Proteins of the kidney microvillar membrane

The 130 kDa protein in pig kidney, recognized by monoclonal antibody GK5C1, is an ectoenzyme with aminopeptidase activity

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The hybridoma GK5C1, secreting a monoclonal IgG₁ antibody, was generated after immunizing a mouse with pig kidney microvillar membranes. An immunoradiometric assay showed that only kidney and intestine contained detectable amounts of the antigen recognized by the antibody, the highest concentration being observed in the ileum. Immunocytochemistry confirmed this observation and revealed that the antigen was associated with renal and intestinal brush borders. By 'Western' blotting, the antigen in kidney microvilli was shown to be a 130 kDa polypeptide. Papain treatment of the membrane before blotting converted the antigen to a 125 kDa polypeptide, no longer associated with membrane. Immunoaffinity chromatography of detergent-solubilized kidney membranes yielded a pure 130 kDa protein. When one purification was monitored by the immunoradiometric assay, the yield was 3.5% and the purification factor was 1000-fold. The antigen constituted about 0.8% of the microvillar membrane protein. The protein could be reconstituted into liposomes, where electron microscopy revealed an asymmetric orientation, similar to that of ectoenzymes in this membrane. The stalk length was about 3 nm. In electron micrographs the purified protein appeared to be dimeric. A search for enzymic activity was rewarded when L-leucyl-L-tryptophan was observed to be hydrolysed. Failure to hydrolyse *N*-blocked peptides and the ability to release the *N*-terminal residue from extended peptides, including Leu-Trp-Leu and Leu-Trp-Met-Arg, showed that the activity was that of an aminopeptidase. The enzyme was maximally active at pH 7.5 and irreversibly inactivated outside the range pH 6–10. This activity could not be attributed to trace contamination with aminopeptidase N. The best substrates so far identified for the 130 kDa protein were those with tryptophan in the P₁' position. This protein is a new microvillar enzyme and it is proposed that it be called aminopeptidase W.

Before the advent of monoclonal antibodies, new enzymes entered the scientific consciousness as activities first perceived in crude preparations of tissues or cells and only later, after laborious fractionation, would the active protein be isolated and the enzyme characterized. Monoclonal antibodies provide an easy means to the isolation of pure antigens and allow their structural characterization to be achieved in advance of any infor-

mation on their activities or functional roles. This approach is a reversal of the way in which information on an enzyme is normally accumulated, but its apparent attractions must be balanced against the frustrations of a possibly unsuccessful search for an activity.

One of the hybridoma lines cloned from a cell fusion using splenocytes from a mouse immunized with a pig kidney microvillar membrane preparation secreted a monoclonal antibody GK5C1. The antigen recognized by this antibody was located in the brush borders of kidney and intestine and by immunoprecipitation was shown to be a 130 kDa subunit protein (Gee & Kenny, 1985). The present

Abbreviations used: i.r.m.a., immunoradiometric assay; Z-, benzyloxycarbonyl; -NMec, 7-amino-4-methylcoumarylamide derivative of an amino acid or peptide; SDS, sodium dodecyl sulphate.

paper describes in detail the isolation of the antigen recognized by GK5C1, assesses its distribution and abundance by i.r.m.a., shows that it reconstitutes in liposomes in a manner comparable with other microvillar ectoenzymes and identifies its activity as an aminopeptidase distinct from those previously described as brush border enzymes. We propose that it be named aminopeptidase W.

Experimental

Many of the procedures used in generating the monoclonal antibody and characterizing it and its antigen were identical with those described for another monoclonal antibody (Gee *et al.*, 1983). These include immunization, cell fusion, cell culture, cloning, metabolic labelling of the antibody, screening of antibodies, production of ascites tumours, immunocytochemistry, preparation and operation of the immunoadsorbent column, preparation of microvillar fraction and polyacrylamide gel electrophoresis in the presence of SDS. 'Western' blotting was modified by substituting 50mM-Tris/HCl/500mM-NaCl, pH7.5, for the pH10.2 buffer.

Production of monoclonal antibody GK5C1

A Balb/c mouse received four intraperitoneal injections of pig kidney microvilli in doses of 80–170 µg of protein on days –110, –85, –37 and –4 with fusion being performed on day 0. GK5C1 was twice cloned on soft agar. The antibody was produced in ascites fluid and purified as follows. The fluid was dialysed against 5mM-Tris/HCl, pH7.8, and then chromatographed on DEAE-cellulose (Whatman DE-52) by elution with a gradient (0–200mM) of NaCl. GK5C1 was desorbed at 60mM-NaCl.

