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Research article

PROFILIN ACTIVATES *BACILLUS THURINGIENSIS* PHOSPHOINOSITIDE SPECIFIC PHOSPHOLIPASE C

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ABSTRACT : Many extracellular signaling molecules including hormones, growth factors, neurotransmitters and immunoglobulins elicit intracellular responses by activating phosphatidylinositol-specific phospholipase C (PI-PLC) upon binding to their cell surface receptors. Activated PLC catalyses the hydrolysis of Phosphotidylinositol 4,5-bisphosphate (PIP_2) to generate DAG and IP_3 , which act as signaling molecules that control various cellular processes. Exploring the mechanism of regulation of PLC activity may lead to understanding various signaling events that regulate cell growth and differentiation. One of the dramatic effects of profilin is inhibition of PIP_2 hydrolysis by PLC-γ in eukaryotic cells. In the present study, the effect of profilin on Phosphotidylinositol specific phospholipase C (PI-PLC) purified from *Bacillus thuringiensis (Bt)* was examined. Assay of PI-PLC activity indicated that Bovine profilin activated the hydrolysis of phosphotidylinositol (PI) by *Bt*PI-PLC in a concentration dependent manner under *in vitro* conditions. A 250 % increase in activity was noted in the presence of profilin but not in presence of phosphoprofilin. In the presence of profilin more proteins are observed in the soluble fraction. In conclusion it can be stated that that profilin activates bacterial PLC activity towards PI hydrolysis.
Key words : Profilin, *Bacillus thuringiensis*, bacterial PI-PLC, phosphatidyl inositol, GPI linkages

Abbreviations

DAG	-	Diacyl glycerol
GPI	-	glycosylphosphatidylinositol
IP_3	-	Inositol 1,4,5-trisphosphate
PI	-	Phosphotidylinositol
PIP_2	-	Phosphatidylinositol 4,5-bisphosphate
PI-PLC	-	Phosphatidylinositol-specific phospholipase C
PLC-γ	-	Phospholipase C-γ
PKC-ζ	-	Protein kinase C-ζ

INTRODUCTION

Many extracellular signaling molecules including hormones, growth factors, neurotransmitters and immunoglobulins elicit intracellular responses by activating phosphatidylinositol-specific phospholipase C (PI-PLC) upon binding to their cell surface receptors. Activated PLC catalyses the hydrolysis of Phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two important secondary messengers- Inositol 1,4,5-trisphosphate (IP_3) and Diacylglycerol (DAG)-which act as signaling molecules that control various cellular processes.

Cell shape and motility during development and differentiation are determined by the cytoskeletal functions and initiated in response to either external or internal stimuli. Profilin is a regulatory component of the actin cytoskeleton in all eukaryotic cells. Profilin is proposed to play a dual role in cells; it regulates the activities of phospholipase C and by binding to actin monomers regulates actin polymerization. In normal eukaryotic cells, profilin by binding to PIP_2 inhibits the production of IP_3 and DAG by the action of unphosphorylated phospholipase C-γ (Goldschmidt-Clermont et al., 1990; Machesky et al., 1990). However, phosphorylation of phospholipase C-γ at specific tyrosine residues during the activation of the cell overcomes the inhibitory effect of profilin on PIP_2 hydrolysis thus resulting in an effective activation of phospholipase C-γ (Goldschmidt-Clermont et al., 1990).

