A monoclonal antibody to kidney endopeptidase-24.11

Its application in immunoadsorbent purification of the enzyme and immunofluorescent microscopy of kidney and intestine

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Hybridoma methodology has been used to produce a monoclonal antibody, GK 7C2, that binds specifically to microvillar endopeptidase-24.11 (EC 3.4.24.11). The antibody (an immunoglobulin G) was generated by fusion of mouse plasmacytoma cells with splenocytes from a Balb/c mouse immunized with pig kidney microvillar membranes. The identity of the antigen recognized by GK 7C2 was established by immunoprecipitation from detergent-solubilized pig kidney microvilli. The protein had an apparent M_r of 90000 and contained endopeptidase activity sensitive to phosphoramidon. The identity was confirmed by immunoadsorbent purification of endopeptidase-24.11 by a column to which GK 7C2 had been attached. The endopeptidase, purified in a yield of 40%, was electrophoretically homogeneous and of specific activity comparable with that purified by other means. Fluorescence microscopy established that GK 7C2 bound specifically to the luminal membranes of kidney tubules and the intestinal mucosa. Thus endopeptidase-24.11 is located in the brush-border membranes of both cell types.

The endopeptidase that is the subject of the present paper was shown to be present in preparations of kidney brush border and microvilli (George & Kenny, 1973; Booth & Kenny, 1974) and was first purified from rabbit kidneys (Kerr & Kenny, 1974a). The Enzyme Commission have designated it as 'kidney brush-border neutral proteinase' (EC 3.4.24.11), but it is becoming increasingly clear that the enzyme has a much wider distribution. The distinctive properties of the rabbit kidney endopeptidase were the presence of Zn^{2+} at the active site, its thermolysin-like specificity and its sensitivity to certain specific inhibitors, such as phosphoramidon (Kerr & Kenny, 1974a, b; Kenny, 1977). The enzyme in pig kidneys has also been studied in some detail and has the same catalytic properties (Mumford *et al.*, 1981; Fulcher & Kenny, 1983; Matsas *et al.*, 1983). Membrane-bound enzymes with similar catalytic and immunological properties have been demonstrated in a variety of organs and tissues in the pig, including intestinal microvilli (Danielsen *et al.*, 1980), brain synaptic membranes (Fulcher *et al.*, 1982; Matsas *et al.*,

1983) and lung, aorta, spleen and myocardium (Kenny & Fulcher, 1983). In other species, similar activity has been found in brain and pituitary (Almenoff *et al.*, 1981; Orlowski & Wilk, 1981; Malfroy & Schwartz, 1982). It follows that the Enzyme-Commission name is too restrictive and will need revision. In the meantime, we have proposed (Matsas *et al.*, 1983) 'endopeptidase-24.11' (from the last two numbers of the E.C. listing) as a short and unambiguous name for a distinctive enzyme that is attracting increased interest.

In the course of a programme designed to generate monoclonal antibodies to kidney microvillar-membrane antigens, we have been successful in characterizing one of them as an antibody to endopeptidase-24.11. In the present paper, we report the properties of this monoclonal antibody, GK 7C2, and describe its application in purifying the enzyme from pig kidneys and in demonstrating its localization to brush borders in kidney and intestine.

Experimental

Materials

Balb/c mice were bred by the University of Leeds Laboratory Animal Services. Yucatan pigs (2–6

Abbreviations used: Ig, immunoglobulin; SDS, sodium dodecyl sulphate.

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weeks old) were from the University of Leeds Field Station. Tissue-culture media and bovine foetal serum were from Flow Laboratories. The myeloma cell line, Sp 2/0-Ag 14 (Shulman *et al.*, 1978) was a gift from Dr. R. A. Killington, University of Leeds, Leeds, U.K. Rabbit anti-(mouse IgG) serum was produced by an immunization schedule as described previously (Fulcher & Kenny, 1983), using mouse IgG that had been purified from normal mouse serum by chromatography on Protein A-Sepharose CL-4B. Rabbit antisera were purchased from Miles Laboratories Ltd. An epithelial-cell line derived from pig kidneys (LLC PK₁) was given by Dr. D. Louvard, European Molecular Biology Laboratory, Heidelberg, Germany.