Aminopeptidase N (EC 3.4.11.2)

This was a gift from Miss S. L. Stephenson. It had been solubilized by papain treatment (George & Kenny, 1973) of pig microvillar membranes (Booth & Kenny, 1974) and purified by chromatography on columns of DEAE-cellulose, Sepharose 6B, and DEAE-cellulose. The enzyme was assayed with Ala-NMec as described (Fulcher & Kenny, 1983). The specific activity was 19.4 µmol/min per mg. Some minor impurities were visible on SDS/polyacrylamide-gel electrophoresis and two other peptidases were detectable by assay. Aminopeptidase A (EC 3.4.11.7) contributed 0.2% and dipeptidyl peptidase IV (EC 3.4.14.5) 3% of the protein.

Leucine aminopeptidase (EC 3.4.11.1)

This was type V from pig kidney, approx. 60units/mg (Sigma Chemical Co.).

Hydrolysis of peptides

Hydrolysis was determined by h.p.l.c. analysis as previously described (Matsas *et al.*, 1983). The hydrolysis of Gly-Pro-NMec was determined as described (Fulcher & Kenny, 1983).

I.r.m.a.

This was performed as described (Gee *et al.*, 1985). The dilution of the GK5C1 ascites fluid used was 1:20000, corresponding to about 20ng of monoclonal antibody in each absorption assay.

Reconstitution of antigen in liposomes

This was performed as described by Kenny *et al.* (1983) using octylglucoside as detergent and pig kidney microvillar lipids. Morphometric studies on the stalk length were made on 58 profiles viewed by electron microscopy and the maximum space between the edge of the liposome and the particle was used to compute the mean length (Kenny *et al.*, 1983).

Treatment of microvillar fraction with Triton X-114

Microvilli (25mg) in 10ml of 10mM-phosphate/150mM-NaCl, pH7.4, were treated for 1h at 4°C with 5ml of 5% (w/v) Triton X-114 (Sigma Chemical Co.) to give a detergent: protein ratio of 10:1. Detergent-insoluble material was pelleted at 31500g for 2h at 4°C before allowing phase separation of the supernatant at 30°C. The pretreatment of the detergent and the phase separation were as described (Bordier, 1981).

Purification of GK5C1 antigen by immunoaffinity chromatography

The affinity column was prepared by coupling partially purified GK5C1 (41mg of protein, estimated to be 14.5mg of antibody) to 3.5ml of CNBr-Sepharose 4B. The coupling efficiency was 96%. Kidney cortex (200g) was homogenized in 0.34M-sucrose (sometimes including 50mM-Tris/HCl, pH8.5) and centrifuged at 8000g for 15min. The supernatant fraction was then centrifuged at 17000g for 2h and the pellet, resuspended in 200ml of 5mM-Tris/HCl, pH7.8, was treated with 100ml of 20% (w/v) Triton X-100. After 1h at 4°C, the extract was centrifuged at 31000g for 1h. The supernatant fraction was concentrated by loading on to 40ml of DEAE-cellulose and, after washing, eluted with 50mM-Tris/HCl/500mM-NaCl, pH7.8, to give a volume of about 40ml. The solubilized proteins were loaded on the immunoaffinity column and, after washing with 25 column volumes, the antigen was eluted by a buffer at pH10.6. Subsequently, elution with 2mM-Tris/HCl, pH8.0, was used for the reasons described below. The eluted antigen was concentrated on a small column (300 µl) of DEAE-cellulose

after preliminary dialysis against 5mM-Tris/HCl, pH7.8. Triton X-100 (0.1%) was present in all buffers.

Hydrophobic chromatography

Octyl- and phenyl-Sepharose 4B (Pharmacia) columns were packed (volume 200 μ l) and washed with 50mM-Tris/HCl/500mM-NaCl, pH7.4. Samples of 130kDa protein (2.5 μ g in 40 μ l) in the same buffer containing 0.1% Triton X-100 were loaded. After washing with 10 column vol. of the buffer (without detergent) the buffer was changed by the addition of Triton X-100 to 2.5%. Binding and elution were monitored by assaying the fractions with Leu-Trp as substrate.

