Proteins of the kidney microvillar membrane

Structural and immunochemical properties of rat endopeptidase-2 and its immunohistochemical localization in tissues of rat and mouse

Kathleen BARNES, Jean INGRAM and A. John KENNY*

M.R.C. Membrane Peptidase Research Group, Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

The phosphoramidon-insensitive endopeptidase-2 in rat renal brush borders was investigated by immunochemical approaches with a rabbit polyclonal antibody raised to the purified enzyme released from the membrane by papain. An immunoaffinity column successfully purified the detergent-solubilized form of endopeptidase-2. This preparation had an apparent subunit M_r of 80 000, and did not show the two subunits, of M_r 80 000 and 74 000, consistently found in the papain-solubilized forms, indicating that the latter resulted from proteolysis by papain. SDS/polyacrylamide-gel electrophoresis of non-reduced samples of the enzyme revealed a band of M_r 220 000, confirming the presence of disulphide-bridged subunits. Treatment with endoglycosidases H and F generated smaller molecular forms, indicating that endopeptidase-2 contained about 30 % asparagine-linked carbohydrate and that a few of these oligosaccharide chains were of the high-mannose type. Treatment with phosphatidylinositol-specific phospholipase indicated that the enzyme did not possess a glycolipid membrane anchor. A survey of rat tissues examined immunohistochemically and by immunoblotting revealed that only the kidney and intestinal tract expressed the antigen in significant amounts. Although some weak staining was seen in salivary glands and thyroid, other organs and tissues including brain and spinal cord were negative by both immunochemical techniques. In the kidney the antigen was confined to the lumen of the proximal tubule and was seen mainly in the population of juxtamedullary nephrons. In the gut, luminal staining was observed throughout its whole length, from duodenum to rectum. Excellent cross-reactivity of the antibody with Balb/c mouse tissues was observed. Immunohistochemistry of mouse kidney and gut revealed a distribution identical with that observed in the rat. Immunopurification of the detergent-solubilized mouse kidney antigen showed it to be a protein containing disulphide-linked subunits of M_r 90 000. It possessed endopeptidase-2-like activity, but was more efficient in hydrolysing azo-casein and less efficient in hydrolysing a model substrate than the rat enzyme. The close similarity between rat endopeptidase-2 and mouse meprin is further supported by these results.

INTRODUCTION

The brush-border membrane of rat kidney contains two endopeptidases, endopeptidase-24.11 and endopeptidase-2; the former is sensitive to inhibition by phosphoramidon, whereas the latter is unaffected by this inhibitor (Kenny *et al.*, 1981; Kenny & Ingram, 1987). In this respect, rat kidney microvilli differ from those of pig, rabbit and human, in which only endopeptidase-24.11 is present. The brush borders of mouse kidneys contain an endopeptidase, known as 'meprin' (Bond *et al.*, 1983), which has a number of properties in common with rat endopeptidase-2 with regard to both structure and specificity (Bond *et al.*, 1986; Butler *et al.*, 1987; Kenny & Ingram, 1987; Stephenson & Kenny, 1988). In brief, both appear to have a tetrameric structure involving disulphide-linked subunits, to contain zinc at the active site and to be capable of hydrolysing peptide bonds flanked by a hydrophobic residue, which, curiously, can occupy either the P_1 or the P_1' position.

In our previous paper (Kenny & Ingram, 1987) on the

properties of the purified endopeptidase-2, the enzyme had been released as a hydrophilic protein by treatment of the membranes with papain. Two subunits of M_r 80 000 and 74 000 were seen on SDS/polyacrylamide-gel electrophoresis in reducing conditions, but it was unclear whether these represented dissimilar native subunits or were the result of limited proteolysis by papain. However, prolonged treatment of the purified enzyme with papain did not increase the yield of the smaller at the expense of the larger subunit, but instead slowly digested both. Attempts to isolate a detergent-solubilized form by using a similar protocol to that which yielded a homogeneous papain-released form were not successful. In the present paper we have exploited a polyclonal antibody generated in a rabbit by immunization with the papain-released form to isolate the detergent-solubilized form of endopeptidase-2 and to explore its localization in kidney and other rat tissues by immuno-peroxidase histochemistry. It also proved to cross-react with a similar antigen in mouse tissues, a property that enabled us to isolate the mouse antigen and to investigate

Abbreviations used: Bz-Tyr-pAB, *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid; -NH-Mec, 7-amino-4-methylcoumarylamide; PI-PLC, phosphatidylinositol-specific phospholipase C; PBS, phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.4).

* To whom correspondence should be addressed.

its immunohistochemical distribution. Our findings further support the close similarity of meprin and endopeptidase-2.

EXPERIMENTAL

Materials

Rat kidneys from male Wistar rats and mouse kidneys from Balb/c mice were stored frozen (-70°C) until required. *Bacillus cereus* phospholipase C (type III) was from Sigma Chemical Co.; PI-PLC from *Bacillus thuringiensis* was the same batch that released renal dipeptidase and aminopeptidase P from pig kidney microvilli (Hooper *et al.*, 1987; Hooper & Turner, 1988) and was a gift from Dr M. G. Low.