Methods

Production of monoclonal antibody GK 7C2. Media. The culture medium (referred to as 'medium' below) was RPMI 1640 containing 1 mM-pyruvate, 4 mM-glutamine, penicillin (50 i.u./ml), streptomycin (50 µg/ml) and foetal bovine serum. The serum concentration was 5% for established cell lines, 10% after fusion and 20% during cloning. HAT medium (Littlefield, 1964) contained 0.4 µM-aminopterin, 0.1 mM-hypoxanthine and 16 µM-thymidine. For freezing cells, the medium contained 6% dimethyl sulphoxide. Phosphate-buffered saline, pH 7.2, used in cell fusion, contained 8 g of NaCl, 0.4 g of KCl, 3.58 g of Na₂HPO₄·12H₂O, 0.78 g of NaH₂PO₄·2H₂O, 2 g of D-glucose, 10 mg of Phenol Red/litre.

Immunization. Balb/c mice received intra-peritoneal injections of purified pig kidney microvilli (Booth & Kenny, 1974) in phosphate-buffered saline in the following schedule (related to the day of fusion=0 day): -56 days in Freund's complete adjuvant, -35 days in incomplete adjuvant, -21 days no adjuvant, -4 days no adjuvant.

Cell fusion. Sp2/0 cells were cultured in logarithmic phase and fused with splenocytes. This and the subsequent stages followed the protocol of Fazekas de St. Groth & Scheidegger (1980). After fusion, the cells were cultured in 6 × 24-well plates (Linbro; catalogue no. 76-033-05).

Feeder cells. Macrophages were obtained from 150 g Wistar rats by peritoneal lavage with 40 ml of 0.34 M-sucrose.

Screening of culture supernatants. This was performed by an immunoradiometric assay in polyvinyl 96-well plates (Dynatech; code 1-220-24). The buffer (referred to as 'saline') contained, in 1 litre, 9.0 g of NaCl, 0.29 g of NaH₂PO₄·2H₂O, 2.9 g of Na₂HPO₄·12H₂O, 0.2 g of NaN₃, pH 7.4. 'Dilute saline' was prepared by diluting 1:10; 'High-salt saline' contained 0.54 M-NaCl; 'albumin/saline' contained 1% bovine serum albumin. Each well received 50 µl of 0.1% poly-L-lysine (Sigma type 1B, P1886)

in dilute saline, mechanically shaken for 5 min and left for 1 h. After washing twice with saline, 50 µl containing 5 µg of pig microvillar membrane protein (Booth & Kenny, 1974) in dilute saline was added to each well and left for 1 h before centrifuging for 20 min at 1400 rev./min (r_{av} , 25 cm) (MSE Coolspin centrifuge). The supernatant was poured off and wells filled with 0.25% glutaraldehyde in saline and left for 10 min. After washing twice with saline, the wells were filled with 1%-albumin/saline and stood for 3 h at 20°C and overnight at 4°C. Plates were washed twice, filled with saline, sealed with Parafilm, and stored until needed at 4°C. When a soluble antigen was used in place of membrane vesicles, it was added directly to untreated plates (approx. 0.1–1 µg in 50 µl of dilute saline), shaken for 10 min, and left for 2 h. Plates were washed twice with saline, then left for 1 h, filled with albumin/saline and washed four times with saline and stored as before. All the steps for the preparation of both types of plate were at room temperature unless otherwise stated. The assay was performed as follows: 50 µl of culture supernatant (or serial dilutions of serum or ascites fluid in albumin/saline) was added to each well, shaken for 10 min and left for 1.5 h at 4°C. Control wells contained albumin/saline or culture medium. Plates were washed twice with saline, twice with high-salt saline, and once with saline before addition of 50 µl of ¹²⁵I-IgG (50 000 c.p.m.) in albumin/saline. After 1.5 h at 4°C the plates were washed as before, dried, and each well assayed for radioactivity in a γ-scintillation counter.

