Addition of G418 and other aminoglycoside antibiotics to mammalian cells results in the release of GPI-anchored proteins

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Abstract Resistance to the neomycin analogue G418 forms the basis of a dominant marker selection system for mammalian cells transfected with the bacterial neomycin gene. We found that COS-1 cells stably transfected with the neomycin resistance gene had a greater than 50% reduction in cell-associated glycosylphosphatidylinositol (GPI)-anchored alkaline phosphatase (AP). A similarly reduced amount of AP was also observed in wild-type COS-1 cells incubated in the presence of G418 or other aminoglycoside antibiotics. The AP was released from cells into the culture supernatant in its GPI-anchored form. Our data suggest that the G418-induced reduction of AP involves a vesiculation process of COS-1 cells.

Key words: G418 (Geneticin); Alkaline phosphatase; Glycosylphosphatidylinositol; Transfection; COS-1 cell; Human erythrocyte

1. Introduction

Chemical modification of foreign molecules represents one of several mechanisms used by bacteria to evade toxic compounds and forms the basis of bacterial resistance to aminoglycoside antibiotics [1]. These compounds have been proposed to act (in part) by binding to the bacterial 30S ribosomal subunit and initiate a complex series of events leading to cell death [2]. The resistance of bacteria to aminoglycoside antibiotics is mainly the result of production of detoxifying enzymes that fall into three classes named according to the site which they modify on the antibiotic, the *O*-phosphotransferases, the *O*-adenylyltransferases, and the *N*-acetyltransferases [3]. The resulting enzymatically modified aminoglycosides lack the toxic properties since they no longer bind with high affinity to ribosomes.

The aminoglycoside G418 is often used in conjunction with eukaryotic expression vectors encoding genes whose products inactivate G418. Resistance to G418 and other aminoglycoside antibiotics forms the basis of dominant marker selection systems of mammalian (and other) cells transfected with the bacterial neomycin (*neo*) gene. This system has been particularly effective because of the low incidence of spontaneous conversion to G418 resistance in mammalian cells [4]. The mechanism by which G418 exerts its slow toxicity in eukaryotes is thought to involve inhibition of protein synthesis by binding to 80S ribosomes [5]. In addition, some aminoglycosides have been reported to interact with various cellular components, including negatively charged membrane phospholipids [6], inositolphospholipids [7–9], and inositolphospholipid-cleaving phospholipases C [10,11].

In the present study, we report on our recent observation that COS-1 cells stably transfected with the neomycin resistance gene as well as wild-type COS-1 cells incubated in the presence of the aminoglycoside antibiotic, G418, showed reduced amounts of cell-associated alkaline phosphatase (AP) activity. Our results suggest that the AP was released from COS-1 cells into the culture supernatant by G418-induced vesiculation.

2. Materials and methods

2.1. Materials

Unless otherwise stated all reagents were of analytical grade and either from Boehringer (Mannheim, Germany), Fluka (Buchs, Switzerland), Sigma (St. Louis, MO), or Merck (Darmstadt, Germany). Eagle's minimal essential medium (MEM), L-glutamine, penicillin/streptavidin, geneticin (G418), neomycin, kanamycin, gentamycin, and streptomycin were purchased from Gibco BRL (Life Technologies, Basel, Switzerland). Fetal calf serum (FCS) was from Seback GmbH (Aidenbach, Germany) and BACTO trypsin from DIFCO Laboratories (Detroit, MI). Acrylamide stock solution was from National Diagnostics (Hull, UK). Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* (600 U/mg) was from Boehringer.

[^{3}H]Myristate-labeled variant surface glycoprotein (VSG) from bloodstream form trypanosomes was prepared following the procedure of Hereld et al. [12], exactly as described before [13]. The membrane-form of acetylcholinesterase (AChE) was purified from bovine erythrocytes as described by Brodbeck et al. [14]. Concentrated erythrocytes in standard anticoagulant buffer were obtained from the ZLB Central Laboratory, Swiss Red Cross Blood Transfusion Service (Bern, Switzerland).