Endoglycosidase H (endo- β -N-acetylglucosaminidase H, EC 3.2.1.96) and endoglycosidase F (endo- β -N-acetylglucosaminidase F, EC 3.2.1.96) were from Boehringer Mannheim. N-Glycanase (glycopeptide N-glycosidase, EC 3.2.2.18) was from Genzyme. Immunohistochemical reagents were those previously described (Barnes *et al.*, 1988).

Methods

Enzyme assays. Endopeptidase-2 and endopeptidase-24.11 (EC 3.4.24.11) were assayed with ^{125}I -labelled insulin B-chain as substrate, the former being defined as the phosphoramidon-insensitive peptidase activity; this assay, and others with Bz-Tyr-pAB and azo-casein as substrates, were performed as previously described (Kenny & Ingram, 1987). Aminopeptidase N (EC 3.4.11.2) was assayed with Ala-NH-Mec as substrate (Fulcher & Kenny, 1983). Alkaline phosphatase (EC 3.1.3.1) was assayed with 4-nitrophenyl phosphate as substrate (Bessey *et al.*, 1946). All units are expressed as μmol of substrate hydrolysed/min at 37°C , except for the azo-casein assay, which is expressed in arbitrary units.

Antibody to endopeptidase-2. The antibody used in these studies (RRt151) was produced by immunizing a rabbit by the protocol described previously (Fulcher & Kenny, 1983) with as the immunogen the preparation of papain-released endopeptidase-2 previously characterized (Kenny & Ingram, 1987). The IgG fraction was obtained by elution from a Protein A-Sepharose column (Danielsen *et al.*, 1980). When titrated against Triton-solubilized rat kidney microvillar proteins, all the endopeptidase-2 activity was precipitated without significant inhibition of the enzymic activity, $4.8 \mu\text{g}$ of IgG being equivalent to $1 \mu\text{g}$ of endopeptidase-2. Monospecificity was demonstrated by crossed immunoelectrophoresis (as previously described by Booth *et al.*, 1979) of rat kidney membranes solubilized by papain treatment. Fig. 1(a) shows a single symmetrical precipitation arc. By Western blotting against microvillar membrane proteins, a single strong band (M_r 82000) with a weak band (M_r 90000) were seen. No band corresponding to aminopeptidase N (M_r 140000) was visible, but, to exclude further any antibodies recognizing this major antigen in microvilli, the IgG preparation was absorbed with CNBr-activated Sepharose to which purified aminopeptidase N had been coupled.

Immunoprecipitation of endopeptidase-2. Rat and mouse microvillar membranes (Booth & Kenny, 1974)

Fig. 1. Specificity of antibody RRt151

See the Experimental section for details. (a) Crossed immunoelectrophoresis. A papain-released fraction of rat microvillar membranes ($20 \mu\text{g}$ of protein in the well marked o) was electrophoresed in the horizontal axis for 1 h at 5 V/cm and in the other axis for 18 h at 2 V/cm into a window containing $200 \mu\text{g}$ of IgG/ml. The gel was stained with Coomassie Blue. (b) Western immunoblot of microvillar membranes. Protein loading was as follows: track 1, $50 \mu\text{g}$; track 2, $20 \mu\text{g}$; track 3, $10 \mu\text{g}$. Track M, M_r markers.

were solubilized in Triton X-100 (see below), and portions ($100 \mu\text{g}$ of protein in $50 \mu\text{l}$) were incubated at 4°C for 18 h with various amounts of the IgG fraction (RRt151) in PBS containing 0.02% NaN_3 . The precipitates were centrifuged and washed four times with PBS/ NaN_3 and analysed by SDS/polyacrylamide-gel electrophoresis.

Purification of endopeptidase-2 from detergent-solubilized rat kidney membranes. The IgG fraction (5 mg) of the immune serum was absorbed for 18 h with $200 \mu\text{l}$ of CNBr-activated Sepharose to which had been coupled rat aminopeptidase N (5 mg/ml). After removal of the beads by centrifugation, the IgG fraction was dialysed for 18 h against 2 litres of $500 \text{ mM-NaCl}/0.25 \text{ M-NaHCO}_3$ buffer, pH 8.7, before being coupled to 1 ml of

CNBr-activated Sepharose (Pharmacia) as described by the manufacturers. Only 4.6 % of the protein remained unbound. A microsomal fraction was prepared from 50 g of frozen rat kidneys (Kenny & Ingram, 1987), solubilized by Triton X-100 (detergent/protein ratio 7:1, w/w) and centrifuged (31 000 *g* for 90 min) to remove insoluble material. The supernatant fraction was chromatographed on DEAE-cellulose as described previously (Kenny & Ingram, 1987) except that the buffers contained 0.1 % Triton X-100. The pooled active fractions were pumped slowly (20 h) on to the immunoaffinity column, which was washed at the same rate (2 ml/h) for 40 h with 500 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4, containing 0.1 % Triton X-100. Elution was effected with 200 mM-NaHCO₃/NaOH buffer, pH 10.6, containing 0.1 % Triton X-100 at 5 ml/h. The eluate was collected in 1 ml fractions into tubes containing 1 ml of 200 mM-Pipes/NaOH buffer, pH 6.2.

