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THE PRODUCTION AND CHARACTERIZATION OF A MONOCLONAL ANTIBODY SPECIFIC FOR THE 94,000 DALTON ENKEPHALIN-DEGRADING PEPTIDASE FROM RABBIT KIDNEY BRUSH BORDER

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SUMMARY: We have prepared a monoclonal antibody specific for a major 94,000 dalton protein from the brush border membrane of rabbit kidney cortex. The monoclonal antibody was used for the immunoaffinity purification of this protein after solubilization of brush border membranes with octylglucoside. The 94,000 dalton protein is a peptidase capable of cleaving the Gly^3-Phe^4 bond of methionine-enkephalin. Identification of this peptidase as a previously described 94,000 dalton enkephalinase of kidney cortex was confirmed by its sensitivity to EDTA and inhibitors such as thiorphan and phosphoramidon.

Several peptidases present in the nervous system have been found to abolish the opiate receptor binding activity of the enkephalins (1). The possibility that one of these hydrolytic activities might be a specific enkephalinase with a physiological role similar to that of acetyl-cholinesterase in cholinergic transmission was suggested by several lines of evidence. Malfroy et al. have described a dipeptidyl-peptidase localized to brain membranes (2), which has a distribution similar to that of the opiate receptor. The enzyme shows a selective and high affinity in the nanomolar range toward the enkephalins, and is increased 60% in morphine addicted mice (3). Crucial biochemical characterization and precise regional distribution of this enzyme

Abbreviations: BBM: brush border membrane; ALM: anteluminal membrane; PBS: phosphate buffered saline; SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis, octylglucoside; N-octyl-β-D-glucopyranoside; HPLC: high pressure liquid chromatography.

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however is still lacking, mainly because of the low concentration of this enzyme in brain tissues and difficulties encountered during its purification.

An enzyme closely related to the enkephalinase originally described in the central nervous system has been detected in brush border membranes (BBM) of the kidney and from membrane fractions of various other organs (1, 4-6). Malfroy and Schwartz (7) have recently reported that the catalytic properties of this enzyme as well as its sensitivity to chelating agents and inhibitors are very similar to those of the cerebral enzyme. Given the relative abundance of this enzyme in the kidney, this tissue would constitute a source of choice for purifying the enzyme in quantities sufficient for a better biochemical characterization. We have recently immunized mice with purified BBM preparations from rabbit kidney cortex and used the spleen of these animals to prepare hybridomas. We report here the isolation of one clone of hybrids that produce immunoglobulins recognizing the previously described renal enkephalin degrading enzyme.

MATERIALS AND METHODS

Monoclonal antibody production: Brush border (BBM) and anteluminal (ALM) membrane fractions of the rabbit kidney were prepared according to Boumendil-Podevin and Podevin (8) using a self-orienting Percoll gradient as the final purification step. BBM were resuspended at the concentration of 1 mg/ml in phosphate saline buffer (PBS). BALB/c mice were immunized by giving intraperitoneal injection of 200 µg of membrane protein in complete Freund's adjuvant. Boost injections (200 µg protein in PBS) were given at three to four weeks intervals. A last boost was always given four days before the fusion. Spleen cells were fused with P3-X63 Ag8.653 myeloma cells (Americal Type Culture Collection, Rockville, MD.) essentially as described by Goding (9). Detection of antibodies specific for BBM antigens was done with an ELISA screening assay. This assay was performed in polyvinylchloride microtiter plates (Dynatech Laboratories, Alexandria, VA) pretreated with poly-L-lysine (10) or gluteraldehyde (11) and coated with either ALM or BBM suspensions (30 µg/ml). Out of 2874 culture supernatants tested, 20 produced immunoglobulins specific for BBM only. When renal cortical BBM proteins were analyzed by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) as described by Laemmli (12) and transferred to nitrocellulose paper sheets according to Towbin et al. (13), four of the hybridoma culture supernatants were found to recognize a 94,000 dalton protein. One of these hybridoma cultures was cloned twice by limiting dilution, expanded and passaged in the peritoneal cavity of pristane treated mice. The ascitic fluid was purified on a protein A-Sepharose CL-4B column (Pharmacia Fine Chemicals) as

described by Ey et al. (14). The monoclonal immunoglobulin could be recovered from the column when the pH of the eluant was brought to 4.5. Solid phase ELISA immunotyping assays showed that it was of the IgG_{2a} class, as expected from its elution position on the protein A-Sepharose column.

