunological techniques performed so far. This monoclonal antibody will be very useful in the elucidation of the biological function of the highly conserved neuroendocrine 7B2 protein.

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