2.2. Cell cultures

COS-1 cells were kindly provided by Dr. E. Sterchi, University of Bern. Cells were maintained in MEM containing 9% (v/v) FCS (heat inactivated for 30 min at 56°C) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (culture medium). Confluent cells were trypsinized, diluted 1:10 in fresh culture medium and seeded onto new cell culture dishes. Cells were harvested by scraping into 12 ml of ice-cold 0.9% (w/v) NaCl in water, pelleted by brief centrifugation (5 min, 250×*g*), washed once and resuspended in 200 μl of buffer (50 mM Mes, pH 6.5) by sonication for 10 s at 50 W.

2.3. Stable transformation of COS-1 cells

To obtain G418-resistant cell lines, wild-type COS-1 cells were transfected with 1 μg of the vector pXT1 carrying the selectable neomycin resistance gene (Stratagene Cloning System, La Jolla, CA), using the calcium phosphate method [15]. G418-resistant clones were grown in culture medium supplemented with 300 μg/ml of the

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Abbreviations: AChE, acetylcholinesterase; AP, alkaline phosphatase; FCS, fetal calf serum; GPI, glycosylphosphatidylinositol; LDH, lactate dehydrogenase; MEM, Eagle's minimal essential medium; neo, neomycin; PI-PLC, phosphatidylinositol-specific phospholipase C; VSG, variant surface glycoprotein

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aminoglycoside antibiotic G418. The obtained resistant clones were designated *neo*-cells.

2.4. Enzyme activity assays

2.4.1. Alkaline phosphatase. Prior to assaying AP activity, COS-1 cells were grown for at least one passage in MEM containing AP-inactivated FCS [16]. Aliquots of sonicated cells (20 μl) or culture supernatants (50 μl) were added to 150 μl of assay solution containing 1.33 mM diethanolamine (pH 9.8), 0.66 mM $MgCl_2$, and 13.33 mM *p*-nitrophenylphosphate, and assayed in a final volume of 200 μl. Conversion of *p*-nitrophenylphosphate to *p*-nitrophenol was measured spectrophotometrically at 405 nm over a period of 5–10 min at room temperature. In some experiments, AP activity in the cell culture supernatants was measured also after high-speed centrifugation (90 min, 90 000 × *g*).

2.4.2. Lactate dehydrogenase (LDH). LDH activity in wild-type COS-1 cells and *neo*-cells was determined exactly as described [17].

2.4.3. Na^+/K^+-ATPase. Na^+/K^+-ATPase activity in wild-type COS-1 cells and *neo*-cells was measured according to Chignell and Titus [18] and calculated as the difference between P_i produced in the presence and absence of ouabain.

2.5. PI-PLC treatment of endogenous AP

Membrane attachment of endogenous AP via glycosylphosphatidylinositol (GPI) in wild-type COS-1 cells and *neo*-cells was analyzed by its sensitivity towards *B. cereus* PI-PLC. Sonicated cell lysates (20 μl) were added to a cocktail containing 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ (to maintain AP activity), 0.5% (w/v) Triton X-100, and 0.03 U PI-PLC. The reaction mixture (50–80 μl final volume) was incubated for 3–4 h at 37°C and the enzymatic reaction was stopped by addition of 400 μl 4% (w/v) Triton X-114. Control samples contained no PI-PLC. PI-PLC-mediated hydrolysis of the GPI anchor of AP was assayed by measuring the distribution of AP activity between aqueous and detergent phase after phase partitioning at 37°C [19]. GPI anchoring of the AP present in the cell culture supernatant was analyzed under identical conditions using 50 μl of culture supernatant.

2.6. Assay for GPI-hydrolyzing activity in COS-1 cells

Aliquots (20 μl) of sonicated cell pellets of wild-type COS-1 cells and *neo*-cells grown in the absence or presence of 750 μg/ml G418 were assayed for endogenous GPI-hydrolyzing activity using purified AChE (400 U/ml) or [^{3}H]myristate-labeled VSG (4000–5000 cpm) as substrates, exactly as described before [16].

