The membrane-anchoring systems of vertebrate acetylcholinesterase and variant surface glycoproteins of African trypanosomes share a common antigenic determinant

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Amphiphilic detergent-soluble acetylcholinesterase (AChE) from *Torpedo* is converted to a hydrophilic form by digestion with phospholipase C from *Trypanosoma brucei* or from *Bacillus cereus*. This lipase digestion uncovers an immunological determinant which crossreacts with a complex carbohydrate structure present in the hydrophilic form of all variant surface glycoproteins (VSG) of *T. brucei*. This crossreacting determinant is also detected in human erythrocyte AChE after digestion with *T. brucei* lipase. From these results we conclude that the glycophospholipid anchors of protozoan VSG and of AChE of the two vertebrates share common structural features, suggesting that this novel type of membrane anchor has been conserved during evolution.

Acetylcholinesterase *Phospholipase C* *Surface glycoprotein* (Trypanosoma brucei) *Crossreacting determinant* *Membrane anchoring*

1. INTRODUCTION

The membrane-bound, amphiphilic form of AChE can be solubilized with detergent, by proteolytic treatment and by the action of PI-specific phospholipase C (review [1]). This has been shown with AChE from *Torpedo* electric organ as well as from red cell membranes. The release of AChE by PI-specific phospholipase C suggests that it is anchored to the membrane via a direct and specific interaction with PI [2,3]. A completely different type of protein, the variant surface glycoproteins (VSGs) of African trypanosomes which are responsible for antigenic variation, seems to be attached to the membranes in a similar manner. They contain at the C-terminus a common glycophospholipid composed of ethanolamine, mannose, glucosamine, galactose, phosphate and dimyristyl-PI which anchors the VSGs to the plasma membrane [4,5]. Following cell lysis an endogenous phospholipase C cleaves dimyristylglycerol from the glycophospholipid and converts the membrane-bound form of VSG into its soluble form [4,5]. This event exposes an antigenic crossreacting determinant on the carbohydrate moiety which is common to all soluble VSGs but is cryptic on the respective membrane form [4,6,7].

The aim of the present study was to determine whether phospholipase C from *T. brucei* could convert amphiphilic DS-AChE into its hydrophilic form and if the crossreacting determinant could then be detected on vertebrate AChE. Such fin-

Abbreviations: DS-AChE, detergent-soluble acetylcholinesterase; VSG, variant surface glycoprotein; PI, phosphatidylinositol

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dings would strongly support the existence of a common membrane-anchoring system for these two genetically distant membrane proteins.

2. MATERIALS AND METHODS

DS-AChE and proteinase K solubilized AChE from *Torpedo marmorata* were purified according to [8] and DS-AChE from human erythrocyte membranes as described in [9]. AChE activity was monitored according to Ellman et al. [10], membrane form VSG (ILTat 1.25) was prepared as described [7]; phospholipase C type III from *B. cereus* was from Sigma. Phospholipase C from MITat 1.6 *T. brucei* trypanosomes (5×10^{10}) was prepared by hypotonically lysing the cells and inducing maximum VSG release. After centrifugation at $100\,000 \times g$ the pelleted material was resuspended in 15 ml of precondensed 2% Triton X-114 in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) at 0°C and centrifuged at $100\,000 \times g$ for 1 h at 4°C. The supernatant was subjected to phase separation [11]. The lipase activity was essentially recovered in the detergent phase containing 12% Triton X-114. It was kept at −20°C until required. Antibodies directed against the crossreacting determinant of soluble VSGs of *T. brucei* were raised in rabbits and purified as described in [4]. To remove a small population of antibodies which could bind the membrane form VSG, the preparation was then applied to MITat 1.6 membrane form VSG immobilized on Sepharose 4B and the flow through of this column was used in the experiments.