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PI-PLC comprise a diverse family of enzymes that are isolated from bacteria, protozoa, yeasts, plants, insects and mammals. Of the well-characterised PI-PLC's, the bacterial enzymes are secreted from cells (extracellular) while those from eukaryotic organisms are intracellular. The eukaryotic PI-PLC's play a central role in most signal transduction, contrarily, bacterial PI-PLC's are interesting as they act as virulence factors in some pathogenic bacteria. They are capable of cleaving phosphatidylinositol (PI) and glycosylphosphatidylinositol (GPI) but not the phosphorylated forms of PI such as PIP or PIP_2. Bacterial PI-PLC's consist of a single domain of 30 to 35 kDa, while the much larger mammalian enzymes (85 to 150 kDa) are organized in several distinct domains. The 10 mammalian PI-PLC isozymes (excluding alternatively spliced forms) identified to date are all single polypeptides that can be divided into three types: β, γ, δ, of which four PLC- β, two PLC- γ and four PLC- δ proteins are known (Rhee et al., 1989; Rhee and Bae, 1997). The catalytic domain of eukaryotic PI-PLC's is assembled from two highly conserved polypeptide stretches (regions X and Y) that are separated by a divergent linker sequence and depend on Ca^{2+} for their catalytic activity. Bacterial enzymes are much simpler and consist of a single domain folded as a (βα)8-barrel (TIM barrel), are calcium-independent, and interact weakly with membranes. Bacterial PI-PLCs are structurally similar to the catalytic domain of mammalian PI-PLCs. Comparative studies of both prokaryotic and eukaryotic isozymes were useful in identifying distinct regions of proteins that are structurally and functionally important (Griffith and Ryan, 1999; Heinz et al., 1998).

Bacterial PI-PLC's are specific towards PI and GPI linked proteins. Hence we purified the enzyme from *Bacillus thurigiensis*, whose PI-PLC has been well characterized previously (Lechner et al, 1989) and PI from Baker's yeast. The effect of profilin on the hydrolysis of PI and GPI linkages by bPI-PLC was studied.

MATERIALS AND METHODS

All the chemicals used were of best quality available. Profilin was purified from bovine spleen. Phosphotidylinositol (PI) was purified from the autolysate of baker's yeast and Phosphatidylinositol-specific phospholipase C, from culture media of *Bacillus thuringiensis*.

Purification of Profilin

Profilin was purified from bovine spleen according to the procedure of Lindberg et al., (1988) with slight modifications. All the steps were carried out at 4°C unless mentioned. Fresh bovine spleen (100 g) obtained from a local slaughterhouse was homogenized in five volumes of homogenization buffer (buffer A containing 0.5% Triton X-100 and 1 mM PMSF). The homogenate was centrifuged at 20,000 rpm (Kubota 6900, Rotor # RA 300G) for 20 min. The supernatant was loaded onto Poly-L-Proline (P-L-P) agarose matrix (15 ml bed volume) previously equilibrated with buffer A (10 mM Tris-HCl, pH 7.8 containing 0.1 M glycine, 0.1 M KCl, 5 mM β-mercaptoethanol). After loading and washing with 50ml of buffer A , the matrix was incubated overnight in 50 ml of wash buffer I (Buffer A containing 0.3 mM $Mgcl_2$ and 20 mM ATP) to favor actin polymerization. The next day the matrix was further washed with 100 ml of wash buffer II (2 mM Tris-Hcl, pH 7.5, containing 1 M KI, 0.4 mM ATP, 0.2 mM NaN_3, 2 mM $MgCl_2$, and 5 mM β-mercaptoethanol) followed by 50 ml of wash buffer III (2 M urea in buffer A) to remove further traces of actin. Profilin was eluted at room temperature with 8 M urea in buffer A. Fractions containing profilin were pooled together and passed through DE-52 column kept at 40 °C before dialyzing against 10 mM Tris-HCl, pH 7.8 containing 1 mM β-mercaptoethanol. Profilin was concentrated by Amicon ultrafilteration unit using 1 kDa cut off filter. The protein concentration was estimated by Lowry method (1951) with BSA as standard. The homogeneity of profilin was analysed by polyacrylamide gel electrophoresis using 10% Tricine-SDS polyacrylamide gels (Schagger et al., 1987). The biological activity of the profilin obtained was checked by studying its inhibitory effect on actin polymerization using pyrene labeled actin monomers (Bhargavi et al., 1998)

Overexpression and partial purification of PKCζ

Sf9 cell line derived from ovarian tissue of *Spodoptera frugiperda* was used in the present study. The cells were grown to 90% confluence in TC-100 medium supplemented with 10% foetal calf serum and were infected with recombinant baculovirus expressing PKCζ (10 multiplicity of infection). After 36 h of infection, the cells were collected by centrifugation at 2000 X g (Kubota 6900, Rotor # RA 300 G) for 10 min and washed with phosphate buffered saline (PBS), pH 7.4. These cells were stored at -70°C until further use.