Preparation of ¹²⁵I-IgG for screening. A rabbit was immunized (Fulcher & Kenny, 1983) with an IgG fraction obtained from mouse serum either by elution from DEAE-cellulose (see below) or by elution from a column of protein A-Sepharose (Pharmacia), the supplier's instructions being followed. The mouse IgG was coupled to glutaraldehyde-activated Ultrogel AcA 22 (Ternynck & Avrameas, 1972) and the rabbit anti-mouse IgG was affinity-purified by binding to this column and elution by 0.2 M-glycine/HCl, pH 2.3. Fractions were neutralized with Tris base immediately after collection. For iodination, 50 µg of the affinity-purified IgG was labelled with 1 mCi of Na¹²⁵I (Amersham International, code IMS 30) by using the chloramine-T method (Brown *et al.*, 1979).

Cloning of hybridomas. This was achieved in 'soft' agar [5 ml of 0.5% Bacto-agar (Difco) in medium with 20% foetal-bovine serum was added to 50 mm-diameter Petri dishes and allowed to set]. Cells were diluted in medium with 20% serum, in the range 100–2500 cells/ml; 0.3 ml samples were mixed with 0.3 ml of agar medium at 43°C and distributed over the base layer and allowed to set at 4°C before transferring to a CO₂ incubator. Clones were picked

by Pasteur pipettes 5–8 days later and expanded in 24-well plates.

Ascites tumours. These were induced by injecting 10^7 cloned cells into the peritoneal cavity of Balb/c or Balb/c $\times$ DBA2 mice, primed with 0.5 ml of Pristane given 1–4 weeks earlier. When ascites was evident, the mice were killed and the fluid was collected by a Pasteur pipette into heparinized tubes. The supernatant, after removing cells, was further purified by DEAE-cellulose chromatography. The sample was loaded in 10 mM-Tris/HCl, pH 7.8 (4°C) and eluted by an NaCl gradient (0–200 mM) in the same buffer.

Large-scale culture of hybridoma cells. The cells were grown to confluence in 200 ml of medium (10% serum) and then transferred to spinner culture in CO₂/air (1:19). The culture was gradually expanded by the addition of serum-free medium until it had reached 2 litres, at which point the cells were permitted to grow to stationary phase. The supernatant was harvested and precipitated by (NH₄)₂SO₄ (50% saturation). The pellet was taken up in 10 mM-Tris/HCl, pH 7.8, dialysed against this buffer and applied to a column of DEAE-cellulose. This was eluted in batches. The fraction eluting at 75 mM-NaCl contained the GK 7C2.

Immunocytochemical methods. PK1 cells were cultured on coverslips in six-well trays in medium containing 10% (v/v) newborn-calf serum until about 60% confluency had been achieved. Buffered saline for all solutions used in immunocytochemistry contained (in 1 litre) 0.29 g of NaH₂PO₄·2H₂O, 4.525 g of Na₂HPO₄·12H₂O, 8.77 g of NaCl, 0.9 mM-CaCl₂, 0.5 mM-MgCl₂, pH 7.4. After washing thrice at 37°C, the cells were fixed in 3% (w/v) paraformaldehyde for 20 min initially at 37°C then at 20°C. After washing, the preparation was quenched with 50 mM-NH₄Cl. Antibodies were diluted in 0.2% gelatin (Sigma G9382) and centrifuged (18000 g for 2 min) before use. Indirect staining was performed for 20 min periods under humid conditions. The second antibody was affinity-purified rabbit anti-mouse IgG and conjugated with Rhodamine (prepared from Lissamine Rhodamine B RB200) after conversion into the sulphonyl chloride form (Brandtzaeg, 1973). The conjugate was purified by chromatography on Sephadex G-50 and DEAE-cellulose.

Tissues were examined in essentially the same way. They were obtained immediately after killing the piglet, frozen in isopentane cooled by liquid N₂, stored in liquid N₂ and sectioned (6 μ m) at –20°C with a Slee cryomicrotome. Sections were transferred to coverslips. After staining, the preparations were mounted in 90% (v/v) glycerol in buffered saline.

Immunoprecipitation of kidney microvillar proteins. Kidney microvillar membrane was solubilized by Triton X-100. A sample containing 1 mg of protein in a buffer containing 0.15 M-NaCl, 40 mM-Na₂EDTA, 40 mM-Tris base, 2% Triton X-100, pH 7.0, was mixed with partially purified GK 7C2 (200 μ g of protein containing about 15 μ g of GK 7C2) and left overnight at 4°C. Affinity-purified rabbit anti-mouse IgG (15 μ g) was added and, after 18 h at 4°C, the precipitate was pelleted at 18000 g for 15 min. It was washed five times in the same buffer containing 0.1% Triton X-100. A control incubation lacked the monoclonal antibody.