Incubation with phospholipase C was performed in TBS containing 0.08% Triton X-100 for 60 min at 27°C in a final volume of 20 μl. Thereafter 200 μl of a 4% solution of Triton X-114 in TBS were added and the phase separation was effected at 37°C [11]. Immunoblots of the same sheets were revealed after overnight incubation with 2 μg/ml of antibodies against the crossreacting determinant followed by protein A-peroxidase conjugate (Sigma) [12,13].

3. RESULTS

We have purified detergent soluble, amphiphilic AChE from the electric organ of *Tor. marmorata* and from human red cell membranes and subjected them to treatment with phospholipase C from *B. cereus* and *T. brucei*. The conversion of the amphiphilic form of AChE to a hydrophilic one was assessed by phase separation using Triton X-114 [11]. In this system amphiphilic proteins are recovered in the detergent phase whereas hydrophilic ones partition into the aqueous phase. Fig.1

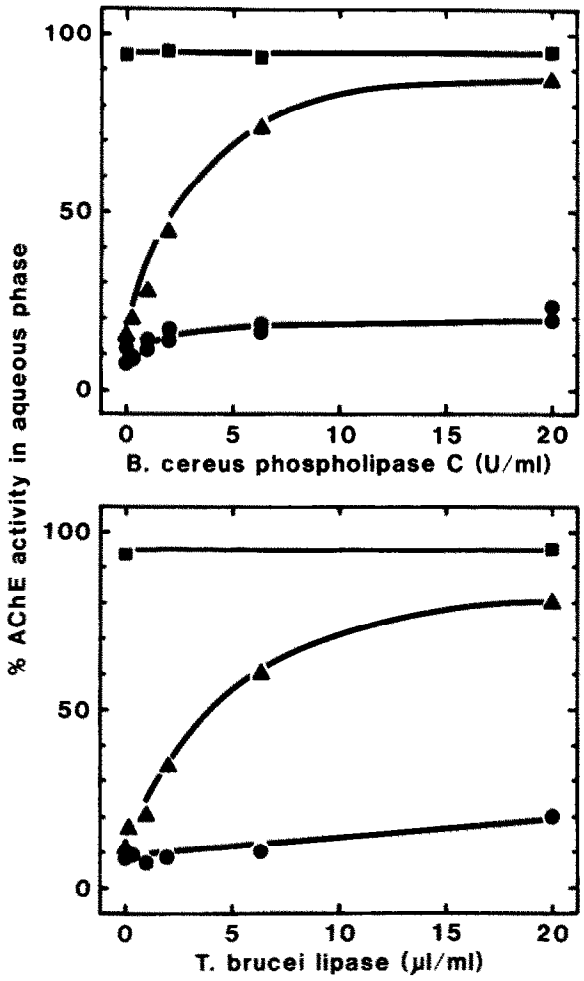

Fig.1. Conversion of amphiphilic DS-AChE to the hydrophilic form by phospholipase C assessed by phase separation in Triton X-114. Pure DS-AChE from *Tor. marmorata* (▲) and from human erythrocyte membranes (●) were incubated with various concentrations of phospholipase C from *B. cereus* (top) and *T. brucei* (bottom). After 60 min at 27°C, the samples were subjected to phase separation in Triton X-114 and the partition of AChE activity was measured. The sum of activities in both phases remained constant. Proteinase K solubilized, hydrophilic AChE from *Tor. marmorata* served as a control (■).

shows that increasing amounts of phospholipase C from *B. cereus* as well as from *T. brucei* progressively converted amphiphilic DS-AChE from *Torpedo* to a catalytically active hydrophilic enzyme. On the other hand, the enzyme from human red cell membranes was less sensitive to phospholipase C treatment as in similar conditions, only a small increase in AChE activity in the aqueous phase was obtained. Proteinase K solubilized, hydrophilic AChE from *Torpedo* served as control. This form of the enzyme was always recovered in the aqueous phase independent of the absence or presence of phospholipase C in the incubation medium. Fig.2 shows the time course of conversion of the amphiphilic DS-AChE to the hydrophilic form by phospholipase C from *T. brucei*. In our conditions about 80% of DS-AChE from *Torpedo* became hydrophilic within 24 h incubation. On the other hand, only a small amount of DS-AChE from human erythrocyte membranes was converted to a form that partitioned into the aqueous phase although the concentration of phospholipase C was 6-fold higher than in the experiment with DS-AChE from *Torpedo*.