Purification of endopeptidase-2 from mouse kidney. Kidneys from Balb/c mice were removed immediately after death and stored frozen at -70 °C. A microsomal pellet was prepared from 3 g of kidney and subjected to the same purification procedure as described above for rat kidney.

Treatment of endopeptidase-2 with endoglycosidases. N-Glycanase was used as described previously (Kenny & Ingram, 1987). Endoglycosidase H (0.1–0.2 munit) was incubated (total volume 50 μ l) with 300 mM-sodium citrate buffer, pH 5.5 (10 μ l), 0.3 % SDS (5 μ l), 1 mM-phenylmethanesulphonyl fluoride (10 μ l) and 300 mM-2-mercaptoethanol (5 μ l). Endoglycosidase F (2–5 munits) was incubated (total volume 30 μ l) with 300 mM-sodium phosphate buffer, pH 7 (10 μ l), and 300 mM-EDTA (5 μ l). Purified samples of detergent-solubilized and papain-released endopeptidase-2 (1 μ g) were treated with the glycosidases for 18 h at 37 °C. In the case of the pH 5.5 incubation, 3 M-Tris base (3 μ l) was then added followed by SDS dissociation buffer for electrophoresis in polyacrylamide gels.

Treatment of rat kidney microvillar membrane fraction with PI-PLC. Rat kidney microvillar membranes were prepared (Booth & Kenny, 1974) and resuspended in 10 mM-Hepes/NaOH buffer, pH 7.4 (10 mg of protein/ml). Two PI-PLC preparations of phospholipase C, from *B. thuringiensis* and *B. cereus*, were used. The membranes (250 μ l) and enzyme (0.005, 0.05 and 0.5 unit in 250 μ l of the same buffer) were incubated at 37 °C for 90 min. The suspensions were cooled and after removal from each of a sample for assay were centrifuged at 31 000 *g* for 90 min at 4 °C. The activities of endopeptidase-24.11, endopeptidase-2 and alkaline phosphatase were determined in the suspension and supernatant fractions after centrifugation.

SDS/polyacrylamide-gel electrophoresis. Slab gels (7–17 % acrylamide unless otherwise stated) were set up, run as described by Laemmli (1970) and stained with Coomassie Brilliant Blue or by silver [Morrissey (1981) as modified by Dubray & Bezdard. (1982)]. Western immunoblotting was performed as described previously (Matsas *et al.*, 1986) except that the peroxidase system was replaced with an alkaline-phosphatase-conjugated

second antibody, the colour being developed with Nitro Blue Tetrazolium (ProtoBlot; Promega Biotec, Madison, WI, U.S.A.).

Immunohistochemical methods. The presence of endopeptidase-2 in cryostat sections was detected by an immunoperoxidase method depending on the use of a biotinylated second antibody and a biotinylated horseradish peroxidase–streptavidin complex (Amersham International) by the use of previously described methods (Matsas *et al.*, 1986; Barnes *et al.*, 1988).

Pathogen-free Wistar rats (body wt. 350–410 g) were anaesthetized with Inactin (Idis, Kingston-upon-Thames, Surrey, U.K.), injected with 200 i.u. of heparin via a cannula in the jugular vein and perfused through the heart with 20 ml of PBS containing heparin, followed by 4 % (w/v) paraformaldehyde. Blocks were cut from the tissues, rinsed in PBS, frozen in isopentane cooled by liquid N₂ and stored in liquid N₂ until required. Cryostat sections (6–10 μ m thick) were thaw-mounted on coverslips previously treated with 3-aminopropyltriethoxysilane (Hauser & Dreyer, 1981).

All antibodies were diluted in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4, containing 2 % (w/v) gelatin and 1.5 % (w/v) rat serum, and the biotinylated second antibody was pre-absorbed with 'rat powder' prepared by centrifugation of a 10 % (w/v) rat kidney homogenate at 30 000 *g* for 30 min and freeze-drying the pellet. Unless stated, all sections were counterstained with Harris' haematoxylin to show up cell nuclei. The RRt151 anti-endopeptidase-2 IgG was also used on cryostat sections (6–10 μ m thick) taken from fresh mouse tissue blocks. In this case the rat serum was replaced by mouse serum in the diluent.