Immunoabsorption of enzyme activity using Sepharose-protein A. Sepharose-protein A (50 μ l) was incubated at 20°C for 1 h, with 100 μ g of affinity-purified rabbit anti-mouse IgG. The gel was washed once with 50 mM-Tris/HCl/0.5 M-NaCl/0.1% Triton X-100, pH 7.4, pelleted and resuspended in 1 ml of this medium. Samples (0.5 ml) were incubated overnight at 4°C with either 4.5 ml of spent supernatant medium from 7C2 cells or fresh medium. After washing three times with the Tris/NaCl buffer, each was incubated overnight with Triton X-100-solubilized kidney microvilli (2 mg of protein) in 1 ml of the same buffer. The pellets were washed five times in buffer, resuspended in endopeptidase assay buffer and assayed with ¹²⁵I-insulin B-chain (Kenny, 1977).

'Western' blotting with nitrocellulose membrane. Microvillar membrane proteins were loaded (100 μ g/track) after dissociation at 100°C for 3 min by SDS, and electrophoresed in a 7–17% polyacrylamide gradient slab gel. Transfer of the polypeptides to nitrocellulose membrane (Schleicher and Schüll GmbH, D-3354 Dassel, Germany) was effected in a Bio-Rad Trans-Blot cell by using the method of Towbin *et al.* (1979) with the Tris/glycine/methanol buffer system, at 60 V for 4 h. Transfer was checked by staining a strip cut from the sheet; the destaining step was modified to minimize changes in size (methanol/acetic acid/water, 10:1:9, by vol.). The rest was treated overnight at 4°C with 0.5% Tween-20 in 50 mM-Tris base/150 mM-NaCl/5 mM-Na₃N, pH 10.2 (unadjusted). The sheet was cut into strips and each incubated overnight in supernatant medium from the monoclonal culture. Strips were washed (five times) in the same buffer without Tween-20 and incubated at 4°C overnight with horseradish peroxidase coupled to rabbit anti-mouse IgG (Sigma; A2028) in the same medium. Strips were washed five times and transferred to staining solution as described by Graham *et al.* (1965).

Preparation of the GK 7C2 immunoabsorbent column. The ascites fluid from 15 mice was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-cellulose chromatography. The antibody was present in a peak eluted between 60 and 100 mM-NaCl. These fractions were pooled and contained 85 mg of protein, of which half was coupled to 4.0 ml of CNBr-activated Sepharose 4B (as described by the manufacturers).

Other methods. SDS/polyacrylamide-gel electrophoresis, determination of protein, assay of endopeptidase activity with ^{125}I -insulin B-chain and immunoabsorbent purification of the enzyme were performed as previously described (Fulcher & Kenny, 1983). The unit of endopeptidase activity is the hydrolysis of 1 μmol of labelled insulin B-chain/min at 37°C under the assay conditions described.

Results and discussion

Production of GK 7C2 monoclonal antibody

GK 7C2 was obtained from fusion 10 with splenocytes from a Balb/c mouse immunized with pig kidney microvilli. After the fusion step, 81 out of 144 wells showed hybridoma growth and 19 wells screened positively by immunoradiometric assay. The supernatant from one well, F10-2B1, gave the highest screening result and this hybrid was cloned in soft agar. All the clones gave similarly positive values on screening. Of these, two clones, 7C2 and 6C4, were selected for culture and the former was recloned with the same findings. Clone 6C4 was indistinguishable in all experiments from the twice-cloned 7C2 (results not shown). These observations are consistent with the view that the hybridoma cells in well 2B1 were derived from a single clone. The 7C2 hybridoma line has proved to be stable during repeated culture over a period of 6 months.