2.7. Treatment of human erythrocytes with G418 and other aminoglycoside antibiotics

Human erythrocytes (2 ml) were pelleted by centrifugation, washed three times with 0.9% (w/v) NaCl, and resuspended in 5 ml of incubation buffer (10 mM Tris-HCl (pH 7.4), 144 mM NaCl, 12.7 mM inosine, 0.54 mM adenine, 2 g/l glucose) supplemented with from 750 μg/ml to 7.5 mg/ml of aminoglycoside antibiotic. After incubation of the cells at 37°C under constant gentle shaking, aliquots were removed and erythrocytes were pelleted by centrifugation (30 s, 6000 rpm) in a microcentrifuge. Total AChE activity in the suspension and in the vesicle-containing supernatant after pelleting the red blood cells was determined following the procedure of Ellmann et al. [20], as described before [21]. Erythrocyte-derived vesicles in the supernatant were pelleted by a subsequent centrifugation step (15 min, 13 000 rpm) in a microcentrifuge. Erythrocyte morphology was examined by light microscopy of cells fixed in 3% (v/v) glutaraldehyde. Erythrocyte ghosts were prepared from washed cells by hypotonic lysis in 10 mM Tris-HCl (pH 7.4), containing 2 mM EDTA.

2.8. Protein analysis

Protein was determined using the BCA reagent kit (Pierce Chemicals, Rockford, IL) with bovine serum albumin as standard. Erythrocyte ghosts and vesicles were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions using 5–15% polyacrylamide gradient gels [22].

3. Results and discussion

Unless otherwise stated, cells were grown in culture medium containing AP-inactivated serum for at least one passage before experiments were started.

3.1. AP in stably transfected cells

In previous studies involving stably transfected COS-1 cells we observed that, independent of the expressed foreign protein, the amounts of endogenous AP were decreased compared to wild type, i.e. non-transfected, cells (results not shown). Since these cell lines all carried the neomycin resistance gene and were cultured in the presence of the aminoglycoside antibiotic G418, we hypothesized that the reduction in cell-associated AP in stably transfected COS-1 cells is a result of the product of the resistance gene and/or the presence of G418 in the culture medium. To study this question we stably transfected COS-1 cells with the vector pXT1 carrying the selectable neomycin resistance gene to generate a cell line (designated *neo*-cells) that is resistant to G418 but otherwise does not express any other foreign protein. When we analyzed these *neo*-cells grown in the presence of 300 μg/ml G418, we found that the amount of cell-associated AP was reduced to 50–60% of control wild-type COS-1 cells. A direct inhibitory effect of G418 on the activity of AP could be excluded since

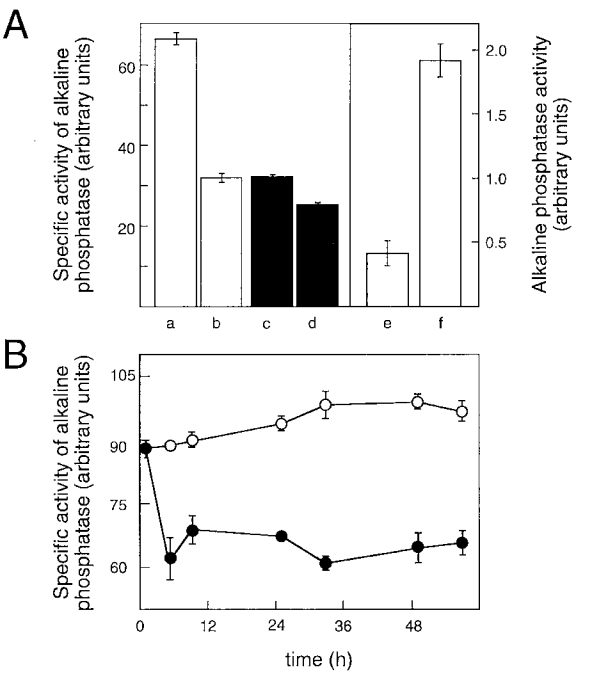

Fig. 1. Effects of G418 addition on AP activity in COS-1 cell cultures. A: Wild-type COS-1 cells (open bars) and *neo*-cells (solid bars) were grown to confluence in 6 cm culture dishes. Subsequently, the culture supernatants were replaced by fresh medium and the cells were cultured for 24 h in the absence (bars a,c) or presence of 750 μg/ml G418 (bars b,d). The specific activity of AP in washed cell pellets (bars a-d) is expressed as mean values of triplicate determinations ± SD from two separate experiments. The AP activity in the culture supernatants of wild-type COS-1 cells cultured in the absence (bar e) or presence (bar f) of 750 μg/ml G418 are expressed as mean values of triplicate determinations ± SD from a typical experiment. B: Confluent wild-type COS-1 cells were cultured for 56 h in the absence (open circles) or presence of 750 μg/ml G418 (closed circles) and the specific activity of AP in the washed cell pellets was measured at the indicated time points. The values represent the means ± SD of triplicate determinations from a typical experiment. Similar results were obtained in two separate experiments.