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Cells were lysed in 20mM Tris-HCl pH 7.5, containing 0.1% β-mercaptoethanol, 10mM benzamidine, 1μg/ml leupeptine, and 2mM PMSF (lysis buffer), and sonicated. The supernatant was collected by spinning at 30,000 rpm (Hitachi 55P-72, Rotor # RP 50-2-268) for 30min at 4°C and purified by subjecting the extract to (i) DE-52 chromatography, (ii) ammonium sulphate fractionation, and (iii) phenyl Sepharose chromatography.

The cell lysate obtained was passed through DE-52 column (2×10 cm-packed volume). The column was washed with 20ml of 20mM KCl in buffer A (40 mM Tris-HCl, pH 7.2, containing 1mM β-mercaptoethanol and 10% glycerol). The bound protein was eluted with 50ml of buffer A containing 150mM KCl, and 5ml fractions were collected. The fractions containing maximal activity were pooled and subjected to ammonium sulphate fractionation. Ammonium sulphate was slowly added to a concentration of 1M with stirring, followed by centrifugation at 10,000 rpm (Kubota 6900, Rotor # RA 300 G) for 10min. The supernatant was loaded onto phenyl Sepharose column (2×10 cm) pre-equilibrated with 1M ammonium sulphate. The column was then washed with 1M KCl, and the bound protein was eluted by running a gradient from 1M KCl to 0 M KCl (50 ml) in buffer A, which was further chased with 25 ml of distilled water.

Profilin Phosphorylation

Profilin was phosphorylated using partially purified PKC over expressed in insect cell lines according to Sathish et al (2004). PKC mediated phosphorylation of profilin was measured in the presence of 10 μg profilin, 10 mM Mg^{2+}, 50 μM ATP (0.2 μCi/nmol) in a final volume of 50μl. The reaction was started by the addition of PKC (one enzyme unit) and incubated at 30 °C for 10 min. The reaction was stopped by the addition of SDS-PAGE sample buffer. The samples were boiled and subjected to electrophoresis on 10% tricine-SDS gel. The gel was autoradiographed.

Large-scale profilin phosphorylation was carried out with cold ATP by the same procedure mentioned above, while running a parallel reaction with labelled ATP to quantitate the extent of phosphorylation. The phosphorylated profilin was separated from ATP and protein kinase by PLP-affinity chromatography. The PLP matrix was thoroughly washed and the protein was eluted from column using 30-50% DMSO. The fractions containing phosphoprofilin were pooled and kept for dialysis against 10mM Tris-HCl, pH 7.8. Phosphoprofilin thus obtained was concentrated and protein concentration was estimated by Lowry method (1951).

Purification of Phosphatidylinositol

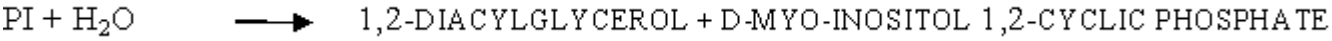
Phosphatidylinositol was purified from the autolysate of baker's yeast according to the method of Trevelyan, 1996. The bacterial enzymes mainly hydrolyse monophosphoinositide during a short incubation period. The liberated water-soluble phosphorus is equivalent to hydrolysed substrate. The purity of the preparation was analyzed by TLC and no contaminating or degradation products were noted.

Purification of *Bt* PI-PLC

Phosphatiylinositol-specific phospholipase C was purified from a culture broth of *Bacillus thurigiensis* by following the procedure of Griffith et al., (1991) in four steps (using ammonium sulphate precipitation, CM-Sephadex column chromatography, DEAE column chromatography and phenyl sepharose column chromatography).