Immunoaffinity purification of the 94,000 dalton protein on monoclonal antibody-protein A-Sepharose CL-4B:

A 50 µl aliquot of protein A-Sepharose CL-4B was saturated with the monoclonal antibody by incubation at 4°C for 45 min. The resin was washed extensively with PBS pH 7.4 and equilibrated in PBS containing 1% N-octyl-β-D-glucopyranoside (octylglucoside, Sigma Chemical Co.). BBM (1 mg/ml in PBS) were solubilized by adding octylglucoside to a final concentration of 1% and leaving for 1 h at 4°C. Residual insoluble material was removed by centrifugation at 100,000 g for 1 h, and the supernatant (1 ml) was gently mixed by rotation with the monoclonal antibody-protein A-Sepharose CL-4B resin. After 16 h at 4°C the resin was washed extensively with PBS, 1% octylglucoside.

For electrophoretic analysis, the resin was then boiled for 2 min in an equal volume of Laemmli sample buffer, removed by low speed centrifugation and the supernatant was analyzed by SDS-PAGE. For determination of enzymatic activity, bound proteins were detached from the resin by a 15 min incubation at 4°C in 1 ml 0.1M sodium citrate buffer pH 4.5, containing 1% octylglucoside and immediately neutralized.

Hydrolysis of [^{3}H]Tyr Met-enkephalin: Digestion of [^{3}H]Tyr Met-enkephalin (Amersham) (20 pmol in 100 µl 50 mM Tris-HCl, pH 7.4, 1% octylglucoside) was carried out for 30 min at 25°C with approximately 1 µl of the immunoaffinity purified 94,000 dalton protein solution. Reaction was stopped by boiling or by adding 50 µl of 0.2N HCl. Identification of the ^{3}H-labeled metabolites was done by high performance liquid chromatography (HPLC) on a C_{18} µBondapak analytical column (Waters) at a flow rate of 1.5 ml/min. Isocratic conditions in 1 mM ammonium acetate buffer (pH 4.3) were used for the first 15 min, followed by a linear gradient from 1 to 30% acetonitrile in 1mM ammonium acetate pH 4.3 over the next 65 min. Fractions (600 µl) were counted in a liquid scintillation spectrometer. Elution of non radioactive peptide standards was monitored by absorbance at 214 nm. For the rapid quantitative analysis of [^{3}H]Tyr Met-enkephalin or its metabolites, step-wise elution of radioactive peptides was performed on C_{18} SEP-PAK cartridges (Waters). Labeled enkephalin metabolites were eluted with 10% acetonitrile in 1mM ammonium acetate pH 4.3 Intact Met-enkephalin was recovered when the acetonitrile concentration was brought to 40%. Total recovery was always better than 95%.

RESULTS AND DISCUSSION

To identify the antigen recognized by our monoclonal antibody, we first separated the BBM proteins from rabbit kidney cortex by SDS-PAGE and transferred them onto nitrocellulose sheets by lateral electrophoresis. The resulting immunoblots are shown in Figure 1 (lanes e-g). It reveals that the antibody recognizes only one protein, and that this protein has an apparent molecular weight of 94,000 (Fig. 1, lane e). There was no reaction when a purified ALM preparation was