Two methods have been used to produce antibody GK 7C2 in quantity. Ascites tumours were readily obtained in mice. Most of the antibody has been produced in this way and partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-cellulose chromatography. The fluid from 15 mice yielded a fraction containing 85 mg of protein. This fraction was by no means pure; analysis of the fraction by SDS/polyacrylamide-gel electrophoresis showed that GK 7C2 was not the main component. A densitometric scan of the stained gel permitted an estimate of its concentration, since one band clearly corresponded to the monoclonal light chain. Assuming staining to be proportional to polypeptide content, the contribution of IgG was assessed as 15%, giving a yield of less than 1 mg per mouse. A later production with five mice yielded 15 mg of

Fig. 1. *Metabolic labelling of GK 7C2 by [^{14}C]leucine* Hybridoma cells (10^7) were washed once with leucine-free Eagle's minimum essential medium containing 5% (v/v) foetal-bovine serum that had been previously dialysed against phosphate-buffered saline (see under 'Media' in the Experimental section). The cells were resuspended in the same medium containing 2 μCi of L-[U- ^{14}C]leucine and incubated for 18 h. The supernatant medium was enriched with 20 μg of mouse IgG and then precipitated by the addition of 200 μg of rabbit anti-(mouse IgG). After washing, the precipitate was subjected to SDS/polyacrylamide-gel electrophoresis and a fluorograph prepared (Bonner & Lasky, 1974). The fluorograph reveals a heavy chain (H) of M_r 50 000 and a single light chain (L) of M_r 25 000.

protein in the peak fractions, half of which appeared to be GK 7C2, i.e. 1.6 mg/mouse.

GK 7C2 was also produced by culturing the hybridoma cells to a final volume of 2 litres. The antibody fraction obtained by DEAE-cellulose chromatography contained 7.1 mg of protein, of which about half appeared to be GK 7C2.

Characterization of GK 7C2 as an IgG

The low yield of secreted antibody from this hybridoma made it difficult to characterize its Ig type by metabolic labelling with [^{14}C]leucine. Most other cloned hybridoma cultures secreted single radioactive products easily identified, by

Fig. 2. *SDS/polyacrylamide gels showing the antigen bound by GK 7C2*

See the Experimental section for details. (a) Immunoprecipitation, by a second antibody, of antigen bound by GK 7C2 from Triton X-100-solubilized microvillar proteins. Tracks 1 and 3, microvillar proteins; track 2, control (GK 7C2 omitted); track 4, GK 7C2; in addition to IgG chains (mainly second antibody) there is a stained band of apparent M_r 90000. (b) Immunoabsorbent chromatography. Track 1, microvilli (100 µg); track 2, markers (Pharmacia) M_r ($\times 10^{-3}$) 94, 67, 43, 30, 20, 14; track 3, endopeptidase-24.11 (10 µg) eluted from column as in Fig. 4.

fluorography of SDS/polyacrylamide gels, as IgM or IgG types. The labelled product of 7C2 cells was not readily discernible from minor radioactive contaminants released from damaged cells. This limitation was overcome by precipitating the labelled Ig with a second antibody. The resulting fluorograph (Fig. 1) confirms GK 7C2 as an IgG, and the single band corresponding to the light chain is also evidence of successful cloning. Ouchterlony diffusion with rabbit antisera against IgG, IgG1, IgM and IgA showed a precipitate against only the first serum. The elution peak from DEAE-cellulose columns of GK 7C2 was consistently observed when the gradient reached 75 mM-NaCl.

Identification of the antigen recognized by GK 7C2

Monolayers of PK1 cells, grown to near confluence on coverslips, were stained with GK 7C2

and a Rhodamine-conjugated second antibody. Some cultures were treated with Triton X-100 to expose internal antigens. The fluorescence observed took the form of a weak punctate surface staining, best seen in the cultures not exposed to detergent.

The first indication of the identity of the antigen was obtained by immunoprecipitation from Triton X-100-solubilized microvilli using a second antibody. The washed immunoprecipitate was analysed by SDS/polyacrylamide-gel electrophoresis. A control omitted the monoclonal antibody. The stained pattern is shown in Fig. 2(a). Apart from the strongly staining IgG heavy chain and the heterogeneous light chains [mainly derived from the rabbit anti-(mouse IgG) antibody], a band of apparent M_r 90000 was visible. The control contained only some traces of IgG bands.