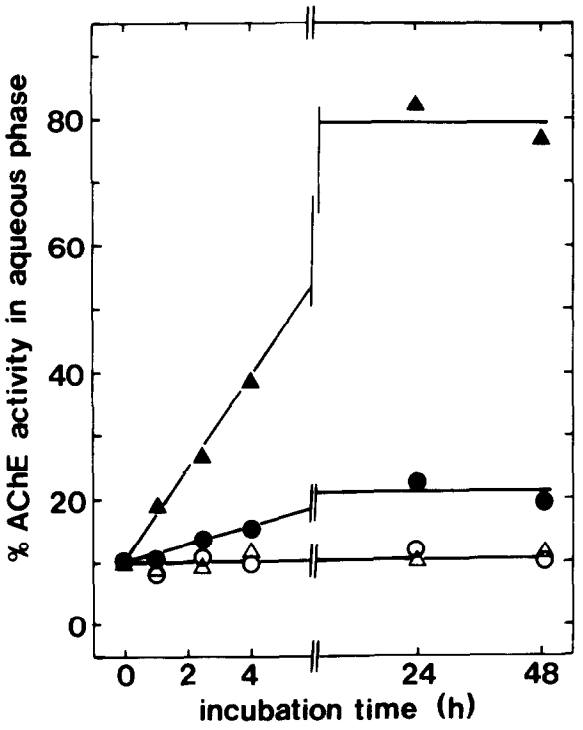

Fig.2. Time course of conversion of amphiphilic DS-AChE to the hydrophilic form by phospholipase C from *T. brucei*. DS-AChE from *Tor. marmorata* was incubated without (△) and with 1.6 μl/ml phospholipase C (▲); DS-AChE from human erythrocyte membranes without (○) and with 10 μl/ml phospholipase C (●). At the times indicated aliquots were subjected to phase separation in Triton X-114 and the partition of AChE activity was measured. The sum of activities in both phases remained constant.

Different samples of AChE were incubated without and with *B. cereus* or *T. brucei* phospholipase C, thereafter analyzed by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Fig.3 (top) shows the protein stain of the blot. The blot was then destained and the exposure of the crossreacting determinant on the different proteins was revealed with anti-crossreacting determinant antibodies (fig.3, bottom). Lane 1 shows that native DS-AChE from *Torpedo* did not react with antibodies against the crossreacting determinant. However, treatment with phospholipase C from *B. cereus* (lane 2) as well as with phospho-

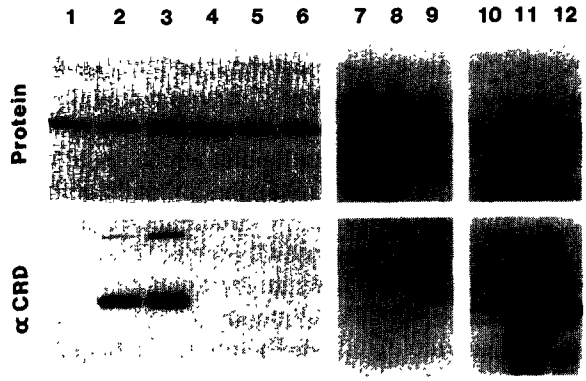

Fig.3. Disclosure of the crossreacting determinant on hydrophilic AChE following phospholipase C treatment. Amphiphilic DS-AChE (lanes 1-3) and proteinase K solubilized, hydrophilic AChE from *Tor. marmorata* (lanes 4-6), DS-AChE from human erythrocyte membranes (lanes 7-9) and membrane form VSG (ILTat 1.25) from *T. brucei* (lanes 10-12) were incubated in absence (lanes 1,4,7,10) or presence of phospholipase C from *B. cereus* (lanes 2,5,8,11) or phospholipase C from *T. brucei* (lanes 3,6,9,12). The samples were then subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and thereon stained for protein with Ponceau S (top). Bottom, corresponding immunoblot obtained by incubation with antibodies against the crossreacting determinant of soluble VSG of *T. brucei*.