its presence in the assay did not affect the AP enzymatic activity (results not shown). Interestingly, the amounts of AP were also found to be decreased in other stably transfected mammalian cell lines. In human carcinoma HT-29 cells and Madin Darby canine kidney (MDCK) cells expressing different foreign proteins, cell-associated AP was reduced to 30–70% of the respective control wild-type cells.

3.2. Effects of G418 and other aminoglycoside antibiotics on COS-1 cells

To further study the reduction of AP activity in COS-1 cells, we treated wild-type COS-1 cells and *neo*-cells with G418 and several other aminoglycoside antibiotics. We found that the cell-associated AP activity in wild-type COS-1 cells grown for 24 h in the presence of 750 μg/ml G418 was reduced to less than 50% of control untreated cells (Fig. 1A, bars a,b). A similarly reduced AP activity was also observed in *neo*-cells grown for more than 4 months in the constant presence of 300 μg/ml G418 (Fig. 1A, bar c). A further reduction in AP activity was observed in these *neo*-cells after increasing the concentration of G418 in the medium from 300 μg/ml, i.e. the typical concentration we used to culture *neo*-resistant COS-1 cells, to 750 μg/ml (Fig. 1A, bar d). Together with the decrease in AP activity in wild-type COS-1 cells we found that the AP activity in the culture supernatant increased (Fig. 1A, bars e,f), indicating that AP was released from the cells into the medium.

In addition, all the other aminoglycosides tested also caused a reduction in AP activity when added to wild-type COS-1 cells in culture. Gentamycin, a close structural analogue to G418, showed a similar AP reduction as G418, while neomycin, kanamycin, and in particular streptomycin, were somewhat less effective in reducing cell-associated AP activity (Table 1; the final concentrations of all antibiotics in the culture media were 750 μg/ml).

As mentioned above, G418 (and other aminoglycoside antibiotics) has been proposed to inhibit protein biosynthesis in mammalian cells by binding to the 80S ribosomes [5,23]. In order to study if G418 reduced the amount of cell-associated AP in COS-1 cells by inhibition of protein biosynthesis, we measured the activities of two other enzymes, i.e. cytosolic LDH and membrane-bound Na^+/K^+-ATPase, in COS-1 cells. We found that the activities of both enzymes in wild-type COS-1 cells grown for 24 h in the presence of 750 μg/ml G418 were unchanged compared to control untreated cells (results not shown). Similarly, the LDH and Na^+/K^+-ATPase activities in *neo*-cells were identical to wild-type COS-1 cells (results not shown).

Table 1
Reduction of AP activity in wild-type COS-1 cells in the presence of different aminoglycoside antibiotics

Antibiotic added	AP activity (% reduction compared to wild-type cells)
G418 (Geneticin)	42–61[a]
Gentamycin	55/63[b]
Neomycin	40/42[b]
Kanamycin	35/42[b]
Streptomycin	19/29[b]

Confluent wild-type COS-1 cells were grown for 24 h in the presence of different aminoglycoside antibiotics (750 μg/ml final concentration) and the activity of AP in the washed cell pellets was measured as described in Section 2.
[a]Range of 13 independent experiments.
[b]Values from two independent experiments.

