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Ectoenzymes of the kidney microvillar membrane

Aminopeptidase P is anchored by a glycosyl-phosphatidylinositol moiety

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The mode of membrane anchorage of three kidney microvillar membrane ectoenzymes has been examined. The release of aminopeptidase P (EC 3.4.11.9) from kidney membranes by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) and the pattern of detergent solubilization of this ectoenzyme implies that it is anchored to the membrane via a covalently attached glycosyl-phosphatidylinositol moiety. As deduced by phase separation in Triton X-114, octyl-glucoside solubilized the amphipathic form of aminopeptidase P, whereas the PI-PLC-released form displayed hydrophilic properties. In contrast, the pattern of detergent solubilization of two microvillar carboxypeptidases and their resistance to release from the membrane by bacterial PI-PLC suggest that these two ectoenzymes are not anchored via phosphatidylinositol.

Aminopeptidase P; Ectoenzyme; Carboxypeptidase; Glycosyl-phosphatidylinositol; Membrane anchor

1. INTRODUCTION

A C-terminal glycosyl-phosphatidylinositol (PI) membrane anchor has been identified on a number of mammalian proteins including enzymes, cell-adhesion molecules and proteins involved in transmembrane signalling (reviews [1,2]). A useful indicator for this type of membrane anchor is the release of a protein from the membrane by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), with the released protein displaying hydrophilic properties due to the loss of the hydrophobic membrane-anchoring domain [2]. Also, we have recently shown that differential solubilization by detergents can predict a glycosyl-PI membrane anchor [3]. Only those detergents with a high critical micellar concentration (CMC) released substantial amounts of PI-anchored microvillar ectoenzymes into the high-speed supernatant, with the resultant solubilized protein displaying amphipathic properties due to retention of the hydrophobic membrane anchor.

The kidney microvillar membrane contains a battery of some 10 or more cell-surface peptidases (reviews [4,5]), most of which are anchored in the lipid bilayer by a membrane-spanning segment of the polypeptide chain, such as endopeptidase-24.11 (EC 3.4.24.11) [5]. However, at least one cell-surface peptidase, renal dipeptidase (dehydropeptidase I, EC 3.4.13.11), is anchored by covalent attachment to a glycosyl-PI moiety in a similar fashion to alkaline phosphatase (EC 3.1.3.1) [6]. Here we show by the criteria of (i) release by bacterial PI-PLC and (ii) the pattern of solubilization by a range of detergents, that aminopeptidase P (microsomal proline aminopeptidase, EC 3.4.11.9) also has the characteristics of

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Abbreviations: CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-1-propane sulphonate; CMC, critical micellar concentration; octyl-glucoside, *n*-octyl-β-D-glucopyranoside; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C

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a glycosyl-PI-anchored membrane protein. The mode of anchorage of carboxypeptidase P (microsomal prolyl carboxypeptidase, EC 3.4.17.-) and of a basic membrane-bound carboxypeptidase resembling carboxypeptidase N [7] has also been examined.

2. EXPERIMENTAL

2.1. *Materials*

Human kidneys were made available by the Department of Surgery, St. James's University Hospital, Leeds, and had been prepared for renal transplantation, but for various reasons were not used for this purpose. PI-PLC from *Staphylococcus aureus* and from *Bacillus thuringiensis* were purified as in [6,8] and were gifts from Dr M.G. Low. Units of PI-PLC activity are μmol/min. Sodium deoxycholate was purchased from BDH (Poole, England). All other detergents, *N*-carbobenzoxy-prolylphenylalanine (N-Cbz-Pro-Phe), glycylprolylhydroxy-proline (Gly-Pro-HyPro) and benzoylglycyllysine (BzGly-Lys) were purchased from Sigma. Triton X-114 was pre-condensed before use [9]. Pig kidneys and all other materials were from sources previously noted.

