Biochem. J. (1989) 262, 33–40 (Printed in Great Britain)

5′-Nucleotidases of chicken gizzard and human pancreatic adenocarcinoma cells are anchored to the plasma membrane via a phosphatidylinositol–glycan

Ursula STOCHAJ,* Karola FLOCKE, Wolfgang MATHES and Hans Georg MANNHERZ
Institut für Anatomie und Zellbiologie, Robert-Koch-Strasse 5, D-3550 Marburg, Federal Republic of Germany

We have analysed the membrane anchorage of plasma-membrane 5′-nucleotidase, an ectoenzyme which can mediate binding to components of the extracellular matrix. We demonstrated that the purified enzyme obtained from chicken gizzard and a human pancreatic adenocarcinoma cell line were both completely transformed into a hydrophilic form by treatment with phospholipases C and D, cleaving glycosyl-phosphatidylinositol (GPI). These data indicate the presence of a glycolipid linker employed for membrane anchoring of the 5′-nucleotidase obtained from both sources. Incubation of plasma membranes under identical conditions revealed that about half of the AMPase activity was resistant to GPI-hydrolysing phospholipases. Investigation of the enzymic properties of purified chicken gizzard 5′-nucleotidase revealed only minor changes after removal of the phosphatidylinositol linker. However, cleavage of the membrane anchor resulted in an increased sensitivity towards inhibition by concanavalin A. After tissue fractionation, chicken gizzard 5′-nucleotidase could be obtained as either a membrane-bound or a soluble protein; the latter is suspected to be released from the plasma membrane by endogenous phospholipases. Higher-molecular-mass proteins immuno-cross-reactive with the purified chicken gizzard 5′-nucleotidase were detected as both soluble and membrane-bound forms.

INTRODUCTION

5′-Nucleotidase is a glycoprotein present on the surface of many eukaryotic cells. It mediates the hydrolysis of AMP and is an ectoenzyme, i.e. its active centre faces the cell exterior (Newby *et al.*, 1975; Baron *et al.*, 1986). Besides the membrane-bound enzyme, cytosolic as well as secreted forms of 5′-nucleotidase exist, which exhibit different enzymic characteristics (Worku *et al.*, 1984; Newby, 1988; Truong *et al.*, 1988). Some forms of the membrane-bound 5′-nucleotidase have been reported to be attached to the lipid bilayer via glycolipid anchors (Panagia *et al.*, 1981; Thompson *et al.*, 1987), whereas other forms of the enzyme seem to contain membrane-spanning protein segments as well as intracellular domains (Zachowski *et al.*, 1981; Grondal & Zimmermann, 1987).

Several glycoproteins involved in cellular adhesion processes have been demonstrated to be anchored to the plasma membrane via phosphatidylinositol (PI)–glycan (Davitz *et al.*, 1986; Dustin *et al.*, 1987; He *et al.*, 1987; Dickson *et al.*, 1987; Yeh *et al.*, 1988). We have recently shown binding of chicken gizzard and human pancreatic adenocarcinoma 5′-nucleotidase to fibronectin and the laminin/nidogen complex (Stochaj *et al.*, 1989), indicating the potential interaction of the enzyme from both sources with different components of the extracellular matrix. The present study was undertaken to characterize further the mode of plasma-membrane anchorage of 5′-nucleotidase from chicken gizzard as well as human pancreatic adenocarcinoma cells.

MATERIALS AND METHODS

Materials

Phospholipase C from *Bacillus cereus* (type III), phospholipase A_2 from *Naja naja* venom, phospholipase C from *Clostridium welchii*, phospholipase D from cabbage, aprotinin, antipain, chymostatin, leupeptin, pepstatin, phenylmethanesulphonyl fluoride, iodoacetamide, concanavalin A and methyl α-D-mannopyranoside were obtained from Sigma. Triton X-114, Triton X-100, sodium deoxycholate and 1,10-phenanthroline were purchased from Serva. PI-specific phospholipase C from *Bacillus thuringiensis* (PIPLC) was kindly provided by Dr. M. G. Low, Columbia University, New York, U.S.A. PIPLC from *Staphylococcus aureus* was a gift from Dr. M. A. J. Ferguson, University of Dundee, U.K.

Methods

Chicken gizzard 5′-nucleotidase was purified and reconstituted into phospholipid vesicles as described by Dieckhoff *et al.* (1987). AMPase activity was determined as previously described (Dieckhoff *et al.*, 1986). 5′-Nucleotidase activities of plasma membranes were measured in the presence of 20 mM-β-glycerophosphate to saturate non-specific phosphomonoesterases (Low & Finean, 1978).

Cell culture. Human pancreatic adenocarcinoma cells (PaTu II; Kern *et al.*, 1988) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) aseptically collected calf serum and 10% (v/v) horse

Abbreviations used: PI, phosphatidylinositol; GPI, glycosyl-PI; GPI-PLD, phospholipase D that cleaves GPI but not PI; PIPLC, PI-specific phospholipase C.

* To whom correspondence and reprint requests should be addressed.

serum. 5′-Nucleotidase was purified essentially as described for the enzyme obtained from chicken gizzard (Dieckhoff *et al.*, 1985).