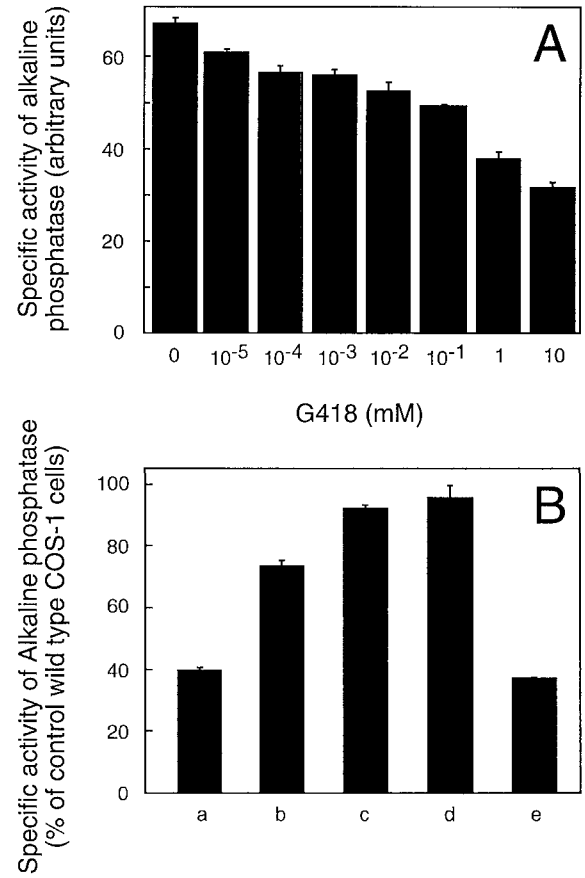

Fig. 2. A: Concentration-dependent decrease of AP specific activity in G418-treated COS-1 cells. Confluent wild-type COS-1 cells were incubated for 24 h in the absence or presence of increasing concentrations of G418 (in mM), and the specific activity of AP in the washed cell pellets was determined. B: AP activity in *neo*-cells cultured for several passages in the absence of G418. *Neo*-cells (bar a) were cultured for 26 weeks (26 passages; 1:10 dilution steps) in the absence of G418 and the specific activity of AP in the washed cell pellets was determined after 7, 12, and 26 passages (bars b, c, d, respectively). Control *neo*-cells were incubated in the presence of 300 μg/ml G418 for 26 passages (bar e). The data represent the mean values ± SD of triplicate determinations from a single experiment.

Together, these results show that G418 and other aminoglycoside antibiotics cause a reduction in cell-associated AP activity in COS-1 cells. Since the G418-induced reduction in AP activity was not only observed in *neo*-cells but also in wild-type COS-1 cells, it was not the result of the expression of the neomycin resistance gene product (i.e. a phosphotransferase) but of the presence of G418 in the culture medium. In addition, the reduced AP activity in G418-treated COS-1 cells was not due to a general effect of G418 on protein synthesis in COS-1 cells since other cell enzymes were not affected by the treatment.

3.3. Time- and concentration-dependent effects of G418 on AP activity in wild-type COS-1 cells

In order to further characterize the G418-induced decrease of cell-associated AP, wild-type COS-1 cells were cultured in the presence of G418 for 56 h and cells and culture media were assayed for AP activity. We found that the AP activity in