Other methods

Ouchterlony immunodiffusion was performed as described (Relton *et al.*, 1983); the antisera to mouse Igs were obtained from Miles Scientific. HIO₄/Schiff staining of gels for carbohydrate was as described by Fairbanks *et al.* (1971). Endopeptidase-24.11 was purified by immunoaffinity chromatography (Gee *et al.*, 1983). The Fast Protein Liquid Chromatography system including a Superose-6 column was operated as recommended by the manufacturers (Pharmacia). The buffer was 20mM-Tris/HCl/100mM-NaCl, pH7.9. The standards were the Pharmacia high-*M_r* calibration standards (thyroglobulin, ferritin, catalase, lactate dehydrogenase and bovine serum albumin).

Results

Purification of monoclonal antibody GK5C1 from ascites fluid

The single ion-exchange step did not yield a pure preparation of the antibody. The main contaminants were transferrin (*M_r* 76000) and an undefined protein, *M_r* 70000. The antibody was assessed to be about 35% of the total protein by staining after electrophoresis in SDS/polyacrylamide gels (Fig. 1). The tracks show the Ig chains of *M_r* 50000 and 27000 and the contaminating transferrin. The yield of monoclonal antibody in this partially purified form was about 2mg/ml of ascites fluid. Ouchterlony immunodiffusion of concentrated (10 $\times$) cell culture medium against antisera to different IgG classes showed GK5C1 be an IgG₁.

Distribution of the GK5C1 antigen in pig tissues

I.r.m.a. The solid-phase assay used for screening monoclonal antibodies was adapted for use as an i.r.m.a. in order to survey the distribution of GK5C1 antigen in pig tissues. Homogenates of tissues were tested for their ability to absorb the monoclonal antibody, any unabsorbed antibody being detected in the solid phase assay by an ¹²⁵I-

Fig. 1. Purification of monoclonal antibody GK5C1 by DEAE-cellulose chromatography

See the Experimental section for details. The region of the SDS/polyacrylamide gel shown (stained with Coomassie Blue) contains fractions (25 μ l) eluted from the column in the 60mM-NaCl region of the gradient. Tracks 4 and 5 contained most of the antibody: heavy chain (H) *M_r* 50000, light chain (L) *M_r* 27000. Transferrin (T) was eluted throughout the gradient, but reached a maximum in the IgG-rich fractions.

labelled second antibody. The results are shown in Table 1. Only two tissues, kidney cortex and small intestine, contained detectable antigen. The antigen in the mucosa from ileum was distinctly higher than kidney cortex; that in jejunum was lower, while it was barely detectable in duodenum. All the other tissues examined were below the limit of detection. They included stomach, lymphoid tissue, various glands, liver and lung.

Immunocytochemical localization in kidney and intestine. Indirect immunofluorescence was employed to locate the GK5C1 antigen in the two tissues, kidney and intestine, which the i.r.m.a. had shown to contain easily detectable amounts. Fig. 2(a) reveals that the antigen was confined to

Fig. 2. Localization of GK5C1 antigen in sections of kidney and intestine (a) Kidney cortex, fixed by perfusion of a 3-week-old piglet with 4% (w/v) paraformaldehyde; G, glomerulus. (b) Ileum, section through villi; LP, lamina propria. (c) Ileum, section through crypts.

the luminal surface of the proximal tubule, i.e. to the region of the brush border. No antigen was detected in glomeruli nor in any of the medullary structures. One feature was the variation in intensity of staining of the tubules, suggesting a nonuniform distribution along the proximal tubule. In Fig. 2(b), it can be seen that the fluorescence is present at the luminal surface of the villi, again associated with the brush border. In addition (Fig. 2c), the crypts also show fluorescence at the luminal pole of the cells, indicating that the antigen is expressed at an early stage in the maturation of the epithelial cells.