Treatment with phospholipase C from *B. cereus* and *B. thuringiensis*. Phospholipid vesicles containing 5′-nucleotidase were incubated with 10 units of *B. cereus* phospholipase C (phosphatidylcholine-specific activity)/ml in 0.1 M-Tris/HCl, pH 7.4, containing 50 mM-NaCl, 0.25 M-methyl α-D-mannopyranoside, 2 mM-1,10-phenanthroline and a cocktail of protease inhibitors (antipain, aprotinin, chymostatin, leupeptin and pepstatin, each at 1 μg/ml). Phospholipase C from *B. cereus* was pretreated with the incubation buffer containing 10 mM-1,10-phenanthroline for 30 min on ice to inactivate phospholipase C specifically hydrolysing phosphatidylcholine.

After incubation for 2.5 h at 30 °C, samples were mixed with protein-free vesicles containing 100 μg of phosphatidylcholine and centrifuged for 45 min at 150000 ***g*** (4 °C). Supernatants and sediments were dialysed against 5 mM-Hepes (pH 7.4)/0.5 mM-NaN_3/150 mM-NaCl and subsequently subjected to SDS/polyacrylamide-gel electrophoresis. Alternatively, 5′-nucleotidase was incubated with pretreated phospholipase C as described above, with omission of 1,10-phenanthroline from the incubation mixture. After 2.5 h at 30 °C, samples were mixed with an equal volume of 5 mM-Hepes, pH 7.4, containing 0.1 mM-$CaCl_2$, 0.5 mM-NaN_3, 150 mM-NaCl and 2% (v/v) Triton X-114, kept for 10 min on ice, incubated for 2 min at 37 °C and centrifuged for 2.5 min at 8000 ***g***. Supernatants were re-extracted with Triton X-114 as described above. AMPase activities were determined before phase separation and for aqueous phases obtained after the second centrifugation step. Control experiments were carried out under identical conditions in the presence of 2.7 mM-$ZnCl_2$ to inhibit the PI-specific enzyme (Ikezawa & Taguchi, 1981). 5′-Nucleotidase was incubated with PIPLC from *B. thuringiensis* in 10 mM-Hepes, pH 7.4, for 1 h at 37 °C.

Treatment with other phospholipases. 5′-Nucleotidase was incubated with phospholipase A_2 (final concn. 45 μg/ml) in 10 mM-Tris/HCl (pH 8.0)/50 mM-$CaCl_2$, with phospholipase D (45 μg/ml) in 10 mM-sodium acetate (pH 6.3)/50 mM-$CaCl_2$/30 mM-NaCl and with phospholipase C from *C. perfringens* (0.9 unit/ml) in 10 mM-Tris/HCl (pH 7.0)/10 mM-$CaCl_2$. Samples containing a cocktail of protease inhibitors (aprotinin, antipain, chymostatin, leupeptin, pepstatin, each at 1 μg/ml) were incubated for 2.5 h at 30 °C and subsequently treated with Triton X-114 as described above.

Partial enrichment of human serum GPI-PLD. Human blood containing 0.4% (w/v) sodium citrate and 0.2 mM-phenylmethanesulphonyl fluoride was centrifuged for 20 min at 30000 ***g*** (4 °C). The supernatant was dialysed against 10 mM-sodium acetate (pH 5.4), centrifuged again (30 min, 10000 ***g***, 4 °C) and dialysed against 50 mM-Tris/HCl, pH 7.8 (Cardoso de Almeida *et al.*, 1988). Samples stored at −20 °C were used for cleavage of the PI–glycan linker.

Incubation with fetal-calf serum or human serum containing GPI-PLD. Purified solubilized 5′-nucleotidase was incubated with serum (2 μl/100 μl reaction volume) in 5 mM-Hepes (pH 7.4), containing 2.6 mM-$CaCl_2$, 10 mM-NaCl, 0.5 mM-NaN_3 and 0.02% (w/v) Triton X-100 for 1 h at 37 °C. Membrane vesicles were incubated with GPI-PLD in 50 mM-Tris/HCl (pH 7.4)/25 mM-NaCl/2.6 mM-$CaCl_2$ for 1 h at 37 °C in the absence or presence of 0.1 % Triton X-100. Cleavage of the PI–glycan anchor was analysed by Triton X-114 phase separation as described above. Neither fetal-calf serum nor human serum contained detectable amounts of AMPase activity.

Preparation of chicken gizzard plasma membranes. This was done essentially as described by Kwan *et al.* (1983).

Preparation of crude membranes from chicken gizzard. This was done as described by He *et al.* (1987). Chicken gizzard was homogenized in 5 mM-Hepes (pH 7.8)/10% (w/v) sucrose in the presence of 5 mM-phenylmethanesulphonyl fluoride, 5 mM-iodoacetamide, 1 μg of aprotinin/ml, 1 μg of antipain/ml, 1 μg of chymostatin/ml and 1 μg of pepstatin/ml. Supernatants obtained after centrifugation for 10 min at 1200 ***g*** were centrifuged again at 10000 ***g*** (10 min). Material recovered from the supernatant was diluted 2-fold in 5 mM-Hepes, pH 7.8, and centrifuged for 2 h at 200000 ***g***. Crude membranes obtained after ultracentrifugation still contained water-soluble 5′-nucleotidase (as judged by partitioning into the aqueous phase after incubation with Triton X-114). They were further incubated with 5 M-urea in 0.1 M-Tris/HCl (pH 7.4)/50 mM-NaCl (10 min on ice) and centrifuged for 45 min at 175000 ***g*** (4 °C). Sediments were resuspended in 0.1 M-Tris/HCl (pH 7.4)/50 mM-NaCl and again ultracentrifuged (30 min, 175000 ***g***, 4 °C). Pellets obtained after this treatment are termed ‘urea-treated crude membranes’.