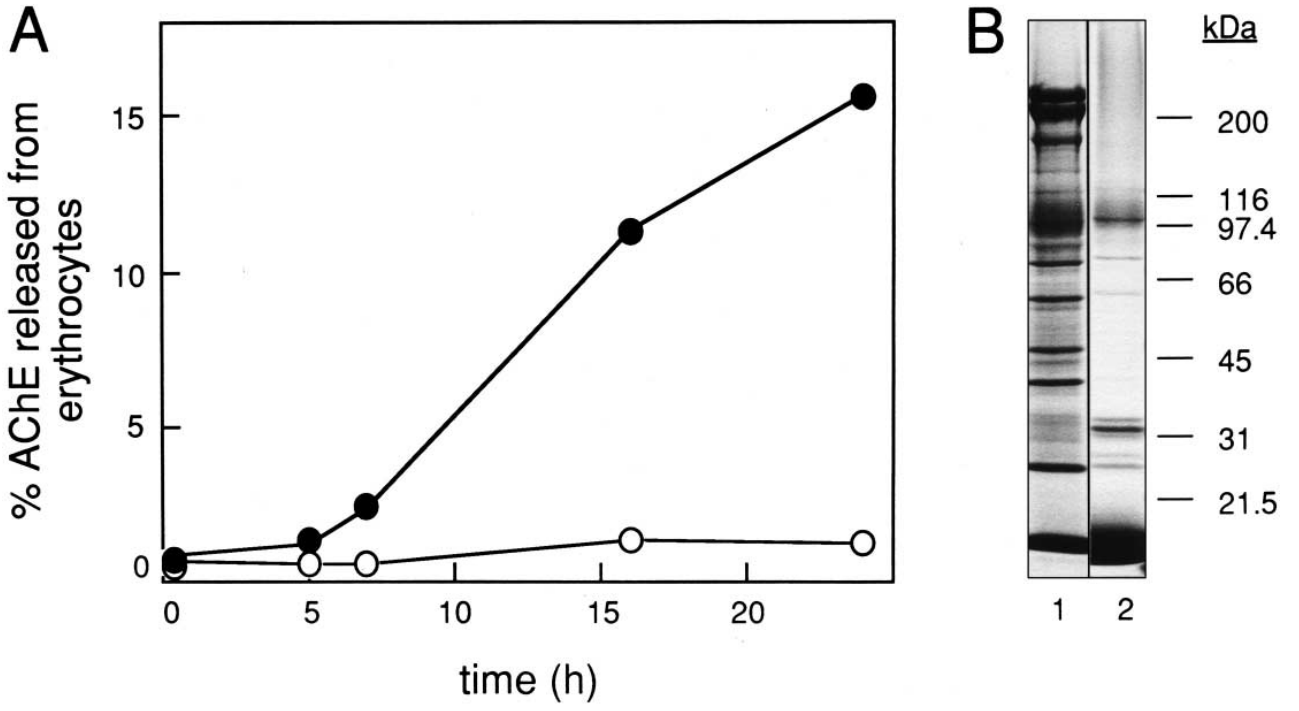

Fig. 3. A: Time-dependent release of AChE from human erythrocytes. Washed human erythrocytes were incubated for 24 h in the absence (open circles) or presence of 7.5 mg/ml G418 (closed circles). At indicated time points erythrocytes were pelleted by centrifugation and the AChE activity in the vesicle-containing supernatant was measured and expressed as the percentage of the total activity in the suspension before centrifugation. The values represent the means of triplicate determinations from a typical experiment. Similar results were obtained in two separate experiments. When lower concentrations of G418 (750 μg/ml to 3.25 mg/ml) were used, the onset of vesiculation was delayed and the extent of AChE release was decreased (results not shown). B: Protein composition of erythrocyte-derived vesicles. Human erythrocyte ghosts (lane 1) and vesicles released from erythrocytes after treatment with G418 for 24 h (lane 2) were analyzed by SDS-PAGE and staining of proteins with Coomassie brilliant blue. Spectrin migrates as a doublet of non-identical subunits with apparent molecular masses of 260 and 246 kDa. Note that AChE is not detectable by protein staining due to its low copy number in human erythrocytes.

the cell pellets rapidly decreased after addition of G418 and remained constant over the entire period of the experiment (Fig. 1B). The reduction in cell-associated AP activity was observed regardless of the time of G418 addition to the COS-1 cells and could not be reversed by a subsequent removal of G418 from the culture medium (results not shown; see also below). Longer incubation times could not be applied since the wild-type COS-1 cells were not viable after 3 days in culture in the presence of G418.

Table 2
Release of AChE from human erythrocytes in the presence of different aminoglycoside antibiotics

Antibiotic added	AChE activity (% of total activity in the suspension)	
	in supernatant 1	in supernatant 2
G418 (Geneticin)	11.8–16.3[a]	0.6[c]
Gentamycin	14.8[b]	1.9[b]
Neomycin	13.1[b]	0.7[b]
Kanamycin	14.8[b]	1.8[b]
Streptomycin	1.8[c]	0.3[c]
no antibiotic	1.2–2.0[a]	0.3[c]

Human erythrocytes were incubated at 37°C in the absence or presence of different aminoglycoside antibiotics (all at a final concentration of 7.5 mg/ml). After 24 h of incubation, the suspensions were pelleted by low-speed centrifugation and the AChE activity in the cell-free supernatant was measured before (supernatant 1) and after (supernatant 2) high-speed centrifugation to pellet the erythrocyte-derived vesicles. The total AChE activity in the suspension stayed constant during the incubation period and was not affected by the presence of the aminoglycoside antibiotics.
[a]Range of seven independent experiments.
[b]Single determinations.
[c]Mean from two independent experiments.