Western blotting on to nitrocellulose also con-

Fig. 3. *Transblotting of microvillar membrane proteins to nitrocellulose membrane*

See the Experimental section for details. Tracks 1 and 3 were stained with Amido Black. Track 2 was stained by the peroxidase reaction, after addition of the second, peroxidase-conjugated antibody. Track 1, marker proteins (Pharmacia) M_r ($\times 10^{-3}$) 94, 67, 43, 30; track 2, GK 7C2 culture supernatant; track 3, microvillar proteins.

firmed the binding of GK 7C2 to a polypeptide of apparent M_r 90000. The results are shown in Fig. 3. The pattern of proteins transferred conformed to that observed in stained gels (cf. Fig. 2) showing efficient transfer. Staining by the peroxidase reaction of the strip incubated with GK 7C2 revealed a single strong band corresponding to a peptide of about 90000 apparent M_r . Some very faint bands were seen, but these were also visible in the controls with other hybridomas (not shown).

Fig. 4. *Immunoabsorbent chromatography of microvillar membrane proteins*

See the Experimental section for preparation of column. The loading, washing and elution was as described previously (Fulcher & Kenny, 1983). The arrow indicates the start of elution by pH 10.6 buffer. The stippled panel shows the endopeptidase-24.11 activity that was unbound, i.e. in the breakthrough and wash volumes; O, endopeptidase activity eluted.

Since kidney endopeptidase-24.11 has an apparent subunit M_r of 89000 (Fulcher & Kenny, 1983), we attempted to confirm the presumed identity by adapting the immunoradiometric assay. Purified endopeptidase-24.11 was immobilized in wells of a polyvinyl plate in a quantity (250 ng/well) that was comparable with the endopeptidase content of 5 μ g of microvillar protein used in the normal screening assay. The addition of supernatant medium from 7C2 cultures to the endopeptidase-containing wells increased the bound radioactivity by a factor of three compared with the controls. This value was less than that bound by wells containing microvillar membrane (6-fold over controls), but was a reproducible result. It is possible that the purified antigen was not quantitatively bound to the plastic and/or that the epitope recognized by GK 7C2 was partially masked by the attachment to the surface.

The enzyme activity of the antigen recognized was subsequently demonstrated by immobilizing the antigen-antibody complex with protein A-Sepharose. This was achieved in three steps: rabbit anti-mouse IgG was first bound to protein A-Sepharose, GK 7C2 was then allowed to bind and finally the complex was mixed with Triton X-100-solubilized microvilli. A control omitted the monoclonal antibody. The two complexes were then assayed for endopeptidase activity with 125 I-insulin B-chain as substrate. The control showed weak activity (0.035 m-unit), the experimental sample contained 0.22 m-unit, and 81% of this activity was inhibited by 1 μ M-phosphoramidon. Thus the information on the subunit M_r of the antigen and the

Fig. 5. *Phase-contrast and fluorescence microscopy of pig kidney cortex*

In each pair the micrograph on the left (a,c,e) is a phase-contrast view of the same area shown on the right by fluorescence with a rhodamine-conjugated second antibody (b,d,f). V, blood vessel; G, glomerulus; A, arteriole; CT, convoluted tubule. The fluorescence is confined to the lumens of the tubules.

Fig. 6. *Phase-contrast and fluorescence microscopy of pig jejunum*

In each pair, the micrograph on the left (a,c,e) is a phase-contrast view of the same area shown on the right by fluorescence with a rhodamine-conjugated second antibody (b,d,f). a and b, section through tips of villi; c and d, section through region near bases of villi; e and f, section through crypts. LP, lamina propria; C, crypt. In b and d, the fluorescence is concentrated at the brush border with some staining of basolateral membranes (in b). In f, only the interior of the crypts is fluorescent. No fluorescence of the lamina propria is visible.

demonstration of enzyme activity strongly pointed to endopeptidase-24.11 as the protein recognized by GK 7C2.