Preparation of plasma membranes from human pancreatic adenocarcinoma cells. Plasma membranes of PaTu II-cells were isolated as described by Lin *et al.* (1987). Vesicles obtained after ultracentrifugation were further purified by centrifugation in sucrose density gradients (Kwan *et al.*, 1983).

SDS/polyacrylamide-gel electrophoresis and Western blotting. A Bio-Rad Minigel apparatus was used for electrophoretic separation of proteins. After transfer on to cellulose nitrate, 5′-nucleotidase was detected with monoclonal or polyclonal antibodies essentially as described by Dieckhoff *et al.* (1986).

RESULTS

Incubation of reconstituted chicken gizzard 5′-nucleotidase with different phospholipases

Purified chicken gizzard 5′-nucleotidase can be reconstituted into phospholipid vesicles (Dieckhoff *et al.*, 1986). These unilamellar vesicles carry the enzyme in either inside-out or right-side-out orientation in about a 1:1 ratio. We investigated the effect of several phospholipases with different specificities on the production of hydrophilic 5′-nucleotidase (Table 1). Phospholipase C obtained from *B. cereus* is known to contain PI-specific activity (PIPLC) as contaminant. Addition of chelators for bivalent cations specifically inhibits the phosphatidylcholine-hydrolysing enzyme, whereas PIPLC remains active (Little, 1981). On the other hand, PIPLC is inactive in the presence of $ZnCl_2$ (Ikezawa & Taguchi, 1981). Phospholipid vesicles incubated with different phospho-

Table 1. Transformation of chicken gizzard 5′-nucleotidase into a hydrophilic form by incubation of proteoliposomes with different phospholipases

Chicken gizzard 5′-nucleotidase reconstituted into phospholipid vesicles was incubated under the conditions described in the Materials and methods section in the absence (−) or presence (+) of different phospholipases listed below. Final concentrations of deoxycholate and $ZnCl_2$ were 0.1% (w/v) and 2.6 mM respectively. The proportion of AMPase in hydrophilic form was assessed by phase separation in Triton X-100 (see the Materials and methods section). 5′-Nucleotidase activity measured before phase separation was taken as 100%.

Phospholipase		Hydrophilic AMPase activity (% of total activity)
A_2 (*Naja naja*)	−	5.5
	+	5.7
D (cabbage)	−	8.7
	+	9.1
C (*C. perfringens*)	−	12.4
	+	12.1
C (*B. cereus*)	−	9.9
	+	52.5
C (*B. cereus*)	−	8.8
+deoxycholate	+	96.3
C (*B. cereus*)	−	8.7
$+ZnCl_2$	+	3.6

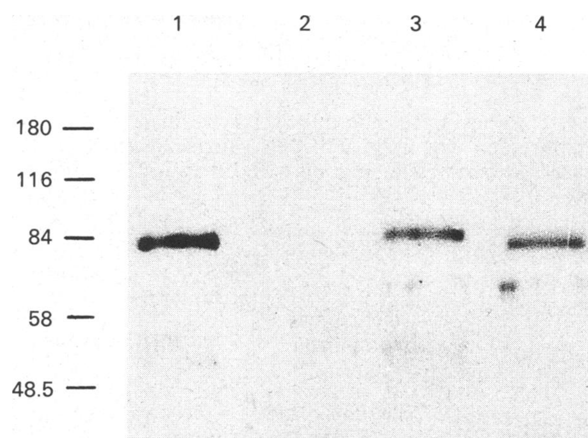

Fig. 1. Release of chicken gizzard 5′-nucleotidase from proteoliposomes by treatment with *B. cereus* phospholipase C

Proteoliposomes harbouring 5′-nucleotidase in either right-side-out or inside-out orientation (ratio approx. 1:1) were incubated in the absence (lanes 1, 2) or presence (lanes 3, 4) of phospholipase C from *B. cereus* (see the Materials and methods section). After addition of protein-free phospholipid vesicles, samples were ultracentrifuged. Comparable amounts of material recovered from sediments (lanes 1, 3) or supernatants (lanes 2, 4) were analysed by Western blotting by using a polyclonal antibody directed against the M_r-79000 protein. M_r values ($\times 10^{-3}$) of marker proteins are indicated on the left margin.

lipases were subjected to Triton X-114 phase separation. Cleavage of the PI linker was monitored by determination of the AMPase activity partitioned into the hydrophilic phase. As shown in Table 1, liberation of 5′-nucleotidase from the lipid bilayer was only obtained by using PIPLC, whereas other phospholipases tested hardly affected the amount of hydrophilic AMPase. In the absence of detergent, about 50% of the total 5′-nucleotidase can be transformed into a water-soluble form by treatment with PIPLC. Addition of detergent (deoxycholate) resulted in an almost complete sequestration of 5′-nucleotidase activity into the aqueous phase. To prove that PIPLC can only attack the outside-out-orientated 5′-nucleotidase, proteoliposomes were incubated in the absence of chelating agents with purified PIPLC from *B. thuringiensis*, followed by ultracentrifugation and determination of the AMPase activity in supernatants and sediments. Only approx. 50% of the enzymic activity was recovered from supernatants. However, the AMPase activity present in sediments could only be measured after addition of detergent, indicating that the active centre of lipid-associated 5′-nucleotidase faces the interior of lipid vesicles. As expected for tight vesicles, PIPLC recognizes only the PI linker resident on the outer leaflet of the lipid bilayer. Similar results were obtained under otherwise identical conditions with purified PIPLC from *Staph. aureus* (results not shown).