2.2. *Preparation of kidney microvillar membranes and solubilization of membrane proteins by phospholipases and detergents*

All operations were carried out at 4°C unless stated otherwise. Microvilli were prepared from pig or human kidney cortex as described [10], except that the 15000 × *g* centrifugation steps were each extended from 12 to 15 min. Before incubation with phospholipase or detergent, the microvillar membranes were centrifuged at 31000 × *g* for 1.5 h and then resuspended in 10 mM Hepes, pH 7.4. Aliquots of phospholipase or detergent were added where appropriate and the membranes incubated for 1 h at 37 or 4°C, respectively. After solubilizing protein, the incubation mixtures were centrifuged at 31000 × *g* for 1.5 h. Enzyme activities were determined in the total incubation mixture and in the supernatant after centrifugation at 31000 × *g*; the solubilized activity was expressed as a percentage of the total (100%) activity in the original incubation mixture.

2.3. *Enzyme assays*

Aminopeptidase P was assayed using Gly-Pro-HyPro (1.0 mM final concentration) as substrate in 0.1 M Tris-HCl, pH 8.0, containing 4.0 mM $MnCl_2$ (0.1 ml final reaction volume). Hydrolysis of Gly-Pro-HyPro was monitored by reverse-phase HPLC on a μBondapak C_{18} column with a UV (214 nm) detector at a flow rate of 1.5 ml/min and isocratic elution for 8 min with 0.9% (v/v) acetonitrile in 0.08% H_3PO_4 at pH 2.5, followed by 5 min elution with 30% (v/v) acetonitrile. The product, Pro-HyPro, was quantified by calibration from a standard curve.

Carboxypeptidase P was assayed using N-Cbz-Pro-Phe (1.0 mM final concentration) as substrate as described in [11]. Hydrolysis of N-Cbz-Pro-Phe was monitored by reverse-phase HPLC using a 15 min linear gradient of 4–45% (v/v) acetonitrile in 0.08% H_3PO_4 at pH 2.5, followed by 5 min elution at the final conditions. The product, Phe, was quantified by calibration from a standard curve.

The basic, membrane-bound carboxypeptidase was assayed using BzGly-Lys (1.0 mM final concentration) as substrate after pre-incubation of the enzyme sample in 0.1 M Tris-HCl, pH 7.4, containing 1.0 mM $CoCl_2$ for 2 h at 4°C (0.1 ml final reaction volume). Hydrolysis of BzGly-Lys and quantification of the BzGly product was monitored as described previously for BzGly-His-Leu [12]. No inhibition of the hydrolysis of BzGly-Lys by the microvillar membranes was observed in the presence of either 0.1 mM *p*-chloromercuribenzoate or 0.1 mM *p*-hydroxymercuriphenylsulphonic acid, discounting any contribution by carboxypeptidase H (enkephalin convertase) to hydrolysis of this substrate.

Alkaline phosphatase, renal dipeptidase, endopeptidase-24.11 and protein were assayed as in [6,12,13].

2.4. *Triton X-114 phase separation of pig kidney microvillar ectoenzymes*

Pig kidney microvillar membranes (65 μg protein), and the 31000 × *g* supernatant after treatment of pig kidney microvillar membranes with *S. aureus* PI-PLC (25 mU/ml) for 1 h at 37°C (cf. fig. 1) or with 60 mM octyl-glucoside for 1 h at 4°C (cf. fig. 2), were made up to 0.2 ml with 10 mM Tris, 150 mM NaCl, 1.0% Triton X-114, pH 7.4, and subjected to phase separation at 30°C for 3 min as in [9]. The detergent-rich and detergent-poor phases were separated through a sucrose cushion by centrifugation at 3000 × *g* and assayed for enzyme activities. The results are presented as means (± SE) for four phase separations.

3. RESULTS AND DISCUSSION

3.1. *Enrichment of ectoenzymes in pig kidney microvillar membranes*

Aminopeptidase P, carboxypeptidase P and the basic carboxypeptidase were all enriched in the pig kidney microvillar membrane preparation to a similar extent as alkaline phosphatase, endopeptidase-24.11 and renal dipeptidase (table 1). This enrichment of aminopeptidase P and carboxypeptidase P is in agreement with previous results suggesting a microvillar location for these two ectoenzymes [14–16]. Likewise, the enrichment of the basic carboxypeptidase is consistent with other reports for a plasma membrane location for this activity [7,17,18]. The specific activity of these enzymes in the human kidney preparation was similar to that in pig kidney (table 1).