***Bt* PI-PLC assay methods**

Bacterial PI-PLCs catalyse the cleavage of the lipid phophatidylinositol (PI), to produce DAG and the water-soluble head group, myo-inositol 1,2-cyclic phosphate.



Mixed micelles of phosphatidylinositol and Triton-X 100 were used the assay. Myo-inositol 1,2 cyclic phosphate, one of the reaction products, were estimated by extraction with chloroform-methanol-Hcl (66:33:1). After decomposition of myo-inositol phosphate by the method of Fiske and Subha Row (1925), phosphorus was determined by the method of Eibl and Lands (1969). One unit of the enzyme is defined as the amount of enzyme that catalyses the hydrolysis of 1mmol of phosphatidylinositol per min at pH 7.5 and 37°C.

Effect of profilin and phosphoprofilin on *Bt* PLC activity

Phosphatidylinositol in 10 mM Tris-Hcl (pH 7.5) was sonicated for 3 min and incubated with different concentrations of profilin or phosphoprofilin (0.5, 1, 5, 10 μg) for 5 min. Reaction assay mixture containing 0.1ml (2 mM final conc.) of PI solution, 0.1 ml of 0.8% Triton-X 100, 0.2 ml of 100 mM sodium borate (pH 7.5) and 0.1ml of the enzyme solution was incubated at 37°C for 20min.

The reaction was terminated by adding and mixing 2.5 ml of chloroform-methanol-Hcl (66:33:1). After centrifugation at 2000 X g for 5min, a 0.4 ml aliquot was transferred from the methanol-water layer onto a test tube. 0.3 ml of 6 N H_2SO_4 was added to each sample and heated at 170 °C for 30 min. A few drops of 60% H_2O_2 were added and the mixture heated at 170 °C for another 120 min. After cooling to room temperature, 2.5ml of distilled water and 0.1 ml of 0.36% Triton-X 100 were added to all the samples and mixed vigorously.

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Approximately 20 min after addition of 0.3 ml of ammonium molybdate, turbidity was measured at 660 nm. The phosphate released (in moles) due to hydrolysis of PI by PI-PLC at different time intervals was calculated from standard graph of absorbance (at 660 nm) as a function of known concentrations (10 to 50 moles) of NaH_2PO_4. The effect of profilin on the hydrolysis was studied.

Effect of profilin on *Bt* PLC Glycosylphosphatidylinositol (GPI) - hydrolytic activity

RBC ghosts were taken as a source of GPI linked proteins and in vitro assay was performed to analyze the effect of profilin on GPI-linkage hydrolysis by *Bt* PLC.

Preparation of erythrocyte membranes

100ml of bovine blood was collected in 3.8 % sodium citrate. The erythrocytes were pelleted by centrifugation at 108 X g for 5 min. The pellet was washed thrice with ice cold isotonic saline. The cells were then lysed at 4 °C by suspending them in lysis buffer (5 mM Tris-HCl, pH 7.5, containing 5 mM di-sodium EDTA, 7 mM β-mercaptoethanol). The membranes were pelleted by centrifugation at 10,000 X g for 20 min and washed (4-6 times) with 20 mM Tris-HCl buffer pH7.5 unit transluscent membrane pellet was obtained.

GPI-Hydrolytic activity

PLC assay was performed as described earlier with erythrocyte membranes as substrate. The hydrolysis of GPI linked proteins in the erythrcyte membranes in the absence and presence of profilin was studied by SDS-PAGE analysis of solubilized proteins. Incubations were performed with 1 µg, 5 µg, 10 µg of profilin and 20µl (18 µg) of PI-PLC at 37°C for 1 hour. The samples were centrifuged and 40 µl of supernatant was loaded onto a 10 % SDS-PAGE gel and run till the tracking dye reached the bottom of the gel and then the bands were visualized by silver staining.