To obtain additional evidence for the release of 5′-nucleotidase from phospholipid vesicles by cleavage of the membrane anchor, proteoliposomes were incubated with PIPLC in the presence of protease inhibitors, followed by ultracentrifugation. Material obtained in sediments and supernatants was further analysed by Western blotting. As shown in Fig. 1 (lane 4), about half of the protein was recovered from the supernatants. This soluble portion of 5′-nucleotidase exhibits a slightly increased electrophoretic mobility with respect to the untreated material or protein co-sedimenting with liposomes. Such a shift in apparent molecular mass after cleavage of the PI anchor has also been reported for other proteins (Davitz *et al.*, 1986). It probably does not result from proteolysis in the vicinity of the *C*-terminal part of the molecule, since this portion of the molecule does not contain preferred target sites for proteolytic attack (U. Stochaj, unpublished work).

Properties of chicken-gizzard 5′-nucleotidase released from proteoliposomes by PIPLC

Comparison of the enzymic properties of chicken gizzard 5′-nucleotidase transformed into a water-soluble form by treatment of either proteoliposomes or detergent-solubilized enzyme with PIPLC revealed only minor changes. As shown in Table 2, there was a 2-fold decrease in the K_m value determined for PIPLC-treated 5′-nucleotidase, a slight increase in $V_{max.}$, as well as increased sensitivity towards the competitive inhibitor ADP. Using different concentrations of a monoclonal antibody, we found an almost identical inhibition of the AMPase activity for both the detergent-solubilized and the PIPLC-treated enzyme. In addition, inhibition of the AMPase activity by adenosine 5′-[$\alpha\beta$-methylene]diphosphate, P^1,P^4-di(adenosine-5′)tetraphosphate and P^1,P^3-di-(adenosine-5′)triphosphate was found to be similar (results not shown).

Table 2. Comparison of purified chicken gizzard 5′-nucleotidase either detergent-solubilized or liberated from proteoliposomes by incubation with PIPLC

Kinetic values were determined for purified detergent-solubilized enzyme and for 5′-nucleotidase liberated from proteoliposomes by incubation with PIPLC of *B. thuringiensis*.

	Enzyme	
	Detergent-solubilized	PIPLC-treated
V_{max}	2.7 μM·min^{-1}·mg^{-1}	4.1 μM·min^{-1}·mg^{-1}
K_m (AMP)	6 μM	3 μM
K_i (ADP)	1.4 μM	0.5 μM
K_i (ATP)	15 μM	14 μM

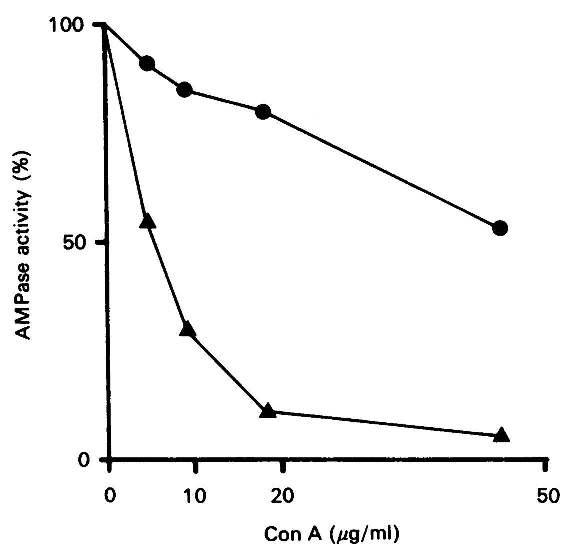

Fig. 2. Incubation of 5′-nucleotidase with concanavalin A

Purified detergent-solubilized 5′-nucleotidase either untreated (●) or incubated with *B. thuringiensis* PIPLC (▲) was incubated for 1 h at 25 °C with different concentrations of concanavalin A (Con A) as indicated in the figure. Residual AMPase activity was determined spectrophotometrically. Controls incubated under identical conditions but without concanavalin A were taken as 100 %.

Concanavalin A is known to inhibit solubilized and reconstituted 5′-nucleotidase (Dieckhoff *et al.*, 1987). Treatment of purified 5′-nucleotidase with *B. thuringiensis* PIPLC, followed by incubation with concanavalin A, revealed a higher sensitivity of the PIPLC-treated enzyme than for the AMPase carrying its hydrophobic membrane-anchor (Fig. 2). Similar results (not shown) were obtained for 5′-nucleotidase treated with PIPLC from *Staph. aureus*.