RESULTS

Immunoprecipitation of proteins from detergent-solubilized microvillar fractions of rat and mouse kidneys

Fig. 2 (track 3) shows a Coomassie Blue-stained gel of the immunoprecipitate of detergent-solubilized rat microvillar proteins, revealing a major band of *M_r* 80 000 and a much weaker one of *M_r* 90 000. A silver-stained gel shows that the antibody precipitated two proteins of *M_r* 90 000 and 100 000 (the latter somewhat weaker) from solubilized mouse kidney membranes (track 4). Immunoprecipitation of the purified enzyme from mouse showed the same pattern (track 5).

Immunoaffinity purification of the detergent-solubilized form of endopeptidase-2

Two batches of Triton X-100-solubilized microsomal fractions were processed as described in the Experimental section, but on the first occasion the fractions eluted from the column were not neutralized at the time of collection and the activities were low, suggesting lability of the enzyme in the pH 10.6 buffer. The results from the second preparation are shown in Table 1. Of the 800 munits loaded, 704 munits were not bound and the yield of activity on elution was only 1 % (6.9 munits) of that bound. On reloading the unbound material in successive cycles, each elution cycle yielded 6–10 munits. The pooled activity from four cycles gave an overall yield of 0.5 % of the homogenate activity with a mean specific

Fig. 2. Immunoprecipitation of proteins from detergent-solubilized microvillar fractions from rat and mouse kidneys

See the Experimental section for details. Tracks 1, 2 and 3 are in a Coomassie Blue-stained gel. Tracks 4, 5 and 6 are in a silver-stained gel. Track 1, rat microvillar membrane proteins (not precipitated); track 2, M_r markers; track 3, immunoprecipitate from rat membranes; track 4, immunoprecipitate from mouse membranes; track 5, immunoprecipitate of purified mouse kidney endopeptidase-2; track 6, M_r markers. A strong 50000- M_r IgG heavy chain is also seen.

activity of 69 munits/mg of protein, comparable with that reported for the papain-released form (Kenny & Ingram, 1987). SDS/polyacrylamide-gel electrophoresis of the detergent-solubilized product revealed a band (Fig. 3, track Det) very slightly retarded compared with the larger band (M_r 80000) of the doublet observed in all the papain-released forms (Fig. 3, track Pap). An immunoblot of another SDS/polyacrylamide gel after

Fig. 3. SDS/polyacrylamide-gel electrophoresis of papain-released and detergent-solubilized forms of endopeptidase-2

See the Experimental section for details. Track M, M_r markers; track Pap, papain-released form (30 µg); track Det, detergent-solubilized form (15 µg). The gel was stained with Coomassie Blue.

electrophoresis is shown in Fig. 4. Under non-reducing conditions a band of M_r 220000 was seen in the track (track 2) containing the detergent-solubilized form. The papain-released form (track 3) migrated slightly faster. The reduced form of the papain-released protein (track 5) exhibited the same pattern as shown in Fig. 2, and a minor band of M_r 90000 in the detergent-solubilized form was also revealed by the immunoblot similar to that shown in Fig. 1.

Treatment of endopeptidase-2 with endoglycosidases

Treatment of the papain-released form with *N*-Glycanase caused extensive proteolysis that was not

Table 1. Immunoaffinity purification of detergent-solubilized rat endopeptidase-2

See the Experimental section for details.

	Protein (mg)	Total activity (munits)	Yield (%)	Specific activity (munits/mg)	Purification (fold)
Homogenate	7380	5550	100	0.75	1
Microsomal pellet	1500	3790	68	2.5	3.4
Triton X-100 supernatant fraction	918	2390	43	2.6	3.4
DEAE-cellulose fraction	287	800	14	2.8	3.7
Immunoaffinity column eluates (four consecutive runs)	0.097	6.9	—	71	95
	0.115	6.1	—	53	71
	0.118	6.1	—	52	69
	0.098	10.3	—	106	141
(Totals/means of four runs)	(0.428)	(29.4)	(0.5)	(69)	(92)

Fig. 4. SDS/polyacrylamide-gel electrophoresis of papain-released and detergent-solubilized forms of endopeptidase-2 from rat kidney and the detergent-solubilized form from mouse kidney

See the Experimental section for details. Western immunoblot of a 4–30% acrylamide gradient gel. Tracks 1, 2 and 3, non-reduced samples. Tracks 4, 5 and 6, dithiothreitol-reduced samples. Tracks 1 and 4, affinity-purified proteins from detergent-solubilized mouse kidney membranes; tracks 2 and 5, detergent-solubilized form of rat endopeptidase-2; tracks 3 and 6, papain-released form of rat endopeptidase-2.

inhibited by chelating agents or di-isopropyl phosphorofluoridate (Kenny & Ingram, 1987). The same fate was suffered by the Triton X-100-solubilized form (results not shown). However, both endoglycosidase H and endoglycosidase F were effective in converting the forms into bands of lower M_r (Fig. 5). With endoglycosidase H the change in M_r was about 3000. Endoglycosidase F converted the papain-released form into a series of products ranging from M_r 70000 to M_r 54000. The detergent-solubilized form responded similarly (results not shown).