3.2. *Release of ectoenzymes from kidney microvillar membranes by bacterial PI-PLC*

When pig or human kidney microvillar mem-

Table 1

Specific activity and enrichment of ectoenzymes in kidney microvillar membranes

Enzyme	Pig kidney		Human kidney
	Specific activity (nmol/min per mg)	Enrichment over homogenate	Specific activity (nmol/min per mg)
Alkaline phosphatase	97.1 ± 4.4	5.58 ± 0.48	80.7
Aminopeptidase P	68.5 ± 8.5	5.82 ± 0.67	43.7
Basic carboxypeptidase	3.7 ± 0.2	5.61 ± 0.58	3.5
Carboxypeptidase P	5.9 ± 0.7	5.09 ± 0.89	1.6
Endopeptidase-24.11	506.3 ± 29.9	5.61 ± 0.49	343.8
Renal dipeptidase	116.3 ± 16.9	6.49 ± 0.59	356.0

Enzymes were assayed as described in section 2. The results for pig kidney are means (± SE) from 3 separate microvillar membrane preparations

branes were incubated with PI-PLC from *S. aureus*, specific release of aminopeptidase P, but not of carboxypeptidase P and the basic carboxypeptidase, was observed (fig. 1). Alkaline phosphatase and renal dipeptidase, but not endopeptidase-24.11, were also released from the pig kidney microvillar membranes, in agreement with previous results [6], as well as from the human kidney membranes (fig. 1). Aminopeptidase P appeared to be more resistant to release from the human kidney microvillar membranes than either alkaline phosphatase or renal dipeptidase (fig. 1b). Identical results were obtained with both membrane sources using PI-PLC from *B. thuringiensis* (not shown).

3.3. *Solubilization of ectoenzymes from pig kidney microvillar membranes by detergents*

The solubilization of aminopeptidase P, carboxypeptidase P and the basic carboxypeptidase from pig kidney microvillar membranes by a range of detergents was examined (fig. 2). Only octylglucoside and CHAPS, detergents with high CMC values, were effective at releasing substantial (>67%) amounts of the membrane-bound aminopeptidase P activity into the high-speed supernatant (fig. 2), whereas all the detergents were effective at solubilizing >80% of the membrane-bound carboxypeptidase P and basic carboxypeptidase activities.

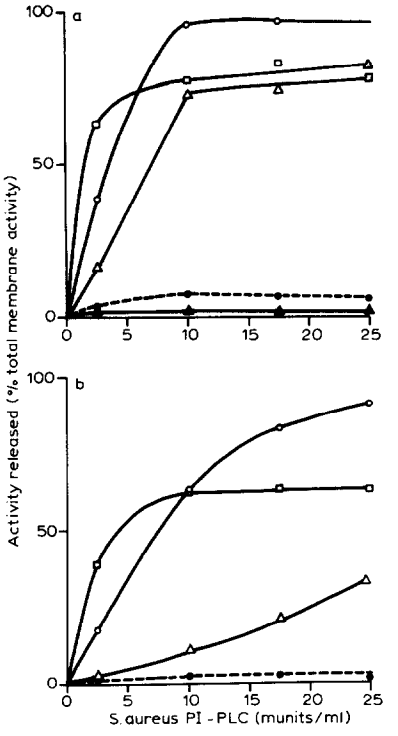

Fig.1. Release of ectoenzymes from kidney microvillar membranes by PI-PLC. (a) Pig kidney microvillar membrane fraction (approx. 3.4 mg protein/ml) or (b), human kidney microvillar membrane fraction (approx. 0.3 mg protein/ml) were incubated in the presence of *S. aureus* PI-PLC as described in Section 2. Results are means of duplicate incubations with PI-PLC. No changes in total activity as a result of incubation in either the presence or absence of phospholipase were observed. No release of endopeptidase-24.11 was observed in either (a) or (b), and no release of carboxypeptidase P and the basic carboxypeptidase was observed in (b). (□) Alkaline phosphatase, (△) aminopeptidase P, (○) renal dipeptidase, (▲) carboxypeptidase P and the basic carboxypeptidase, (●) protein.