Liberation of 5′-nucleotidase from plasma membranes

We have analysed the liberation of 5′-nucleotidase from plasma membranes of adult chicken gizzard and a human pancreatic adenocarcinoma cell line containing high concentrations of AMPase activities. Purified plasma membranes from both sources contained about 90 % vesicles in right-side-out orientation. These membrane vesicles were treated with different phospholipases as described for the reconstituted chicken gizzard enzyme. The results given in Table 3 demonstrate that a significant production of hydrophilic AMPase from both membrane preparations is only obtained after incubation with *B. cereus* phospholipase C. As shown in Table 3, treatment with this enzyme liberated only about half of the membrane-bound AMPase activity even after incubation in the presence of detergent. The partial transformation of 5′-nucleotidase into a hydrophilic form does not simply reflect limiting amounts of PIPLC present in the incubation mixture. Tenfold higher concentrations of the enzyme did not result in an increased production of water-soluble 5′-nucleotidase (results not shown). Different preparations of membrane vesicles showed some variation in the amount of 5′-nucleotidase that could be liberated from the plasma membrane with PIPLC or GPI-PLD (see below). However, the maximal production of hydrophilic 5′-nucleotidase was about 70 %, and none of the experiments gave a complete transformation of 5′-nucleotidase into a hydrophilic form.

Table 3. Incubation of plasma membranes obtained from chicken gizzard or human pancreatic adenocarcinoma cells (PaTu II) with different phospholipases

Treatment with phospholipases was carried out as described for Table 1. Amounts of AMPase activity present in the hydrophilic form, assessed as described for Table 1, are expressed as a percentage of the total.

Phospholipase		Hydrophilic AMPase activity (%) Chicken gizzard	PaTu II
A_2 (*Naja naja*)	−	5.0	4.1
	+	3.2	3.2
D (cabbage)	−	3.6	0.9
	+	6.1	1.8
C (*C. perfringens*)	−	3.2	1.3
	+	3.6	1.9
C (*B. cereus*)	−	9.0	3.2
	+	46.5	41.0
C (*B. cereus*)	−	6.5	4.7
+deoxycholate	+	44.2	48.8
C (*B. cereus*)	−	3.5	0.5
+$ZnCl_2$	+	4.8	4.6

Treatment of plasma membranes and purified 5′-nucleotidase with phospholipase D from mammalian sera

Fetal-calf and human serum contain a phospholipase D (GPI-PLD) which can hydrolyse PI linkers of different proteins (Davitz *et al.*, 1987; Cardoso de Almeida *et al.*, 1988). We have tested the accessibility of 5′-nucleotidase, either purified or present in plasma membranes of chicken gizzard and human adenocarcinoma cells, towards GPI-PLD of human and fetal-calf serum. In the absence of detergents, only small amounts of AMPase were liberated from intact proteoliposomes (results not shown) or plasma membranes (Table 4). However, after addition of non-ionic detergents, 5′-nucleotidase from chicken giz-

Table 4. Treatment of 5′-nucleotidase from chicken gizzard or human pancreatic adenocarcinoma cells (PaTu II) with GPI-PLD

Chicken gizzard and human pancreatic adencarcinoma 5′-nucleotidase, either purified and detergent-solubilized or present in plasma membranes, was incubated with fetal-calf serum or partially purified human GPI-PLD (see the Materials and methods section). Cleavage of the PI anchor was monitored by measurement of the transformation of 5′-nucleotidase into a hydrophilic form, as in Table 1. Abbreviation: n.d., not done.

			Hydrophilic AMPase activity (%)			
			Chicken gizzard		PaTu II	
	GPI-PLD	Triton X-100	Purified	Membranes	Purified	Membranes
(*a*) Fetal-calf serum	−	−	n.d.	1.3	n.d.	5.9
	+	−	n.d.	4.0	n.d.	7.9
	−	+	8.5	2.2	1.9	11.8
	+	+	93.4	53.8	90.0	76.4
(*b*) Human serum	−	−	n.d.	6.8	n.d.	8.6
	+	−	n.d.	15.1	n.d.	18.5
	−	+	2.6	13.0	4.3	14.7
	+	+	93.6	50.0	101.7	53.8

zard or adenocarcinoma cells was transformed into a hydrophilic form to a similar extent (approx. 50%), as determined for treatment with *B. cereus* phospholipase C (Table 4). In accordance with previous results (Davitz *et al.*, 1987; Cardoso de Almeida *et al.*, 1988; Low & Prasad, 1988), the transformation of 5′-nucleotidase to a water-soluble form was not affected by inclusion of 1 mM-phenylmethanesulphonyl fluoride. In contrast, addition of EGTA or 1,10-phenanthroline decreased the production of hydrophilic 5′-nucleotidase (Table 5). Moreover, the liberating activity present in human serum was not sensitive to LiCl or low concentrations of dithiothreitol, but was inhibited by $NiCl_2$ (cf. Cardoso de Almeida *et al.*, 1988). These data support the idea that the production of hydrophilic 5′-nucleotidase during treatment with human or fetal-calf serum results from cleavage of the PI linker mediated by GPI-PLD. Similar results to those described for chicken gizzard 5′-nucleotidase (Table 5) were obtained for the AMPase from human pancreatic adenocarcinoma cells (results not shown).

Table 5. Effect of different reagents on the production of hydrophilic 5′-nucleotidase during incubation with fetal-calf or human serum

Purified chicken gizzard 5′-nucleotidase was incubated with fetal-calf or human serum as described for Table 4. Final concentrations of the reagents added to the reaction mixtures are indicated in parentheses. Incubations containing EGTA or 1,10-phenanthroline were carried out in the absence of $CaCl_2$. Before determination of the enzymic activity in the aqueous phase (as in Table 1), these samples were incubated with 40 mM-$CaCl_2$ and 40 mM-$MgCl_2$ for 45 min at room temperature. Abbreviation: n.d., not done.