Incubation of rat kidney microvillar membranes with PI-PLC

The release of alkaline phosphatase, an enzyme known to be anchored to microvilli by a glycolipid anchor (Low, 1987), was demonstrated with the PI-PLC preparations from both *B. cereus* and *B. thuringiensis* (Fig. 6). Each preparation released more than 90% of the membrane activity at the highest phospholipase concentration. In contrast, less than 3.5% of either endopeptidase-24.11 or endopeptidase-2 was released.

Immunoprecipitation of endopeptidase activity from detergent-solubilized mouse kidney microvilli

Increasing amounts of the RRt151 IgG fraction were added to 100 μ g quantities of mouse microvillar membrane protein, solubilized with Triton X-100. After incubation at 4 °C for 18 h, peptidase activity was assayed with ^{125} I-labelled insulin B-chain and azo-casein as substrates, the former in the presence of 1 μ M-phosphoramidon. In the resuspended mixtures both activities were inhibited in parallel to 40% of the control and, after centrifugation, the precipitation curves were

Fig. 5. SDS/polyacrylamide-gel electrophoresis of endopeptidase-2 after treatment with endoglycosidases

See the Experimental section for details. Western immunoblot. (a) Detergent-solubilized form; (b) and (c) papain-released form. Abbreviations: Endo-H, endoglycosidase H; Endo-F, endoglycosidase F. Endoglycosidase H converted the 80000- M_r band of both forms into one of M_r about 77000. Endoglycosidase F generated a family of deglycosylated products, the smallest of which was a 54000- M_r band.

Fig. 6. Effect of incubation of rat microvillar membranes with PI-PLC

See the Experimental section for details. ○, □ and △, incubation with *B. cereus* PI-PLC; ●, ■ and ▲, incubation with *B. thuringiensis* PI-PLC; ○ and ●, alkaline phosphatase; □ and ■, endopeptidase-2; △ and ▲, endopeptidase-24.11 (i.e. phosphoramidon-sensitive hydrolysis of ¹²⁵I-labelled insulin B-chain).

also identical, only 10 % of the activities remaining in the supernatant.

Immunoaffinity purification of a mouse endopeptidase by the anti-endopeptidase-2 IgG

Since antibody RRt151 precipitated the endopeptidase-2 activity from detergent-solubilized mouse microvillar membranes and yielded polypeptides of M_r 90000 and 100000 (Fig. 2, tracks 4 and 5), a small-scale preparation of the endopeptidase activity was attempted from 3 g of Balb/c mice kidneys, following the same steps employed for the purification of the detergent-solubilized form of rat endopeptidase-2. Although only 6.4 munits were present in the DEAE-cellulose fractions, 5.4 munits remained unbound by the RRt151 immunoaffinity column and, after extensive washing, elution of the column at pH 10.6 yielded only 0.10 munit. Assay of a sample of the column material revealed 1.1 munits to be still bound. The eluted material had a specific activity of 6.46 munits of protein, and was not as pure as the rat preparations in that endopeptidase-

24.11 (i.e. phosphoramidon-sensitive) activity was detectable with an activity of 0.3 munit/mg of protein. The immunoblot (Fig. 4) revealed a single band of M_r 90000 (track 4) when subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions and a major band of M_r 250000 with a minor one of M_r 220000 in non-reducing conditions (track 1).

The endopeptidase activity isolated by the immunoaffinity column from mouse kidneys was compared with rat endopeptidase-2 in the three assay systems with ¹²⁵I-labelled insulin B-chain, Bz-Tyr-pAB and azo-casein as substrates (Table 2). The papain-released and detergent-solubilized forms of rat endopeptidase-2 were similar in the relative values for the three assays, but the mouse preparation hydrolysed Bz-Tyr-pAB very weakly and azo-casein very strongly in comparison with the rat preparations.

Immuno-peroxidase staining of rat tissues

In the kidney (Fig. 7), the immunostaining for endopeptidase-2 was confined to the cortex, where a characteristic pattern was revealed. The frequency of positively stained tubules was highest in the juxtamedullary zone (Fig. 7a), indicating that the long looped nephrons with their glomeruli in this zone express this antigen on the surface of the proximal tubule. The outermost zone of the cortex was unstained, but small groups of tubules, sectioned transversely, were arrayed in a radial pattern between the outer zone and the consistently stained juxtamedullary zone. At higher magnifications (Figs. 7b and 7c) it is apparent that the staining is confined to the brush border and includes both the convoluted and straight portions of the proximal tubules. Other components of the nephron, including glomeruli and distal tubules, were unstained.

A study along the length of the gastro-intestinal tract is shown in Figs. 8(a)–8(h). No staining was detected in the stomach (Fig. 8a), but throughout the small intestine the luminal surface of the villi was stained, including the duodenum (Fig. 8b), jejunum (Fig. 8c) and ileum (Fig. 8d), with some positive staining extending into the crypts. The luminal surface of the colon was also stained (Fig. 8e), and the staining continued into the rectum (Figs. 8f and 8g), again on the mucosal surface, and the ducts of some mucous glands were also stained. The last panel (Fig. 8h) shows that immunostaining of the rectum was abolished if the antibody was absorbed with purified endopeptidase-2.