Purification of the GK5C1 antigen

The i.r.m.a. provided a means for assessing the yield and purification of the antigen from pig kidney cortex, and one such purification was

monitored in this way (Table 2). The antigen units are arbitrary, being related to that amount of antigen (relative to kidney homogenate) in a sample required to absorb 50% of the monoclonal antibody in the i.r.m.a. The overall yield of antigen, only about 1 mg of protein from 200 g of kidney cortex, was 3.5%. The enrichment of 1000-fold makes it a minor, but not an insignificant, antigen in the membrane. Indeed, in another experiment, the antigen content of homogenate and microvillar fraction were directly compared. Fig. 3 shows the absorption curves of these fractions in comparisons with that for the purified antigen. A direct comparison with the latter is valid in this i.r.m.a. because detergent solubilization of the antigen had little effect on the assay. The i.r.m.a. value for microvilli was only 17% lower after detergent treatment. This is in contrast

Table 1. Distribution of the GK5C1 antigen in pig tissues
Homogenate were subjected to an i.r.m.a. depending on the preabsorption of the antibody by tissue antigen (Gee *et al.*, 1985). Results are expressed as arbitrary units/mg of protein relative to kidney cortex (= 100). Abbreviation used: n.d., not detectable (in 10%, w/v, homogenate).

Tissue	Relative activity
Kidney cortex	100
Duodenum mucosal scraping	4
Jejunum mucosal scraping	72
Ileum mucosal scraping	162
Stomach	n.d.
Spleen	n.d.
Lymph node	n.d.
Salivary gland	n.d.
Pancreas	n.d.
Thyroid	n.d.
Adrenal (whole gland)	n.d.
Liver	n.d.
Lung	n.d.
Heart (ventricle)	n.d.
Aorta	n.d.

Fig. 3. Absorption curves (i.r.m.a.) of GK5C1 by kidney homogenate, microvillar membranes and the purified, detergent-solubilized antigen

See the Experimental section for details. ●, Homogenate (undiluted = 20mg of protein/ml); ○, microvillar membranes (undiluted = 0.6mg of protein/ml); ▲, purified, detergent-solubilized antigen (undiluted = 0.6mg of protein/ml). The dilutions of all samples were made in 10mM-phosphate/150mM-NaCl/0.1% bovine serum albumin, pH 7.4, without the addition of detergent.

Table 2. Purification of the GK5C1 antigen, monitored by i.r.m.a.
See the Experimental section for details. The units are arbitrary, relating to the concentration of each sample giving 50% absorption of the antibody.

Fraction	Protein (mg)	10 ⁻³ × Activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Homogenate	27500	1905	69.3	(100)	1
8000g supernatant	20000	1390	69.5	73	1
17000g fraction	5625	635	113	33	1.6
Triton-solubilized pellet after DEAE-cellulose chromatography	1600	241	151	13	2.2
Unbound by immunoaffinity column	1462	<12	<0.3	—	—
Eluted from immunoaffinity column	0.98	67	68050	3.5	982

to the i.r.m.a. for endopeptidase-24.11, where detergent reduced the sensitivity of the assay about 6-fold (Gee *et al.*, 1985). The enrichment value for the 130 kDa antigen in the microvillar fraction was 8.5 (that for a marker enzyme, aminopeptidase N, was 8.4) and the further enrichment of the purified protein was 121-fold. This implies that the antigen constitutes about 0.8% of the microvillar membrane protein.

Identification of GK5C1 antigen as a 130 kDa protein

When pig kidney microvillar proteins were transferred on to nitrocellulose, after SDS/poly-

Fig. 4. Immunoblotting of GK5C1 on kidney microvillar proteins

See the Experimental section for details. (a) Protein stained by Coomassie Blue; (b) GK5C1 bound to antigen, stained by peroxidase reaction. Tracks: 1, microvillar membrane; 2, Triton X-114 detergent phase; 3, supernatant fraction after papain treatment of microvillar membrane.

acrylamide-gel electrophoresis, the monoclonal antibody bound to a polypeptide of M_r 130000 (Fig. 4). It corresponded in migration to the blurred band in the Coomassie Blue stained track that is known to include dipeptidyl peptidase IV, the subunits of which are also of M_r 130000 (Booth & Kenny, 1976) and carboxypeptidase P (Hedeager-Sorensen & Kenny, 1985). The detergent phase obtained after solubilization of membranes with Triton X-114 also revealed the same 130 kDa polypeptide. However, after the papain-solubilization, a slightly smaller protein (approx. 125 kDa) was detected in the supernatant, indicating that the proteinase had removed part of the polypeptide chain, presumed to include the hydrophobic anchor.