In several mammalian cell types, AP has been shown to be anchored to the plasma membrane via covalently attached GPI. Accordingly, we found that more than 70% of AP in wild-type COS-1 cells was susceptible to PI-PLC treatment indicating that the endogenous AP in COS-1 cells is membrane-anchored via GPI. In addition, we noticed that the AP in the culture supernatant from G418-treated COS-1 cells quantitatively partitioned into the detergent phase after phase partitioning in Triton X-114. In contrast, after treatment of the supernatant with bacterial PI-PLC, more than 55% of the AP activity was recovered in the aqueous phase. These results demonstrate that the AP released from COS-1 cells into the supernatant contained a GPI anchor. This observation was important since it had previously been reported that G418 can activate PI-PLCs capable of cleaving protein GPI anchors [24]. However, we were unable to detect such an activity in G418-treated COS-1 cells using either endogenous AP or exogenously added GPI-anchored AChE or VSG as substrates (results not shown). Together these results exclude the possibility that the appearance of the AP in the culture supernatant resulted from a possible (transient) activation of a GPI-hydrolyzing phospholipase in COS-1 cells.

Usually, stably transfected COS-1 cells are maintained in culture in the presence of 300–500 μg/ml G418. In order to study the effects of different concentrations of G418 on cell-

associated AP activity, we incubated wild-type COS-1 cells in the presence of 7.5 ng–7.5 mg/ml (equaling 10 nM–10 mM) G418 for 24 h. Our results showed that the reduction in cell-associated AP activity strictly correlated with the increase in the concentration of G418 in the culture medium (Fig. 2A). At a very low concentration of 100 nM G418, the AP activity in COS-1 cells was reduced by ~10% whereas a ~50% reduction was observed in the presence of 10 mM G418 in the culture medium.

In order to study if the G418-induced reduction in cell-associated AP activity was reversible, we cultured *neo*-cells in the absence of G418 over a period of 26 weeks (Fig. 2B). As mentioned above, *neo*-cells grown in the presence of 300 μg/ml G418 showed a reduction in AP activity of more than 50% compared to wild-type COS-1 cells (Fig. 2B, bar a). When these cells were subsequently cultured in the absence of G418, the cell-associated AP activity slowly increased to ~70% of control wild-type cells after 7 passages (Fig. 2B, bar b). An almost complete recovery of AP activity in *neo*-cells to 92% and 95% of control wild-type COS-1 cells was observed after 12 and 26 passages, respectively, in the absence of G418 (Fig. 2B, bars c,d). In contrast, in control *neo*-cells cultured for the same period of time in the presence of G418, the AP activity remained unchanged at less than 50% of control wild-type cells (Fig. 2B, bar e). If G418 was added back to *neo*-cells grown for 12 passages in the absence of the antibiotic, the AP activity immediately decreased again to less than 50% of control wild-type cells (results not shown). Thus, in order to restore the cell-associated AP activity in *neo*-cells to wild-type COS-1 cell levels, the cells need to be cultured for at least 12 passages in the absence of G418.

Together these findings demonstrate that the addition of G418 to COS-1 cells in culture results in a rapid release of cell-associated AP into the culture supernatant. Interestingly, we found that the GPI-anchored AP in the supernatant could be quantitatively pelleted by high-speed centrifugation (results not shown), suggesting that it may be associated with membranous material that was released from COS-1 cells together with the AP. Unfortunately, the limited amount of material obtained from the supernatants did not allow a detailed analysis of its composition.

3.4. *Effect of different aminoglycoside antibiotics on human erythrocytes*

It has been reported that human erythrocytes can be induced to release membrane vesicles enriched in GPI-anchored proteins. Depletion of intracellular ATP stores [25], loading with calcium [26], and incubation in the presence of dimyristoylphosphatidylcholine [21] all lead to dramatic shape changes of human erythrocytes followed by a release of vesicles highly enriched in the GPI-anchored proteins AChE [21,25,26], decay accelerating factor [27], and membrane inhibitor of reactive lysis [28]. The extent of vesiculation could be modulated by a series of amphiphilic compounds that intercalate into the plasma membrane [21]. Since the vesiculation process of human erythrocytes has been relatively well characterized, we decided to use human erythrocytes as a model system to study if the G418-induced release of GPI-anchored AP from COS-1 cells may involve a vesiculation event.