In other tissues, weak positive staining was observed in

Table 2. Comparison of rat and mouse peptidase activities in different assays

See the Experimental section for details. The activities are shown relative to that in the insulin B-chain assay, the experimental values for which are given in parentheses (munits/mg of protein).

Peptidase preparation	Assay substrate...	Relative peptidase activity		
		¹²⁵ I-labelled insulin B-chain (phosphoramidon-insensitive)	Bz-Tyr-pAB	Azo-casein
Rat endopeptidase-2 (papain-released form)		100 (40.1)	144	914
Rat endopeptidase-2 (detergent-solubilized form)		100 (85.9)	146	819
Mouse peptidase eluted from immunoaffinity column (detergent-solubilized form)		100 (6.46)	15	9070

Fig. 7. Immuno-peroxidase staining for endopeptidase-2 in rat kidney

Cryostat sections ($6\ \mu\text{m}$ thick), cut from perfused tissue blocks, were immunostained with the polyclonal antibody RRt151 and counterstained with Harris' haematoxylin. (a) shows positive staining in the proximal tubules of the juxtamedullary zone and in some other nephrons with tubules radiating from the juxtamedullary zone towards the unstained outer cortex (C, cortex; M, medulla). (b) and (c) are micrographs at higher magnifications showing the strong luminal (brush border) staining of the tubules and the absence of staining in glomerulus (G). Bars in (a) and (b), $250\ \mu\text{m}$; bar in (c), $100\ \mu\text{m}$.

Fig. 8. Immuno-peroxidase staining for endopeptidase-2 in rat gastrointestinal tract

Cryostat sections ($10\ \mu\text{m}$ thick) were cut from perfused rat tissue blocks and immunostained with the polyclonal antibody RRt151 and counterstained with Harris' haematoxylin. Positive staining of the luminal surface of the villi and of the crypts can be seen in each region of the small intestine: duodenum (b), jejunum (c) and ileum (d). In micrographs (e), (f) and (g), staining of the mucosal luminal surface of the distal gut is shown: colon (e) and rectum (f and g), including mucosal glands of the rectum (shown in g). No staining was detected in stomach (a) or in section of rectum (h) with the same antibody pre-absorbed with purified endopeptidase-2. Bar, $250\ \mu\text{m}$.

Fig. 9. Immuno-peroxidase staining for endopeptidase-2 in kidney and intestine of a Balb-c mouse

Cryostat sections (8 μ m thick) were cut from fresh mouse tissues and stained with the polyclonal antibody RRt151 to rat endopeptidase-2. (a) Mouse kidney showing positively stained tubules of the juxtamedullary nephrons and some other nephrons radiating outwards into the outer cortex; C, cortex; M, medulla (no counterstaining). (b)–(f) (counterstained sections) demonstrate the staining of the luminal surface of duodenum (b), jejunum (c), ileum (d), colon (e) and rectum (f). Bar, 250 μ m.

some of the duct cells in the salivary gland and at the luminal surface of the acini of the thyroid gland. Fresh and perfused-fixed samples of rat brain and spinal cord were prepared and blocks from many regions were sectioned and examined, but no endopeptidase-2 staining was observed.

Immunoblotting of membrane fractions of rat brain and other tissues

Our finding that the kidney and intestine were the only organs in which endopeptidase-2 was unequivocally

detected by immunohistochemistry led us to examine a number of tissues by Western immunoblotting with the same antibody, RRt151. Membrane pellets were prepared from post-mitochondrial supernatant fractions by centrifugation at 31000 g for 2 h and solubilized by heating in SDS dissociation buffer containing 2-mercaptoethanol. Samples (100 μ g of protein) of spleen, brain, spinal cord, lymph nodes, salivary glands, thyroid and kidney were subjected to SDS/polyacrylamide-gel electrophoresis, blotted and immunostained. In a track containing kidney an 80000- M_r band stained positively.

No polypeptide bands with M_r greater than 30 000 were stained in the other tracks, and these low- M_r bands also appeared in a control blot immunostained with pre-immune serum (results not shown).

Immuno-peroxidase staining of mouse kidney and intestine

Fresh organs were obtained from a Balb/c mouse, and cryostat sections were prepared from kidney and intestine and immunostained with the antibody RRt151 generated to rat endopeptidase-2. The micrographs are shown in Figs. 9(a)–9(f). The pattern of staining of the kidney (Fig. 9a) mirrors that seen in the rat: tubules of juxtamedullary nephrons were positive, together with those of other nephrons radiating some distance into the otherwise unstained outer cortex. The mucosal surface along the whole length of the intestine was also positively stained, including the duodenum (Fig. 9b), jejunum (Fig. 9c) and ileum (Fig. 9d) and the surfaces of the colon (Fig. 9e) and rectum (Fig. 9f).