3.4. *Phase separation of soluble and membrane forms of ectoenzymes in Triton X-114*

When samples of pig kidney microvillar membranes were analysed by phase separation in Triton X-114, the three ectoenzymes partitioned predominantly (>83%) into the detergent-rich phase (aminopeptidase P, 87.4 ± 5.8%; carboxypeptidase P, 89.0 ± 1.0%; basic carboxypeptidase, 83.1 ± 1.9%), indicative of an amphipathic nature for each ectoenzyme. When the soluble fraction released by *S. aureus* PI-PLC was subjected to phase separation in Triton X-114, the aminopeptidase P activity partitioned predominantly (95.5 ± 3.3%) into the detergent-

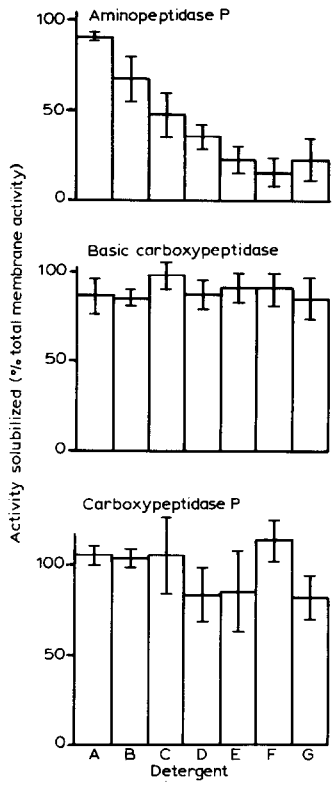

Fig.2. Solubilization of pig kidney microvillar peptidases by detergents. Pig kidney microvillar membrane fraction (approx. 2.5 mg protein/ml) was incubated with detergent as described in Section 2. Results are means (± SE) of four separate experiments with each detergent, and have been corrected for any activation or inhibition caused by individual detergents in the enzyme assays. Detergents (and their concentrations) were: (A) octyl-glucoside (60 mM), (B) CHAPS (20 mM), (C) sodium deoxycholate (8.0 mM), (D) Nonidet P-40 (6.1 mM), (E) Triton X-100 (5.9 mM), (F) Triton X-114 (6.9 mM), (G) Emulphogene BC-720 (0.37% (w/w)).

poor phase. Thus, aminopeptidase P, like other proteins anchored in the membrane by a glycosyl-PI moiety [3,6] is converted into a soluble, hydrophilic form upon treatment with PI-PLC. In contrast, when the supernatant released from the membranes by solubilization with octyl-glucoside was subjected to phase separation in Triton X-114, the aminopeptidase P activity partitioned predominantly (68.0 ± 11.9%), into the detergent-rich phase, consistent with retention of the hydrophobic membrane-anchoring domain.

3.5. *General conclusions*

Aminopeptidase P, carboxypeptidase P and a basic carboxypeptidase which resembles carboxypeptidase N are all present in the kidney microvillar membrane (table 1) as amphipathic proteins consistent with a cell-surface location. Carboxypeptidase P and the basic carboxypeptidase do not appear to have the characteristics of glycosyl-PI anchored enzymes (fig.1,2). In contrast, the release of aminopeptidase P from both pig and human kidney microvillar membranes upon incubation with bacteria PI-PLC (fig.1) and the pattern of solubilization by a range of detergents (fig.2), indicates that this cell-surface peptidase is anchored in the lipid bilayer by a covalently attached glycosyl-PI moiety, in a similar fashion to alkaline phosphatase and renal dipeptidase. In this context it is interesting to note that when aminopeptidase P was first isolated, the purified enzyme exhibited a large M_r ($1-2 \times 10^6$) and contained a number of lipid components, including phosphoinositide [19]. However, the significance of these observations was unclear at the time.

The release of a substantial proportion of aminopeptidase P from the membrane in a hydrophilic form by PI-PLC, or in an amphipathic form by certain detergents, should prove useful as initial steps in the purification and characterization of this enzyme.

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