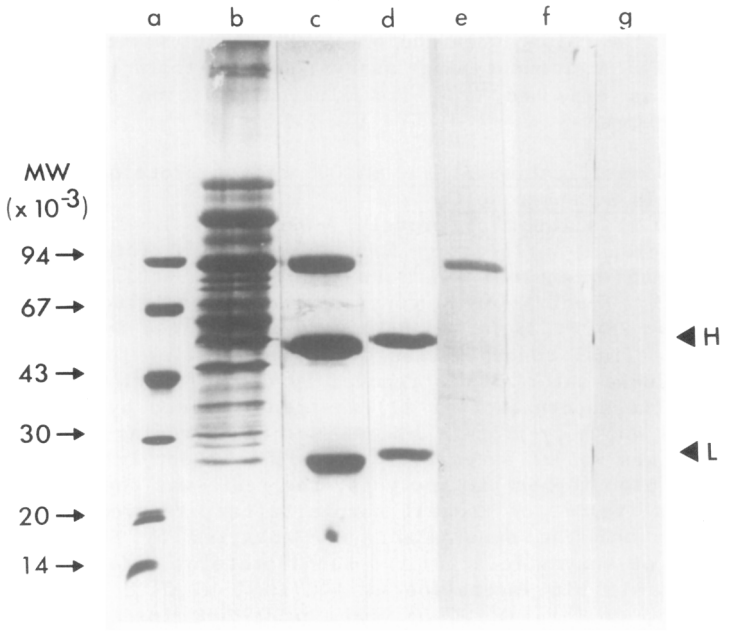

Figure 1 **Identification after SDS-PAGE electrophoresis of the protein antigen recognized by the monoclonal antibody.** Lanes a-d represent Coomassie blue stained proteins corresponding to a) standards with the indicated molecular weight b) 50 µg BBM c,d) immunopurified BBM on protein A-Sepharose CL-4B saturated with either the specific anti-BBM monoclonal antibody (c) or an unrelated antibody (d). Electrophoresis and immunopurification were as described under Materials and Methods. Lanes e-g are immunoblots corresponding to 50 µg BBM (e,g) or ALM (f) and incubated with either the specific anti-BBM antibody (e,f) or an unrelated antibody (g). The antigen-antibody complex was revealed with rabbit anti-mouse immunoglobulins conjugated to horseradish peroxydase. Arrows H and L point the position of heavy and light chains of mouse IgG respectively.

used instead of BBM (Fig. 1, lane f). Moreover an unrelated monoclonal antibody failed to recognize the 94,000 dalton protein (Fig. 1, lane g).

Further proof of the specificity of the monoclonal antibody was obtained by binding solubilized BBM proteins to an immunoaffinity resin consisting of the purified monoclonal antibody adsorbed to protein A-Sepharose CL-4B. This was possible because our monoclonal antibody, being of the Ig_{2a} class, interacts strongly with protein A. As shown in Figure 1 (lane c), this immunoaffinity gel was capable of binding selectively a 94,000 dalton protein from solubilized BBM extracts. This protein was not retained by protein A-Sepharose CL-4B

in the absence of the monoclonal antibody (not shown) or if the specific antibody had been replaced by an unrelated antibody (Fig. 1, lane d). Taken together, these results indicate that our monoclonal antibody is specific for a 94,000 dalton protein, which appears to be a major constituent of the BBM (Fig. 1, lane b) but is absent in the ALM (Fig. 1, lane f).

We next investigated the possibility that this protein could be identical to the 94,000 dalton membrane-bound carboxydipeptidase originally described in BBM by Fulcher et al. (6) and recently purified to homogeneity by Malfroy and Schwartz (7). Since these authors also showed that the enzyme was capable of cleaving the enkephalins at their Gly^3-Phe^4 bond, we attempted to hydrolyze [^{3}H]Tyr Met-enkephalin with the 94,000 dalton protein. As shown in figure 2, the protein was able to cleave [^{3}H]Tyr Met-enkephalin into tritiated metabolites which co-eluted with the Tyr-Gly-Gly standard after HPLC. No cleavage

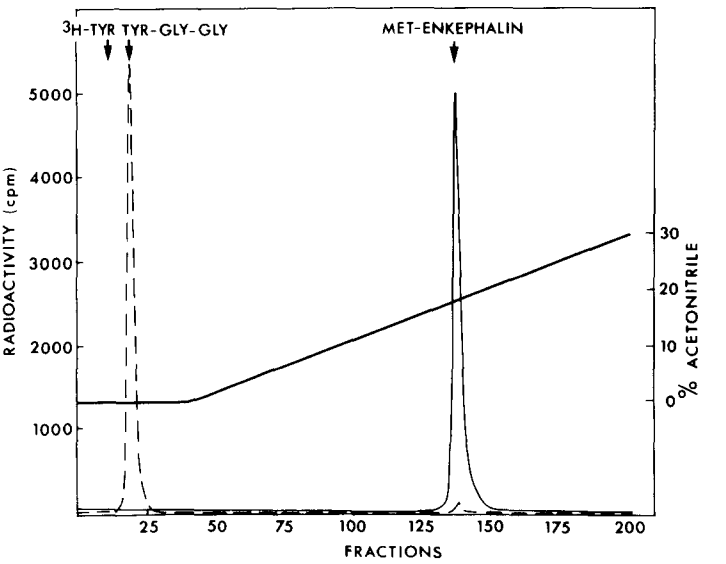

Figure 2 **Hydrolysis of [^{3}H]Tyr Met-enkephalin by the 94,000 dalton protein.** Digestion and HPLC were performed as described under "Materials and Methods". The chromatogram drawn as a broken line represents the elution profile of the labeled material recovered after incubation with the immunoaffinity purified enzyme. A control is included (solid line) to show the position of the radioactive substrate incubated in the same conditions but with the heat inactivated enzyme. The positions of standard non radioactive peptides and of [^{3}H]tyrosine are also indicated.

