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Rapid Report

Release of GPI-anchored membrane aminopeptidase P by enzymes and detergents has some peculiarities

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Abstract

The enzyme aminopeptidase P (APP) of rat small intestine is an integral membrane protein. It is not released from the membrane by proteinases and is also resistant to bacterial inositol-specific phospholipases C (PI-PLC's). We show that this resistance is due to a hindered accessibility of the bond split by PI-PLC and not to a modified glycosylphosphatidyl inositol (GPI)-anchor.

Key words: GPI-anchored enzyme; Membrane enzyme; Glycosylphosphatidyl inositol; Aminopeptidase P; Brush-border membrane; (Rat intestine)

There exist a number of well established methods to find out whether or not a given protein is anchored to the membrane via a transmembranal peptide or a glycosylphosphatidyl inositol (GPI) moiety [1]. The most frequently used approach to prove the latter possibility is to check the sensitivity of a protein anchor to bacterial inositol-specific phospholipases C (PI-PLC's). If there is no release of the protein, modification of the anchor, mostly an additional fatty acid esterified to inositol is discussed. This could be proved for some human erythrocyte proteins [2]. Molecules bearing this modified anchor can be split from the membrane by mammalian serum inositol-specific phospholipase D (GPI-PLD) or by treatment with hydroxylamine at high pH followed by bacterial PI-PLC.

We have shown that the enzyme aminopeptidase P (APP) of the rat small intestine is an integral membrane protein not released from the membrane by proteinases [3]. In this paper we report results which confirm the existence of an unmodified GPI anchor of APP only accessible to PI-PLC's after disturbance of the membrane structure.

Brush-border membrane vesicles (BBMV's) of rat small intestine were prepared as described in [3]. *Bacillus cereus* PI-PLC was purchased from Sigma, Deisenhofen, Germany, *Bacillus thuringiensis* PI-PLC from ICN Flow Biochemicals, Meckenheim, Germany. Papain was from Fluka, Buchs, Switzerland, n-octyl glycoside, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), Triton X-100 and Triton X-114 from Serva, Heidelberg, Germany, triethylamine, hydroxylamine and Nonidet P-40 from Sigma, Deisenhofen, Germany.

(G)PI-PLD was partially purified from pig plasma according to [4]. Triton X-114 was used after precondensation [6] for phase separations as described in [1].

APP-activity was assayed in a continuous spectrophotometric assay according to [5] using L-Ala-Pro-Pro-4-nitroanilide as substrate and purified DP IV as auxiliary enzyme. (We thank Prof. Dr. K. Neubert, Martin-Luther-University, Fachbereich Biochemie/Biotechnologie for providing us the substrate and Dr. J. Rahfeld, Max-Planck-Arbeitsgruppe 'Enzymologie der Peptidbindung', Halle for purified DP IV.)

Hydroxylamine treatment. BBMV's (10 mg protein/ml) were treated with 1 M hydroxylamine in Tricine (final concentration) at pH 9.5–10.5 and 37°C for 2 h. After either dialysis against Tricine buffer (pH 7.4) or dilution to pH 7.4 the mixture was centrifuged or further treated with PI-PLC.

Treatment of BBMV's with triethylamine. BBMV's (10 mg protein/ml) were treated with 0.25 M (final

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concentration) of triethylamine in Tricine at pH 10.8 for 2 or 4 h at 37°C. After that time the pH was readjusted to 7.4 by addition of 0.2 M Hepes-buffer (pH 7.0). The mixture was either centrifuged (1.5 h 31 000 × *g*) or incubated with PI-PLC for further 4 h at 37°C. Aliquots of the mixtures and the supernatants were subjected to phase separation in Triton X-114.

Papain treatment. BBMV's (40 μg protein) were incubated with 6 units of activated papain for 6 h at room temperature. The reaction was stopped by addition of iodoacetamide in ice and the material was centrifuged for 45 min at 23 000 × *g*. The pellet was washed three times with Tricine buffer (50 mM, 150 mM NaCl (pH 7.4)) and resuspended in the same buffer. This suspension was incubated for 4 h at 37°C with *Bacillus thuringiensis* PI-PLC (1.2 units/ml). Thereafter the mixture was centrifuged again (1.5 h, 31 000 × *g*). APP activity was assayed in the suspensions and supernatants.

Sonication of BBMV's. Sonication was done in ice with a Branson sonifier B12 in intervals (30 s sonication, 30 s pause) for 5 or 10 min. The material was then centrifuged (1.5 h, 31 000 × *g*). Supernatant and resuspended pellet were incubated with *Bacillus thuringiensis* PI-PLC (280 mU/ml) for 4 h at 37°C.

Hooper [1] holds the view that differential solubilization by detergents is a first hint whether a protein is bound to the membrane by a transmembranal polypeptide or a GPI moiety. In the first case the protein is solubilized to the same extent by all surfactants whereas GPI-anchored proteins are efficiently solubilized only by detergents with a high critical micellar concentration (i.e., octyl glycoside or CHAPS).

Fig. 1 shows the pattern of differential solubilization of APP by various detergents. This pattern is not the typical one proposed for GPI-anchored proteins. As our results show, conclusions drawn from such pattern as in [7] for pig intestinal APP have to be regarded with caution.

Table 1 shows the results of incubation of BBMV's with PI-PLC of *Bacillus cereus* and *Bacillus thuringiensis*, respectively. Treatment of the vesicles with hydroxylamine prior to PI-PLC incubation did not increase the amount of APP in the supernatant, pointing to the fact that there is no additional fatty acid esterified to inositol.