	Hydrophilic AMPase activity (% of total activity)	
Reagent	Fetal-calf serum	Human serum
None	97.6	95.1
Phenylmethanesulphonyl fluoride (1 mM)	98.3	97.6
EGTA (2.5 mM)	11.6	12.5
1,10-Phenanthroline (250 μM)	0	0
Dithiothreitol (0.1 mM)	n.d.	100.0
$NiCl_2$ (1 mM)	n.d.	97.6
Control without serum	4.8	3.4

Presence of soluble 5′-nucleotidase in chicken gizzard

Anchorage of a protein via a PI linker could indicate that the linker plays an important role in the release of this polypeptide from the membrane. To analyse the presence of hydrophilic 5′-nucleotidase, we prepared crude membranes as well as membrane-free high-speed supernatants from chicken gizzard. As shown in Fig. 3, the M_r 79000 5′-nucleotidase recovered from membrane-free supernatants partitions completely into the aqueous phase after incubation with Triton X-114. Parallel analysis of urea-treated crude membranes reveals only small amounts of the membane-bound AMPase to be hydrophilic. This result supports the idea that chicken gizzard 5′-nucleotidase present in the membrane-free supernatants is devoid of its PI linker. Additional immuno-cross-reactive material of higher M_r can be detected in high-speed supernatants as well as in the crude membrane fraction of chicken gizzard. A prominent hydrophilic protein of M_r 85000 was restricted to the membrane-free fraction of chicken gizzard. In contrast, immuno-cross-reactive material of M_r 120000 could be detected predominantly as hydrophobic membrane-bound protein (Fig. 3). To study the membrane linkage of this M_r-120000 protein, crude membranes were incubated with phospholipase C from *B. cereus*, followed by analysis of water-soluble proteins produced by this treatment. Fig. 4 demonstrates the production of hydrophilic M_r-79000 5′-nucleotidase after incubation with *B. cereus* phospholipase. In contrast, the M_r-120000 protein could not be transformed into a water-soluble form, but partitioned completely into the hydrophobic phase. In addition, a

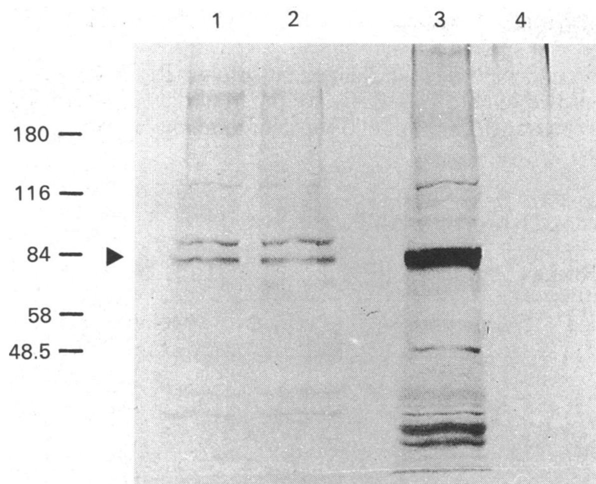

Fig. 3. Distribution of chicken gizzard 5′-nucleotidase in membrane and soluble fractions of the tissue

Chicken gizzard was fractionated into a membrane-free supernatant (lanes 1, 2) and urea-treated membrane vesicles (lanes 3, 4). Equal amounts of protein were separated in lanes 1 and 3. Comparable amounts of hydrophilic proteins present in membrane-free supernatants (lane 2) or urea-treated membrane vesicles (lane 4) were separated in parallel. Hydrophilic proteins were obtained after phase separation with Triton X-114 (see the Materials and methods section). Proteins were detected by Western blotting with a monoclonal antibody directed against the purified M_r-79000 protein (position marked by an arrow). M_r values ($\times 10^{-3}$) of marker proteins are indicated on the left margin.

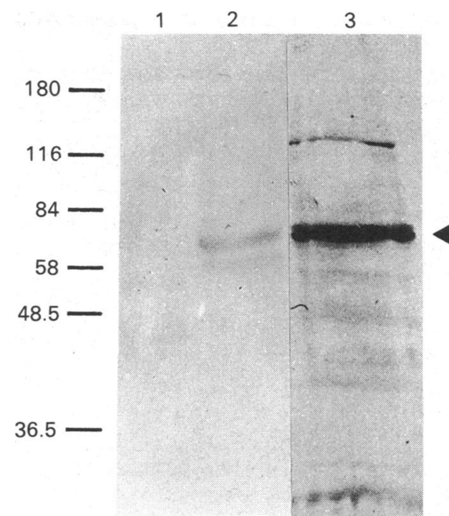

Fig. 4. Incubation of crude membranes with phospholipase C

Crude membranes were incubated with Triton X-114 to remove residual amounts of hydrophilic proteins. Hydrophobic polypeptides recovered after this treatment were incubated with 125 units of *B. cereus* phospholipase C/ml (see the Materials and methods section) in the presence of 0.1% (w/v) deoxycholate. Proteins transformed into a hydrophilic form were obtained after phase separation with Triton X-114 (lane 2). Water-soluble proteins of controls incubated in the absence of phospholipase C are shown in lane 1. Hydrophobic proteins recovered after incubation with phospholipase C are separated in lane 3. Proteins were detected by Western blotting with an affinity-purified polyclonal antibody directed against the purified M_r-79000 chicken gizzard 5′-nucleotidase protein (position marked by an arrow). M_r values ($\times 10^{-3}$) of marker proteins are indicated on the left margin.

portion of the M_r-79000 5′-nucleotidase was recovered as hydrophobic protein, indicating incomplete cleavage of the PI linker. Similar results were obtained for PIPLC from *B. thuringiensis* (not shown).