GK 7C2-immunoadsorbent purification of endopeptidase-24.11

The column was prepared by coupling half of the fraction obtained from ascites fluid. This amounted to 42 mg of protein containing about 6 mg of GK 7C2. The column was very effective in purifying a single protein from detergent-solubilized kidney microvilli. One operation of the column is shown in Fig. 4, in which the column was fully saturated by loading a large excess of endopeptidase-24.11 (1166 m-units). Of this, 764 m-units were recovered in the breakthrough and wash fractions, indicating that the binding capacity was 402 m-units. The unbound activity was not antigenically different from the bound enzyme; it could be purified by immuno-adsorbent chromatography in subsequent runs of the column. Elution at pH 10.6 yielded 146 m-units, 36% of the bound activity. Since the endopeptidase activity in this assay is slightly exaggerated by the presence of aminopeptidases in the microvillar fraction (Fulcher & Kenny, 1983), the corrected value for the bound fraction was 368 m-units and the corrected recovery 40%.

The specific activity was 142 m-units/mg of protein (mean of two batches) comparable with the value of 177 m-units/mg for that purified by a polyclonal immunoadsorbent column (Fulcher & Kenny, 1983). The GK 7C2-purified enzyme is shown in Fig. 2(b); it is homogeneous by polyacrylamide-gel electrophoresis and has an apparent M_r of 90 000.

The elution curve shows a prompt elution of activity when 1 column volume of pH 10.6 buffer had passed through. It also shows considerable trailing over several volumes. This was unexpected for a monoclonal antibody, but is probably explained by the anomalous binding properties of some of the antibody molecules caused by their covalent attachment to the column matrix.

Immunocytochemical staining of pig kidney and intestine with GK 7C2

The localization of the endopeptidase-24.11 in brush borders of kidney and intestine had been established by subcellular fractionation of the two tissues (George & Kenny, 1973; Booth & Kenny, 1974; Danielsen *et al.*, 1980). The availability of GK 7C2 has permitted us to confirm the localization by immunofluorescence microscopy. Frozen sections of kidney and intestine, obtained immediately after death from a 4-week-old Yucatan piglet, were fixed and stained by using a Rhodamine-conjugated

second antibody. Controls were prepared by omission of the first antibody. The results are shown in Figs 5 and 6, in which identical fields are observed by phase-contrast and fluorescent microscopy. In kidney cortex (Fig. 5) the fluorescence (b, d and f) is confined to the luminal aspects of the tubules. Glomeruli, connective tissue and blood vessels showed no fluorescence. The tubules are collapsed (since no attempt was made to fix by arterial perfusion) and the brush borders fill the lumens, with consequent distortion of the tubule cells, but the fluorescent tubules are typical of the proximal convoluted portion.

The intestinal villi (Fig. 6b and 6d) are intensely fluorescent at the luminal surface of the mucosal cells. Although there is some fluorescence of the basolateral membranes (Fig. 6a), it is much less intense than the brush border and barely visible at the base of the villus (Fig. 6d). In Fig. 6f, the fluorescence is seen to be confined to the crypts. There is no staining of the lamina propria at any level. Thus, in intestine, the endopeptidase is expressed in the luminal membrane at all stages in maturation of the mucosal cells as they migrate from crypt to villus tip.

Conclusions

Our current interest in many aspects of endopeptidase-24.11 made the acquisition of a monoclonal antibody to this enzyme a matter of high priority. To this end, several cell fusions have been done using splenocytes from mice immunized with highly purified preparations of the enzyme. None of these fusions yielded any useful antibodies. In the event, GK 7C2 was obtained after immunizing a mouse with a membrane preparation that contained about a dozen major proteins and many more minor components, not all of them necessarily associated with microvilli. The appearance of GK 7C2 in one of the cultures soon after fusion, coupled with its high screening value and apparently in a 'self-cloned' form, was a very welcome event. The only cause for regret is that this hybridoma is a relatively poor producer of antibody. Nevertheless it has already produced enough to permit rapid purification of 1 mg batches of pig kidney endopeptidase-24.11. It has been equally effective in purifying the enzyme from intestine (I. S. Fulcher & A. J. Kenny, unpublished work) and brain (J. Relton, A. J. Turner & A. J. Kenny, unpublished work). In immunocytochemistry, GK 7C2 has demonstrated the location of the antigen in the brush borders of kidney and intestine and may be expected to have applications in exploring the distribution of endopeptidase-24.11 in other organs and tissues.

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