DISCUSSION

Structural aspects

The use of papain to release endopeptidase-2 from the membrane had the important advantage of generating a hydrophilic form that could be successfully purified in reasonable yield by a series of chromatographic steps. However, the doublet (M_r 80 000 and 74 000) revealed by SDS/polyacrylamide-gel electrophoresis in reducing conditions suggested that limited proteolysis may have occurred (Kenny & Ingram, 1987), and one objective in seeking to isolate the detergent-solubilized form was to clarify this point. The immunopurified detergent-solubilized form revealed a single major band of M_r 80 000 in reducing conditions, but by Western blotting the antibody revealed a second faint band of M_r 90 000. The antibody also stained the doublet bands of the previously purified papain-released form as well as another preparation immunopurified after release by papain treatment. These results argue strongly that the doublet is an artifact of papain treatment of endopeptidase-2 and does not indicate a heterodimeric protein. The native protein in non-reducing conditions migrated in SDS/polyacrylamide-gel electrophoresis as a polypeptide of M_r 220 000 consistent with an oligomeric structure requiring disulphide bridges. Gel filtration gave an M_r value of 436 000 for the native enzyme (Kenny & Ingram, 1987). If we assume that the apparent M_r is underestimated in non-reducing conditions, it seems likely that the native enzyme is tetrameric. Although we cannot exclude the existence of dissimilar subunits of M_r 80 000, it is probably that the structure can be represented as α_4 rather than $\alpha_2\beta_2$.

It is clear that endopeptidase-2 is a glycoprotein, since treatment with endoglycosidase F converted the subunit into a series of lower- M_r forms, the smallest of which had M_r 54 000, suggesting about 30 % *N*-linked carbohydrate. It was noteworthy that the glycoprotein was also sensitive to endoglycosidase H, with a diminution in size of about M_r 3000, indicating that 10–15 % of the *N*-linked carbohydrate chains are of the high-mannose type. Endopeptidase-24.11 and aminopeptidase W from pig kidney show no such residual sensitivity to endoglycosidase H. However, pig aminopeptidase N is similar to endopeptidase-2 in this respect (K. Barnes, J. Ingram &

A. J. Kenny, unpublished work). The nature of the membrane anchor of endopeptidase-2 is not yet established, but a glycolipid anchor seems to be excluded since treatment of membranes with PI-PLC in conditions where alkaline phosphatase was completely released failed to solubilize any endopeptidase-2.

Distribution of endopeptidase-2 in rat organs and tissues

In the kidney, endopeptidase-2 was confined to the luminal membrane of proximal convoluted tubules, including the convoluted and straight portions, being undetectable in other components of the nephron, including glomeruli. However, a very characteristic staining pattern was observed in that the proximal tubules of juxtamedullary nephrons were selected, whereas most of those in cortex were unstained. The outer cortical staining was restricted to a pattern of positive tubules seen as radiating spokes interspersed with negative areas. This pattern is identical with that reported for the immuno-peroxidase staining of meprin in mouse kidneys (Craig *et al.*, 1987). Endopeptidase-2-positive staining was detected along the whole length of the intestine from duodenum to ileum in the small intestine and from colon to rectum in the lower gut. The staining at each level was confined to the luminal surface. This pattern is unusual among other microvillar peptidases, such as endopeptidase-24.11 and aminopeptidase W, which are found only in the small intestine, being undetectable in the colon. Since there are few markers for the apical membrane of the colonic epithelium, this observation may be of wider importance. In the absence of either specific substrates or specific inhibitors for endopeptidase-2, activity determinations in intestinal brush-border membranes are not conclusive, but it is of interest that rat intestinal brush borders have been reported to contain several metallo-endopeptidase activities, including one hydrolysing azo-casein (Guan *et al.*, 1988).

The immunohistochemical survey did not reveal other major locations for the enzyme, including brain and spinal cord, an important point in distinguishing endopeptidase-2 from 'substance P-degrading enzyme' (Stephenson & Kenny, 1989).

Immunochemical studies of endopeptidase-2 in mouse tissues

The polyclonal antibody raised to the rat enzyme recognized a similar peptidase in mouse kidney and intestine. In summary, when applied to studies with mouse, the RRt151 antibody had the following properties. (a) It precipitated a protein exhibiting 90 000- M_r and 100 000- M_r bands on SDS/polyacrylamide-gel electrophoresis in reducing conditions. The identity of the latter band is unclear, but may relate to 'meprin b', which has a slightly larger subunit than 'meprin a' (Butler & Bond, 1988). (b) It immunoprecipitated 90 % of the activity hydrolysing ^{125}I -labelled insulin B-chain and azo-casein from detergent-solubilized mouse renal microvillar membranes. (c) When covalently attached to CNBr-activated Sepharose, it bound endopeptidase-2-like activity to the immunoaffinity support, some of which could be eluted under alkaline conditions. All three endopeptidase-2 substrates, ^{125}I -labelled insulin B-chain, Bz-Tyr-pAB and azo-casein, were hydrolysed by the bound and the eluted activities. The eluted material analysed by SDS/polyacrylamide-gel electrophoresis was single-banded (M_r 90 000) in reducing conditions and

Table 3. Comparison of properties of rat endopeptidase-2 with mouse meprin

Key to references: (1), present paper; (2), Kenny & Ingram (1987); (3), Stephenson & Kenny (1988); (4), Butler *et al.* (1987); (5), Beynon *et al.* (1981). Abbreviation: PAGE, polyacrylamide-gel electrophoresis.