DISCUSSION

The experiments described above were designed to study the membrane anchorage of plasma-membrane 5′-nucleotidase present in chicken gizzard smooth muscle and a human adenocarcinoma cell line. We demonstrated that at least half of the AMPase activity present in plasma membranes of smooth-muscle or pancreatic-tumour cells can be transformed into a hydrophilic form by incubation with phospholipases hydrolysing PI–glycan linkers.

Comparison of detergent-solubilized chicken gizzard 5′-nucleotidase with the AMPase liberated from proteoliposomes with PIPLC revealed that both forms share similar enzymic properties. The different extent of inhibition by concanavalin A might result from a decreased tendency of the AMPase to associate to high-M_r aggregates after removal of the PI linker. In support of this idea, we found that electrophoresis under non-denaturing conditions revealed a significantly higher electrophoretic mobility of chicken gizzard 5′-nucleotidase after cleavage of its membrane anchor (results not shown). The hydrophobic PI linker might increase the non-covalent interactions between subunits of the enzyme, thereby protecting it against binding of concanavalin A. Similarly, Ogata *et al.* (1988) have reported that the electrophoretic mobility of alkaline phosphatase in native gels, and possibly its hydrophobic interactions, depends on the presence of its PI anchor. It remains to be studied whether the release of 5′-nucleotidase from plasma membranes by cleavage of its PI linker alters the interaction with other high-M_r molecules such as components of the extracellular matrix.

Purified chicken gizzard as well as human pancreatic 5′-nucleotidase can be transformed almost completely into a water-soluble form after removal of their PI anchors by PIPLC or GPI-PLD. In contrast, treatment of membrane vesicles with PIPLC or GPI-PLD released only about half of the AMPase activity even in the presence of detergent. In principle, the partial resistance of 5′-nucleotidase present in plasma membranes to these phospholipases might reflect the presence of different kinds of PI anchors (Ferguson *et al.*, 1988; Menon *et al.*, 1988), which are not recognized by the PI-specific phospholipases used in the present study. However, it seems unlikely that the procedures employed for the purification of 5′-nucleotidase should result in a selective accumulation of a subpopulation sensitive to PIPLC and GPI-PLD, whereas resistant molecules are lost. Analysis of hydrophobic material obtained after incubation of chicken gizzard crude membranes with PIPLC demonstrated the presence of M_r-79000 5′-nucleotidase. The

incomplete release of AMPase activity from plasma membranes might in part be attributed to steric hindrance for the attack of PIPLC and GPI-PLD. Jemmerson & Low (1987) have shown that binding of several monoclonal antibodies to alkaline phosphatase decreases the amount of enzyme accessible to PIPLC. Similarly, interaction of 5′-nucleotidase with components present on the surface of plasma membranes might result in a partial resistance to cleavage of the PI anchor. In addition, one might assume the plasma membrane to harbour additional forms of 5′-nucleotidase anchored to the lipid bilayer by a protein portion of the enzyme rather than by a PI linker. The presence of such heterogeneous forms of membrane proteins is well established for N-CAM, the Q_a2-antigen or the lymphocyte function associated antigen 3 (Rutishauser & Goridis, 1986; Stroynowski *et al.*, 1987; Dustin *et al.*, 1987). The immuno-cross-reactive protein(s) of M_r 120000 present in plasma membranes might represent such isoforms. Under the conditions used in our assays, the M_r-120000 protein(s) was not detected to be transformed into a hydrophilic form after incubation with PIPLC. It will be interesting to characterize this polypeptide(s) further and to analyse its relationship to the M_r-79000 protein. At present, we cannot explain the origin of hydrophilic M_r-85000 protein(s) restricted to the membrane-free fraction of chicken gizzard. This material might represent proteolytic products obtained from the M_r-120000 proteins. Alternatively, one has to consider the possible production of secreted forms of 5′-nucleotidase, which has been observed for other PI-linked proteins such as N-CAM (Gower *et al.*, 1988). After tissue fractionation the M_r-79000 5′-nucleotidase of chicken gizzard was detected as a membrane-bound as well as a soluble form. Similarly, Thompson *et al.* (1987) have reported crude extracts of human placenta to contain significant amounts of 5′-nucleotidase in a non-membrane-bound form. This distribution possibly reflects the liberation of the AMPase into the extracellular space owing to cleavage of its PI linker by endogenous phospholipases. Release of PI-anchored proteins from the cell surface has been reported for several proteins, such as N-CAM 120 (He *et al.*, 1987), a M_r-120000 protein of the central nerve system (Mikol & Stefansson, 1988) or the FcRIII-protein of neutrophils (Huizinga *et al.*, 1988). Several findings seem to indicate that liberation of PI-linked proteins or heparan sulphate proteoglycan can occur after exposure of the cells to different stimuli (Huizinga *et al.*, 1988; Ishihara *et al.*, 1987). It is tempting to speculate that this kind of regulated release can take place also for 5′-nucleotidase exposed on the surface of chicken gizzard smooth-muscle cells or human adenocarcinoma cells. Cleavage of the membrane anchor of chicken gizzard 5′-nucleotidase does not prevent its successful interaction with fibronectin or the laminin/nidogen complex (Stochaj *et al.*, 1989), indicating that 5′-nucleotidase released from the plasma membrane might still bind to components of the extracellular matrix. Assuming a specific liberation of chicken gizzard 5′-nucleotidase from the cell surface, this mechanism might control the adhesive properties of the cell by decreasing the potential binding sites for fibronectin and/or the laminin/nidogen complex.