lipase C from *T. brucei* (lane 3) yielded a form of AChE that crossreacted with these antibodies. On the other hand, proteinase K-treated AChE from *Torpedo* did not crossreact independent of the absence (lane 4) or presence of phospholipase C (lanes 5 and 6) in the incubation mixture. Lane 7 shows native AChE from human red cell membranes prior to treatment with phospholipase C. It also revealed the crossreacting determinant after treatment with phospholipase C from *T. brucei* (lane 9) but no reaction could be detected after treatment with phospholipase C from *B. cereus* (lane 8). VSG from *T. brucei* served as control. In the absence of phospholipase C only a faint band in the immunoblot was obtained (lane 10) whereas a strong reaction was observed after treatment with the two phospholipases C (lanes 11 and 12).

4. DISCUSSION

The glycophospholipid which anchors the membrane form VSGs to the membrane of *T. brucei* is cleaved by the action of an endogenous phospholipase C thereby converting the membrane form VSG to the soluble VSG and concomitantly exposing the crossreacting determinant [4,6,7]. The membrane-bound amphiphilic form of AChE is solubilized by PI-specific phospholipase C from different sources [14–16]. In addition other membrane proteins such as alkaline phosphatase, 5′-nucleotidase [14], Thy-1 glycoprotein [17] and p63 from *Leishmania major* [18] are also converted to a hydrophilic form by PI-specific phospholipase C. It appears likely that the attachment of these different proteins to membranes involves a common anchoring mechanism, i.e. a glycophospholipid covalently attached to the protein moiety [19].

Our results show that *Torpedo* DS-AChE is efficiently transformed into a hydrophilic molecule by the action of *T. brucei* phospholipase C. Human erythrocyte AChE, on the other hand, is a poor substrate for the enzyme. The difference in susceptibility may be due to a specificity of the amphiphilic *T. brucei* lipase for the length of the fatty acids of the lipid anchor which are different in the erythrocyte AChE [20] and in the membrane form VSG [5]. Alternatively, a slightly different or heterogenous crossreacting determinant or a different accessibility of the cleavage site to phospholipase C could also influence the rate and the extent of the reaction. The results obtained with proteinase K-digested DS-AChE from *Torpedo* suggest that the 3 kDa hydrophobic entity removed by proteinase K [21] contained the phospholipid and the crossreacting determinant. The presumed C-terminal location of this moiety is consistent with the known location of the glycophospholipid in the membrane form VSG [6].

In contrast to Ferguson et al. [5] who failed to show that the crossreacting determinant was exposed on AChE from *Torpedo* after digestion with PI-specific phospholipase C from *Staphylococcus aureus,* we found that the anti-crossreacting determinant antibodies reacted strongly with AChE after digestion with both *T. brucei* and *B. cereus* phospholipase C. The same result was obtained with human erythrocyte AChE digested with *T. brucei* lipase indicating that an analogous glycophospholipid can also be found in mammalian cells. It further suggests that the complex biosynthetic pathway, proposed for *T. brucei* VSG [22] may also be found in higher organisms.

From our results we conclude that the anchoring of proteins to membranes via a glycophospholipid moiety has been conserved through evolution. A possible advantage of such a protein-membrane attachment may be that membrane proteins could be specifically released by the action of an endogenous phospholipase C. This is of importance for trypanosomes that lose their surface coat during differentiation into procyclics [23]. In cells of neuronal origin a secretory form of AChE has been described [24]. It is tempting to speculate that this secretory process might involve a phospholipase C-mediated cleavage of the hydrophobic anchor of AChE.

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