The identification of the antigen was confirmed by purifying it by immunoaffinity chromatography from a Triton X-100 extract of kidney membranes. Starting with 200 g of kidney cortex and eluting the immunoaffinity column with the pH 10.6 buffer, the yield was 1.5 mg of protein. The yield was slightly less (1.1 mg) when the hypo-osmotic buffer

Fig. 5. Immunoaffinity purification of the antigen recognized by GK5C1

See the Experimental section for details. Tracks: 1, standards, $M_r \times 10^{-3}$ shown; 2, eluted protein from kidney (20 μ g); 3, kidney microvillar membrane (100 μ g).

Fig. 6. *Negatively stained electron microscopy of the 130 kDa protein and endopeptidase-24.11 reconstituted in liposomes* See the Experimental section for details. (a), (b), (c), 130 kDa protein. Particles are visible projecting from the edge of the liposomes as well as on their surface. Some of the former and many of the latter suggest a dimeric structure, e.g. those indicated by arrows in (c). (d), (e), Endopeptidase-24.11 reconstituted under the same conditions, shown for comparison. The subunit M_r of this protein is 89000 and is a dimer when purified (Fulcher & Kenny, 1983). All micrographs are at same magnification; the bar represents 100 nm.

at pH 7.5 was used. SDS/polyacrylamide-gel electrophoresis revealed a single band of M_r 130 000 (Fig. 5).

Reconstitution of the 130 kDa protein in liposomes

One of the properties of integral membrane proteins is their ability to reconstitute in artificial lipid bilayers. Other microvillar enzymes that have been studied in this way are aminopeptidase N (Hussain *et al.*, 1981) and endopeptidase-24.11 (Kenny *et al.*, 1983). The 130 kDa protein in octylglucoside was also reconstituted into liposomes prepared from pig kidney microvillar lipids. Electron microscopy of the vesicles (Fig. 6a, b, c) showed the presence of particles protruding from the edge of liposomes, and separated from it by a distinct gap. This space indicated a stalk (or junctional peptide) length of 2.95 ± 0.61 nm (S.E.M., $n = 14$). This appearance is very similar to that observed when another microvillar protein, endopeptidase-24.11, reconstituted under the same conditions (Fig. 6d, e). This protein has a median stalk length of 2 nm (Kenny *et al.*, 1983). The surface of the vesicles shows some of the 130 kDa protein *en face*: these appeared, as did some of those seen at the edges, to be dimers, with dimensions 5.5 ± 0.26 nm $\times$ 3.8 ± 0.17 nm (S.E.M., $n = 13$).

Other properties of the 130 kDa protein

The 130 kDa band in polyacrylamide gels stained positively for carbohydrate with the HIO_4 /Schiff reagent. The protein revealed amphipathic properties when subjected to hydrophobic chromatography. Under the loading conditions (500 mM-NaCl, 0.1% Triton X-100) 99.7% of the enzyme activity (see below) was bound to octyl- and phenyl-Sepharoses. Increasing the concentration of Triton X-100 to 2.5% eluted 60% of the activity from each column.

The oligomeric state of the protein was investigated by chromatographing the protein on a column of Ultrogel AcA-22. In the run illustrated in Fig. 8(a), a single symmetrical peak emerged corresponding to a protein of M_r 520 000. Occasionally two partially resolved peaks were observed from samples that had been stored at -20°C which, in the case of that in Fig. 8(a), had given a single peak previously. The two peaks corresponded to M_r values of 520 000 and 452 000. An independent estimate using a Superose-6 column attached to a Pharmacia Fast Protein Liquid Chromatography system, monitored by A_{280} , also revealed two peaks, assessed as M_r 514 000 and M_r 408 000. The possibility that the smaller component in this separation represented a hydrophilic form of the protein was not supported by analysis of the material from the pooled peaks by hydropho-

bic chromatography. As before, phenyl- and octyl-Sepharoses bound 99.5% of the peptidase activity and 61% was recovered by elution with detergent.