We thank Mrs. G. Wener and Mr. J. Koch for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

Baron, M. D., Pope, B. & Luzio, J. P. (1986) Biochem. J. **236**, 495–502

Cardoso de Almeida, M. L., Turner, M. J., Stambuk, B. B. & Schenkman, S. (1988) Biochem. Biophys. Res. Commun. **150**, 476–482

Davitz, M. A., Low, M. G. & Nussenzweig, V. (1986) J. Exp. Med. **163**, 1150–1161

Davitz, M. A., Hereld, D., Shak, S., Krakow, J., Englund, P. J. & Nussenzweig, V. (1987) Science **238**, 81–84

Dickson, G., Gower, H. J., Barton, C. H., Prentice, H. M., Elsom, V. L., Moore, S. E., Cox, R. D., Quinn, C., Putt, W. & Walsh, F. S. (1987) Cell **50**, 1119–1130

Dieckhoff, J., Knebel, H., Heidemann, M. & Mannherz, H. G. (1985) Eur. J. Biochem. **151**, 377–383

Dieckhoff, J., Lietzke, R. & Mannherz, H. G. (1986) J. Biol. Chem. **261**, 922–927

Dieckhoff, J., Niggemeyer, B., Lietzke, R. & Mannherz, H. G. (1987) Eur. J. Biochem. **162**, 451–459

Dustin, M. L., Selvaraj, P., Mattaliano, R. J. & Springer, T. A. (1987) Nature (London) **329**, 846–848

Ferguson, M. A. J., Homans, S. W., Dwek, R. A. & Rademacher, T. W. (1988) Science **239**, 753–759

Gower, H. J., Barton, C. H., Elsom, V. L., Thompson, J., Moore, S. E., Dickson, G. & Walsh, F. S. (1988) Cell **55**, 955–964

Grondal, E. J. M. & Zimmermann, H. (1987) Biochem. J. **245**, 805–810

He, H.-T., Finne, J. & Goridis, C. (1987) J. Cell Biol. **105**, 2489–2500

Huizinga, T. W. J., van der Schoot, C. E., Jost, C., Klaassen, R., Kleijer, M., von dem Borne, A. E. G. K., Roos, D. & Tetteroo, P. A. T. (1988) Nature (London) **333**, 667–669

Ikezawa, B. & Taguchi, R. (1981) Methods Enzymol. **71**, 731–741

Ishihara, M., Fedarko, N. S. & Conrad, H. E. (1987) J. Biol. Chem. **262**, 4708–4716

Jemmerson, R. & Low, M. G. (1987) Biochemistry **26**, 5703–5709

Kern, H. F., Bosslet, K., Sedlacek, H. H. & Schorlemmer, H. U. (1988) Pancreas **3**, 2–10

Kwan, C. Y., Lee, R. M. L. W. & Grover, A. K. (1983) Mol. Physiol. **3**, 53–69

Lin, P. H., Selinfreund, R., Wakshull, E. & Wharton, W. (1987) Biochemistry **26**, 731–736

Little, C. (1981) Methods Enzymol. **71**, 725–731

Low, M. G. & Finean, J. B. (1978) Biochim. Biophys. Acta **508**, 565–570

Low, M. G. & Prasad, R. S. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 980–984

Menon, A. K., Mayor, S., Ferguson, M. A. J., Duszenko, M. & Cross, G. A. M. (1988) J. Biol. Chem. **263**, 1970–1977

Mikol, D. D. & Stefansson, K. (1988) J. Cell Biol. **106**, 1273–1279

Newby, A. C. (1988) Biochem. J. **253**, 123–130

Newby, A. C., Luzio, J. P. & Hales, C. N. (1975) Biochem. J. **146**, 625–633

Ogata, S., Hayashi, Y., Takami, N. & Ikehara, Y. (1988) J. Biol. Chem. **263**, 10489–10494

Panagia, V., Heyliger, C. E., Choy, P. C. & Dhalla, N. S. (1981) Biochim. Biophys. Acta **640**, 802–806

Rutishauser, U. & Goridis, C. (1986) Trends Genet. **2**, 72–76

Stochaj, U., Dieckhoff, J., Mollenhauer, J., Cramer, M. & Mannherz, H. G. (1989) Biochim. Biophys. Acta, in the press

Stroynowski, I., Soloski, M., Low, M. G. & Hood, L. (1987) Cell **50**, 759–768
Thompson, L. F., Ruedi, J. M. & Low, M. G. (1987) Biochem. Biophys. Res. Commun. **145**, 118–125
Truong, V. L., Collinson, A. R. & Lowenstein, J. M. (1988) Biochem. J. **253**, 117–121
Worku, Y., Luzio, J. P. & Newby, A. (1984) FEBS Lett. **167**, 235–240
Yeh, E. T. H., Reiser, H., Bamezai, A. & Rock, K. L. (1988) Cell **52**, 665–674
Zachowski, A., Evans, W. H. & Praf, A. (1981) Biochim. Biophys. Acta **644**, 121–126

Received 19 October 1988/6 March 1989; accepted 21 April 1989