Human erythrocytes release vesicles after prolonged incubation in nutrient-poor buffer leading to ATP-depletion of the cells. In contrast, in the presence of glucose, inosine, and adenine, vesiculation is virtually inhibited [25]. The released vesicles are enriched in GPI-anchored AChE and, in addition, contain several membrane-bound proteins but are virtually devoid of the major skeletal protein, spectrin [25]. Interestingly, we found that when human erythrocytes were incubated in the presence of G418 under conditions to maintain intracellular ATP levels, they released AChE-containing vesicles into the cell-free supernatant (Fig. 3A). After 24 h of incubation at 37°C, 12–16% of total AChE activity in the suspension was recovered in the vesicle fraction whereas less than 2% of AChE activity was released from erythrocytes incubated in the absence of G418. The release of AChE from human erythrocytes was also observed after the addition of other aminoglycoside antibiotics to the cells (Table 2). While the extent of AChE release in the presence of gentamycin, neomycin, and kanamycin was similar to that observed in the presence of G418, little release of AChE was observed after the addition of streptomycin to human erythrocytes (Table 2). The isolated G418-induced vesicles showed the typical protein pattern of erythrocyte-derived vesicles, notably the complete absence of the major skeletal protein, spectrin (Fig. 3B; see also ref [25]).

In summary, we found that the addition of G418 and other aminoglycoside antibiotics to COS-1 cells in culture resulted in a reduction of cell-associated endogenous GPI-anchored AP. In wild-type COS-1 cells this decrease could not be reversed by short-term removal of G418 from the incubation medium. In contrast, in *neo*-cells the AP activity could be restored to normal, i.e. wild-type COS-1 cell levels after at least 12 continuous cell passages in the absence of G418. The AP present in the culture supernatant was shown to contain a GPI anchor and could be pelleted by centrifugation suggesting that it was associated with membranous material released from COS-1 cells. Since the aminoglycoside antibiotics were found to induce a release of vesicles enriched in GPI-anchored AChE from human erythrocytes, we speculate that the G418-induced release of GPI-anchored AP from COS-1 cells may also occur via vesiculation. Furthermore, since it has been shown that erythrocyte-derived vesicles are not only enriched in AChE [21,25,26] but also other GPI-anchored proteins [27,28], it is possible that additional GPI-anchored proteins besides AP are released from COS-1 cells after treatment with the aminoglycoside antibiotics. Unfortunately, the activity of 5′-nucleotidase which represents another GPI-anchored cell surface enzyme often studied in mammalian cells, was below the limit of detection in wild-type COS-1 cells and could not be used as an additional marker for the release of GPI-anchored proteins (results not shown). At present, the mechanism of how the aminoglycoside antibiotics induce vesiculation in mammalian cells is completely unclear.

Resistance to aminoglycoside antibiotics forms the basis of a widely used dominant selectable marker system in animal [29,30] and plant [31] cells. Over the past years, a number of G418-resistant cell lines have been used to study the expression and sorting of GPI-anchored proteins [32–34]. Although our present findings may not have direct implications for these reports, they at least strongly suggest that the results from studies involving mammalian cells cultured in the presence of G418 (or other aminoglycoside antibiotics) need to be interpreted with great caution, in particular if they relate to GPI-anchored proteins. The possibility that the presence of

G418 causes the cells to release vesicles enriched in GPI-anchored proteins has to be considered and should be accounted for by using G418-resistant cells cultured for at least 12 passages in the absence of any antibiotic. Alternatively, the results from G418-resistant cells expressing a given protein to be studied should be compared with mock transfected, i.e. G418-resistant cells, and *not* with wild-type cells as it is usually done. Since a G418-induced reduction in cell-associated AP was not only found in COS-1 cells but also in HT-29 and MDCK cells, our findings may be applicable to cultured (mammalian) cells in general.

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