Peptidase activity of the 130 kDa protein

Hydrolysis of L-leucyl-L-tryptophan (Leu-Trp). In

Fig. 7. Effect of pH on hydrolysis of Leu-Trp by 130 kDa protein

(a) Stability of peptidase. Samples of the pH 10.6-eluted enzyme (O) and pH 7.5-eluted enzyme (●) were diluted with 9 vol. of various buffers (0.2 M) in the range pH 4 to pH 11 (pH 4, 5, sodium acetate/acetic acid; pH 6, Mes/NaOH; pH 7–9, Tris/HCl and pH 10, 11, $\text{NaHCO}_3/\text{NaOH}$) and incubated at 4°C for 1 h. Each sample was then brought back to pH 7.0 by dilution with at least 9 vol. of 0.2 M-Tris/HCl, pH 7.0, before assay. (b) pH-activity curve. Samples of the pH 10.6-eluted enzyme (O) and pH 7.5-eluted enzyme (●) were assayed in the buffers listed above.

the course of a long search for possible substrates for the putative enzyme, Leu-Trp was the first substance found to be attacked by the 130kDa protein. Other assay substrates tested with negative results included the following compounds: α -Glu-NMec, γ -Glu-NMec, Gly-D-Phe, Gly-Pro-Hyp, Z-Pro-Met, Hip-His-Leu, 125 I-insulin B chain, luliberin (LH-RH), substance P, 5'-AMP, acetylcholine, maltose and trehalose. Weak hydrolysis was observed with a substrate for aminopeptidase N (Ala-NMec, 7nmol/min per mg of protein) and for dipeptidyl peptidase IV (Gly-Pro-NMec, 5nmol/min per mg of protein). However, these activities were too low to encourage the view that either represented the primary activity of the protein. In the absence of a superior peptide substrate, 0.5mM-Leu-Trp has been used in the standard assay. Tryptophan release was monitored at 215nm by using h.p.l.c. The specific activity of the protein eluted at pH10.6 from the immunoaffinity column was 0.84 μ mol/min per mg of protein. We later realized that the enzyme activity was unstable outside the pH range 6 to 10, as shown in Fig. 7(a). It proved possible to elute the 130kDa protein from the column by a neutral buffer of low ionic strength (2mM-Tris/HCl, pH7.5). Two such preparations possessed much higher activities: 18.4 and 17.9 μ mol/min per mg. Both the pH10.6- and pH7.5-eluted forms were maximally active at neutral pH, the former at pH 7, the latter at pH8 (Fig. 7b).

The rather weak activity first observed made it necessary to consider the possibility that the hydrolysis of Leu-Trp might be the result of traces of other peptidases copurifying with the 130kDa protein. Aminopeptidase N (EC 3.4.11.2) has a broad specificity towards many peptides and is also a major component of the microvillar mem-

brane. Any significant contribution of this enzyme was excluded in experiments in which the activity of the 130kDa protein was compared with a sample of aminopeptidase N purified from pig kidneys (Table 3). Both preparations hydrolysed Leu-Trp, but the 130kDa protein gave the higher rate. The tripeptide, Leu-Trp-Leu, was hydrolysed (at the Leu-Trp bond) more slowly by the 130kDa protein but much faster by aminopeptidase N. The same trend was observed with the pentapeptide, Tyr-Gly-Gly-Phe-Leu ([Leu]enkephalin). Only aminopeptidase N hydrolysed Ala-NMec efficiently. Pro-Trp and Gly-Trp were very poor substrates for aminopeptidase N, but fairly good for the 130kDa protein.

Leucine aminopeptidase is an abundant cytosolic enzyme in kidney. Although it hydrolysed Leu-Trp and Leu-Trp-Leu, it did not attack Pro-Trp and Gly-Trp (results not shown).

Evidence that the 130kDa protein is an aminopeptidase. Since the two N-blocked peptides (Z-Leu-Trp and phosphoramidon) were not hydrolysed by the 130kDa protein and Leu-Trp-Leu was hydrolysed only at the Leu-Trp bond (Table 3), the results overall showed that the 130kDa protein was an aminopeptidase. The series Leu-Trp, Leu-Trp-Leu and Leu-Trp-Met-Arg indicate a preference for small peptides, and also show that the enzyme is not a dipeptidase. [Leu]enkephalin was hydrolysed at the Tyr¹-Gly² bond, but at a rate less than that observed for Leu-Trp-Met-Arg. So far, the best substrates have been peptides with Trp in the P₁' position. Trp-Leu was a poor substrate in comparison with Leu-Trp.