Property	Rat endopeptidase-2	(Reference)	Mouse meprin	(Reference)
M_r				
SDS/PAGE (reducing conditions)	80000 (detergent-solubilized form)	(1)	85000	(4)
	80000 + 74000 (papain-released form)	(2)		
SDS/PAGE (non-reducing conditions)	220000	(1)	320000	(4)
Gel filtration	436000	(2)	210000	(5)
Endoglycosidase F treatment, SDS/PAGE (reducing conditions)	70000–54000	(1)	70000	(4)
Endoglycosidase H treatment, SDS/PAGE (reducing conditions)	77000	(1)	–	
Activity				
Bond specificity	Prefers hydrophobic residue in P_1 or P_1'	(3)	Prefers hydrophobic residue in P_1 or P_1' (but also hydrolyses Gly–Glu and Gly–Ser bonds)	(4)
Antigenic				
Antiserum to endopeptidase-2	Immunoblots, immunopurifies and precipitates antigen; immunostains kidney tubules and intestine	(1)	Immunoblots, immunopurifies and precipitates antigen; immunostains kidney tubules and intestine	(1)

revealed evidence of disulphide-linked subunits when run in non-reducing conditions. (d) In sections of mouse kidney it immuno-peroxidase-stained the luminal membranes of a distinct set of proximal tubules, similar in distribution to that of the rat kidney and to that reported for meprin in mouse kidney (Craig *et al.*, 1987). We conclude that the antigen in mouse kidney recognized by the RRt151 antibody is meprin.

Relationship of rat endopeptidase-2 to mouse meprin

The present paper has added to the list of similar properties of these two rodent enzymes. These include the structural aspects that both have subunits of M_r about 80000–90000 in disulphide-linked oligomeric structure and that both are glycoproteins sensitive to endoglycosidase F. The similarities in bond specificity with a variety of peptides between meprin (Butler *et al.*, 1987) and endopeptidase-2 have already been noted (Stephenson & Kenny, 1988). The evidence for the immunological identity outlined above is a conclusive argument that meprin and endopeptidase-2 are closely related proteins. However, there are some differences in the activities towards different types of substrates. Meprin has a higher activity towards azo-casein and very weak activity towards Bz-Tyr-pAB compared with endopeptidase-2. Indeed, meprin has been reported to have little or no activity towards Bz-Tyr-4-nitroanilide, 2-Phe-Arg-NHMec and Bz-Phe-Val-Arg-NHMec (Butler & Bond, 1988). Another apparent difference concerns our clear immunohistochemical demonstration of the antiserum on the luminal surface throughout the length of the Balb/c-mouse and Wistar-rat intestine. The only previously published comment on this point in the mouse is that meprin could not '...be detected in other tissues, including the brush-border-rich intestinal mucosa by enzymological or immunological means' (Beynon &

Bond, 1985). We believe this conclusion may be in error and deserves re-investigation.

The name 'meprin' (metallo-endopeptidase from renal tissue; Shannon *et al.*, 1981) is at the same time too restrictive in emphasizing the renal location and too general in embracing all metallo-endopeptidases in kidney of whatever subcellular localization. The genetics of meprin have been studied in some detail (see, e.g., Bond & Beynon, 1986) and meprin-deficient mouse strains have been identified, one interesting aspect of which is that the latent activity in one such strain (C3H/HcJ) can be activated by treatment of the microvillar membranes with trypsin (Butler & Bond, 1988). It is not clear whether rats exhibit a comparable genetic variation in endopeptidase-2, though one study showed that among seven strains of rats there was a 2-fold variation in azo-casein hydrolysis by rat kidney homogenates (Stolc, 1986). It should be noted that this activity was not shown to be associated with a membrane fraction and cannot yet be equated with endopeptidase-2 or meprin.

The other enzyme that may be grouped with endopeptidase-2 and meprin is 'PABA peptide hydrolase' (Sterchi *et al.*, 1982, 1983, 1988a,b) present in the human intestinal brush border. The topological, structural and catalytic similarities of all three membrane peptidases have been recently discussed (Stephenson & Kenny, 1989). It is our view that all three should be classified under the same EC number 3.4.24.x to indicate their close relationship and to encourage the acceptance of a common nomenclature. The latest Supplement to the Enzyme Nomenclature Recommendations [(1989) Eur. J. Biochem. 179, 489–533] has confused matters by including endopeptidase-2 under EC 3.4.24.11.

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