Table 1 Action of various inhibitors on the activity of the purified enzyme

Inhibitor	Percent hydrolysed
Phosphoramidon (1 μM)	6.6 ± 1.5
Thiorphan (1 μM)	3.1 ± 1.7
EDTA (5mM)	2.9 ± 1.1
Puromycine (100 μM)	101.7 ± 5.5
Captopril (1 μM)	98.2 ± 4.8

An identical amount of enzyme purified on monoclonal antibody protein A-Sepharose was used in all the samples. Enzyme activities were assayed as described under Materials and Methods. Two series of independent experiments were performed and each sample was run in duplicate. For each series, a control sample containing the peptidase and no inhibitor was included and the amount of ^{3}H-labeled Met-enkephalin metabolites recovered (accounting for at least 85% of the total amount of radioactive substrate used) was taken as 100%. The values represent the mean (± S.D.) of the amount of ^{3}H-labeled metabolites recovered, expressed as percent of the control without inhibitor. A sample where HCl (0.07N, final concentration) was added prior to the enzyme was used as a blank (always less than 2% of the total substrate added).

of labeled enkephalin occurred if there was no enzyme present during the incubation (not shown) or if the enzyme had been denatured by boiling for 15 min or by HCl addition before the incubation with the substrate.

As shown in Table 1, the peptidase activity was almost completely inhibited by 1 μM phosphoramidon or 1 μM thiorphan. These two compounds had been previously shown to completely inhibit the enkephalinase activity at the concentration used (7). Similarly, EDTA which is also known to inhibit the Zn-containing enkephalinase (7) blocked completely the hydrolysis activity of our 94,000 dalton protein. By contrast, puromycin (100 μM) which blocks the aminopeptidase activity capable of hydrolyzing the Tyr^{1}-Gly^{2} bond of enkephalin (7) was without effect. Captopril, a potent inhibitor of the angiotensin converting enzyme, which also cleaves the enkephalins at the Gly^{3}-Phe^{4} bond (15,16) has no effect on the activity of our enzyme preparation.

These results strongly suggest that the protein recognized by the monoclonal antibody is the 94,000 dalton enkephalinase previously described (7). Our results also show that our monoclonal antibody can

be used to purify the enzyme activity in one step with a high yield from proteins solubilized from BBM preparations of the rabbit kidney. The availability of this monoclonal antibody should allow us to better characterize the kidney peptidase and eventually study the homology between the enkephalin-degrading enzymes extracted from different tissues.

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REFERENCES

1. Schartz, J.-C., Malfroy, B. and De La Baume, S. (1981) Life Sci. 29, 1715-1740.
2. Malfroy, B., Swerts, J.P., Llorens, C. and Schwartz, J.-C. (1979) Neurosci. Lett. 11, 329-334.
3. Malfroy, B., Swerts, J.P., Guyon, A., Roques, B.P. and Schwartz, J.C. (1978) Nature, 276, 523-526.
4. Almenoff, J., Wilk, S. and Orlowski, M. (1981) Biochem. Biophys. Res. Commun. 102, 206-214.
5. Benuk, M. and Marks, N. (1979) Biochem. Biophys. Res. Commun. 88, 215-221.
6. Fulcher, I.S., Matsas, R., Turner, A.J. and Kenny, A.S. (1982) Biochem. J. 203, 519-522.
7. Malfroy, B. and Schwartz, J.-C. (1984) J. Biol. Chem. 259, 14365-14370.
8. Boumendil-Podevin, E.F. and Podevin, R.A. (1983) Biochim. Biophys. Acta, 735, 86-94.
9. Goding, J.W. (1980) J. Immunol. Methods, 39, 285-308.
10. Kennett, R.H., in "Monoclonal Antibody" (1980) (Kennett, R.H., McKearn, T.J. and Bechtol, K.B. eds) p. 376, Plenum Press, New York.
11. Evan, G.I. (1984) J. Immunol. Methods, 73, 427-435.
12. Laemmli, U.K. (1970) Nature, 277, 680-685.
13. Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
14. Ey, P.L., Prowse, S.J. and Jenkin, C.R. (1978) Immunochemistry 15, 429-436.
15. Ondetti, M.A., Rubin, B., Cushman, D.W. (1979) Science, 196, 441-444.
16. Erdös, E.G., Johnson, A.L. and Boyden, N.T. (1978) Biochem. Pharmacol. 27, 843-848.