Aminopeptidase activity could not be dissociated from the 130kDa protein by chromatography. Although the purified 130kDa protein possessed consistently high aminopeptidase activity, it was

Table 3. Hydrolysis of peptides by 130kDa protein and aminopeptidase N

Hydrolysis was monitored by h.p.l.c. except for that of Ala-NMec, which was assayed fluorometrically. Enzyme preparations were diluted appropriately for a 20 min incubation. A sample of the pH7.5-eluted 130kDa protein was used. Abbreviation used: n.d., not determined. Phosphoramidon is N-(α -L-rhamnopyranosyloxyhydroxyphosphinyl)Leu-Trp.

Peptide	Concn. (mM)	Specific activity (μ mol/min per mg of protein) (relative activities shown in parentheses)	
		130kDa protein	Aminopeptidase N
Leu-Trp	0.5	18.0 (100)	2.6 (100)
Leu-Trp-Leu	0.5	7.4 (41)	23.9 (919)
Leu-Trp-Met-Arg	0.5	2.1 (12)	17.5 (673)
[Leu]enkephalin	0.5	0.9 (5)	32.2 (1238)
Ala-NMec	0.2	0.007 (0.004)	19.4 (746)
Pro-Trp	0.5	1.8 (10)	0.02 (0.8)
Gly-Trp	0.5	7.7 (43)	0.3 (11)
Trp-Leu	0.5	0.4 (2)	n.d.
Z-Leu-Trp	0.5	0 (0)	n.d.
Phosphoramidon	0.5	0 (0)	n.d.

Fig. 8. *Chromatography of the immunoaffinity-purified aminopeptidase activity on Ultrogel AcA-22 and DEAE-cellulose* (a) A sample of the 130 kDa protein (250 μ g in 200 μ l of 50 mM-Tris/HCl/500 mM-NaCl/0.1% Triton X-100, pH 7.5) was loaded on a column of Ultrogel AcA-22 (55 cm $\times$ 1 cm) and developed at 3 ml/h with the same buffer. Fractions (0.36 ml) were collected, a portion being assayed with Leu-Trp as substrate and another portion (150 μ l) was analysed by SDS/polyacrylamide-gel electrophoresis. The upper region only of the gel is shown. Alternate fractions were analysed, numbered as indicated on the elution curve. Track M contained β -galactosidase as a marker, M_r 130 000. ●, Aminopeptidase activity. (b) Samples (150 μ l) of 12 of the peak fractions from the Ultrogel AcA-22 column were pooled, dialysed against 5 mM-Tris/HCl/0.1% Triton X-100, pH 7.5, and applied to a small column (0.3 ml) of DEAE-cellulose, equilibrated in the same buffer. It was eluted with a gradient of NaCl. Fractions (0.5 ml) were assayed and analysed as above. The gel tracks correspond to fraction numbers. ●, Aminopeptidase activity; —, [NaCl].

necessary to exclude the possibility that traces of a very highly active peptidase might have copurified with the protein. The purified 130 kDa protein was therefore subjected to two additional chromatographic steps. A sample of the 130 kDa protein (obtained by elution from the antibody column at pH 7.5) was loaded on to a column of Ultrogel AcA 22. The aminopeptidase activity emerged as a symmetrical peak ($V_e/V_t = 0.34$). Polyacrylamide-gel electrophoresis in SDS of the same fractions showed that the intensity of the stained 130 kDa band correlated with the peak of aminopeptidase activity (Fig. 8a). The peak fractions were then pooled, dialysed against a buffer of low ionic strength and loaded on a column of DEAE-cellulose. The aminopeptidase activity was eluted by a gradient of NaCl, emerging between 25 and 60 mM-NaCl. Polyacrylamide gel electrophoresis in SDS again showed that the enzyme activity remained associated with the eluted 130 kDa protein (Fig. 8b).