Incubation of the vesicles with partially purified PI-PLD from pig plasma, however, released a substantial amount of APP as a hydrophilic protein (Table 2).

These results led us to the conclusion that APP in the brush-border membrane of rat small intestine has an unmodified GPI-anchor but the site of PI-PLC attack is hidden.

Several methods were used to expose this site.

Detergent-lipid-protein mixed micelles formed by treatment with octylglycoside are susceptible to PI-PLC

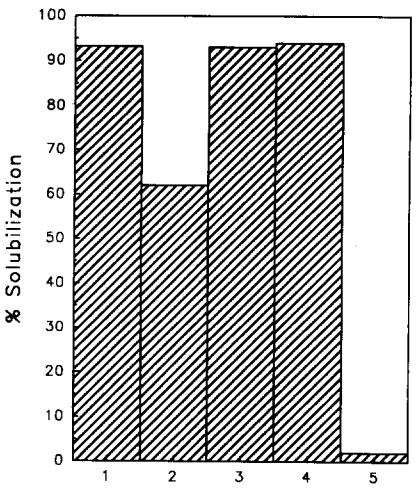

Fig. 1. Differential solubilization by detergents. BBMV's (0.54 mg/ml) were mixed with the following detergents: 1, octyl glycoside (60 mM); 2, CHAPS (20 mM); 3, Triton X-100 (6.1 mM); 4, Nonidet P-40 (5.9 mM); 5, control without detergent. After shaking for 1 h at 4°C aliquots were removed for activity assays and the remaining suspensions were centrifuged for 1.5 h at 31 000 × *g*. Enzyme activities were measured in the supernatants.

Table 1
Incubation of BBMV's with PI-PLC

PI-PLC source	units/ml	% release of APP
Bacillus cereus	0	0.5 ± 0.5
	0.10	1.4 ± 0.4
	0.35	1.2 ± 1.0
	0.50	1.4 ± 0.6
Bacillus thuringiensis	0	0.1 ± 0.1
	0.06	0.5 ± 0.4
	0.29	4.7 ± 0.7
	0.58	7.6 ± 0.3

BBMV's (3 mg protein/ml) were incubated with the given amounts of bacterial PI-PLC for 1 h at 37°C. Thereafter the material was centrifuged for 1.5 h at 31 000 × *g*. % release = % of total enzyme activity in the supernatant. $n = 4$.

Table 2
Incubation of BBMV's with PI-PLD from pig plasma (partially purified)

Plasma added (μl)	% release of APP
0	13.5 ± 3.9
2	88.0 ± 2.2
5	93.4 ± 3.5
10	93.5 ± 4.5
20	95.5 ± 1.5

20 μl BBMV's (10 mg protein/ml) in 0.2 M Hepes buffer (pH 7.0) were incubated with plasma in a total volume of 50 μl in the presence of 1% Nonidet P-40 for 2 h at 37°C. Thereafter the samples were subjected to phase separation in Triton X-114. % release = the fraction of total enzyme activity found in the detergent-depleted (aqueous) phase. Plasma without added membranes (control) contributed 0–2% to the total enzyme activity. $n = 4$.

Table 3
Release of APP from BBMV's

Treatment	% of APP released	% of released APP in the aqueous phase after Triton X-114 phase separation
Papain	1.0	n.d.
Papain followed by *B. thur.* PI-PLC (0.16 units/ml)	66.0	96
Octyl glycoside	80.0	13
Octyl glycoside followed by *B. thur.* PI-PLC (0.16 units/ml)		65
Triethylamine at pH 10.8, 37°C	55.6	2
Triethylamine at pH 10.8 followed by *B. thur.* PI-PLC (0.1 unit/ml)	55.0	90
Sonication	38.0	2
Supernatant after sonication incubated with *B. thur.* PI-PLC (0.58 units/ml)		83

For details see text. Values given are means of at least four different experiments.

(Table 3). Stieger et al. [8] reported a similar observation for the GPI-anchor of acetylcholinesterase from bovine erythrocytes which could only be cleaved if present in detergent/lipid micelles or in an artificial lipid bilayer.

Exposure of membrane vesicles to high pH (pH 10–11) did not release APP as was shown before [3]. By which mechanism triethylamine at alkaline pH brings APP into a soluble form is not clear up to now (Table 3). There are some hints from freeze-fracture electron microscopy (pictures not shown) pointing to micelles in the supernatant after this treatment, but it is difficult to understand how a molecule like triethylamine should act like a detergent.

Papain which releases peptide anchored proteins from BBMV's leaving "proteolytically shaved" vesicles [3] still containing APP on their surface also brings this enzyme in a state accessible to PI-PLC action (Table 3).

After sonication (5 min) a fraction of APP becomes soluble. This fraction is hydrophobic as proved by its partition into the detergent phase after Triton X-114 phase separation. PI-PLC transforms it into the hydrophilic form (Table 3). Prolonged sonication does not increase the amount of APP in the supernatant but leads as expected to a loss of total enzyme activity (81% after 5 min sonication, 66% after 10 min). Physical destruction of the membrane by sonication produces small vesicles or fragments which evidently expose a more favorable surface for PI-PLC action. Wong et al.[9] made recently a similar observation in connection with the action of GPI-PLD on alkaline phosphatase at cell surfaces.

In summary, a missing or modified GPI-anchor should be postulated only if a hindered accessibility of cleavable bonds to the corresponding enzymes has been definitively excluded.

This is especially important if the membranes have a "screening" glycocalix.

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