Discussion

The GK5C1 antigen appears to be restricted to the brush borders of kidney and intestine with its highest concentration in the ileum. The isolation of

the antigen from kidney and its structural characterization as a 130 kDa subunit protein presented few technical problems. The purified protein had properties typical of an amphipathic membrane protein: it partitioned in the detergent phase when extracted with Triton X-114, bound to octyl- and phenyl-Sepharose and could be reconstituted into liposomes. Electron microscopy of the reconstituted protein showed it to be an ectoprotein, particles protruding outwards from the liposome edge, with stalk length of 3 nm. Papain treatment released the antigen from the microvillar membrane, a result that implies sufficient space between membrane and the globular domain of the protein for the proteinase to attack susceptible bonds in the junctional peptide. By way of comparison, endopeptidase-24.11 is an ectoenzyme that resists release by papain: its stalk length assessed morphometrically was only 2 nm (Kenny *et al.*, 1983). The hydrophilic, papain-released, form of the antigen appeared to be about 5 kDa smaller than the detergent-solubilized form and this may give some indication of the size of the peptide comprising the stalk, hydrophobic anchor and any transmembrane domain. Although electron micrographs revealed profiles suggesting a

dimeric structure, the results of gel filtration experiments were difficult to interpret. Some preparations yielded a single symmetrical peak (M_r 520000), others contained an additional smaller component, M_r 450000, even though these too were single-banded by SDS/polyacrylamide-gel electrophoresis. The expected M_r for the Triton X-100-solubilized protein would be about 360000, if the protein is assumed to be dimeric and to bind only one detergent micelle. It is possible that two micelles were bound, giving a predicted M_r closer to 500000. However, in the absence of any direct determination of bound detergent, it is not possible to distinguish this model from one implying a more complex oligomeric state.

These structural attributes and localization are typical of many microvillar hydrolases (for review, see Kenny & Maroux, 1982; Semenza, 1985) and a search for a hydrolase activity was pertinent. Two other microvillar peptidases are known to be 130kDa subunit proteins in pig kidney microvilli dipeptidyl peptidase IV (Kenny *et al.*, 1976; Macnair & Kenny, 1979) and carboxypeptidase P (Hedeager-Sorensen & Kenny, 1985), but no significant hydrolysis was observed with the assay substrates (Gly-Pro-NMec and Z-Pro-Met) for these enzymes. Other substrates for phosphatases, esterases and glycosidases were also tested, with negative results. Endopeptidase activity would be expected to be revealed by using a limited number of oligopeptides such as insulin B chain, substance P and luliberin, but these, too, gave no hint of such activity. An exopeptidase exhibiting restricted specificity is much more difficult to expose since it may depend on a fortunate selection of peptides from the very large number now commercially available. Leu-Trp was the first peptide observed to be hydrolysed, but the relatively weak activity raised initial doubts about the significance of the observation, in particular the possibility that the activity was the result of a minor contamination by other highly active exopeptidases. Differences in the rates of hydrolysis of a series of peptides showed that pig aminopeptidase N could not account for the activity in our preparation. Similar arguments excluded the participation of leucine aminopeptidase. Renal dipeptidase (Campbell, 1970; Armstrong *et al.*, 1974; Kozak & Tate, 1982; Campbell *et al.*, 1984) is a strict dipeptidase, subunit M_r 50000, with a rather broad specificity that tolerates a D-amino acid as the second residue. However, the 130kDa protein did not attack Gly-D-Phe, nor was it restricted to a dipeptidase attack. In any case, the experiments that demonstrated that Leu-Trp hydrolysing activity remained associated with the 130kDa protein argue strongly against a contribution from this and other enzymes with different subunit M_r values.

The use of a pH 10.6 buffer to desorb enzymes from immunoaffinity columns has previously yielded fully active peptidases, e.g. endopeptidase-24.11 (Fulcher & Kenny, 1983; Gee *et al.*, 1983) and aminopeptidase A (Danielsen *et al.*, 1980). The 130kDa peptidase was not so tolerant. When a hypo-osmotic buffer (2mM-Tris/HCl, pH 7.5) was substituted, the eluted protein had a specific activity 20-fold higher than the earlier preparations.

The full potential of this enzyme has yet to be explored. In kidney microvilli it constitutes about 0.8% of the brush border membrane protein, and presumably over 1% of that of the ileum. The results reported here establish that it is an aminopeptidase capable of hydrolysing peptides containing two to five residues. It had no activity against N-blocked peptides, nor has any endopeptidase activity been demonstrated. Peptides with tryptophan as the second residue have, so far, been much the best substrates and we propose that aminopeptidase W would be a convenient name for such an enzyme, in line with other names such as aminopeptidase P and carboxypeptidase P for enzymes hydrolysing bonds adjacent to proline residues.

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