RESULTS

Purification of PI-PLC from Bt and PI from baker's Yeast

Bacillus thuringiensis are rod shaped gram-positive bacteria that are nonpathogenic to humans. These bacteria excrete PI-PLC enzyme in relatively large quantities into the growth medium. This facilitates the purification of PI-PLC because intracellular proteins are removed with the cells during an initial centrifugation step. The enzyme (PI-PLC) when purified yielded 6 mg from 4 litres of *Bt* culture. Figure 1 shows the 10% SDS-PAGE separation of the purified enzyme, thus indicating no contaminating protein in the final preparation. Preparation of PI from baker's yeast according to the protocol described by Trevelyan (1966) resulted in 5 mg PI per gram of yeast. The purity of the preparation was analyzed by thin layer chromatography and no contaminating or degradation products were noted (data not shown).

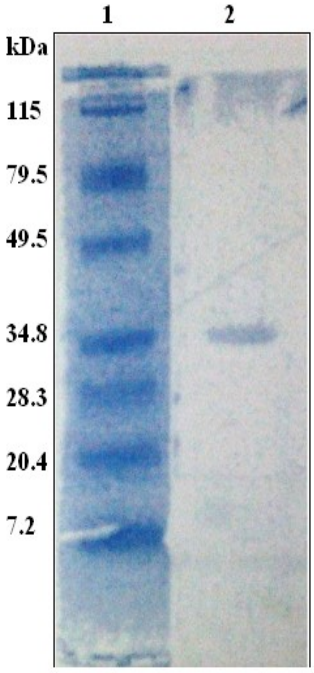

Figure 1 : Purification of PI-PLC from *Bacillus thuringiensis*

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Effect of profilin and phosphoprofilin on the hydrolysis of PI by Bt PLC

The amount of phosphate released (in nmoles) due to hydrolysis of PI by PI-PLC was plotted against time (Fig 2). The PLC activity in presence of profilin/phosphoprofilin is shown in Figure 6. The results indicated that both profilin and phosphoprofilin activated the hydrolysis of phosphatidylinositol (PI) by PI-PLC purified from *Bacillus thuringiensis* in a concentration dependent manner under *in vitro* conditions. However, profilin activated the hydrolysis more than phosphoprofilin. A 250% increase in activity was noted in the presence of profilin. The amount of phosphate released at different time intervals was calculated from the standard graph of NaH_2PO_4 shown in Figure 3.

Effect of profilin on GPI Hydrolytic activity

Bacterial PI-PLCs are known to cleave PI and PI linkages in GPI linked membrane proteins. An attempt was made to study whether profilin triggers GPI linkage hydrolysis using RBC ghosts. Figure 4 indicates that in the presence of profilin more proteins are observed in the soluble fraction. The results further confirmed that profilin activates bacterial PLC activity towards PI hydrolysis

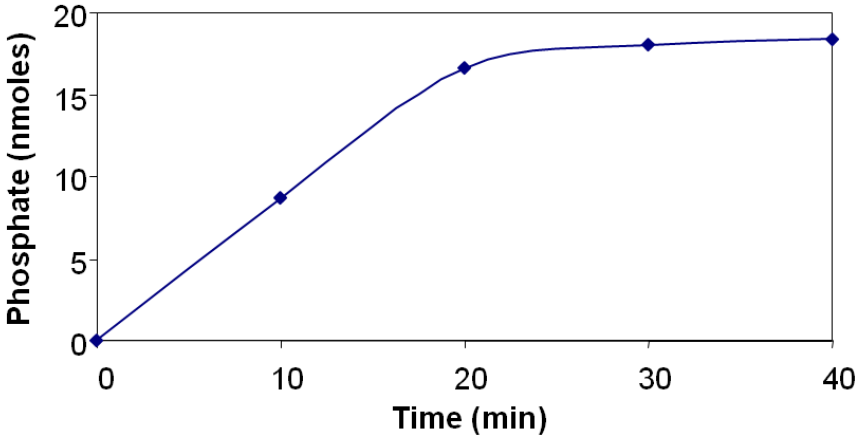

Figure 2: Time course of PI hydrolysis of *Bt* PI-PLC

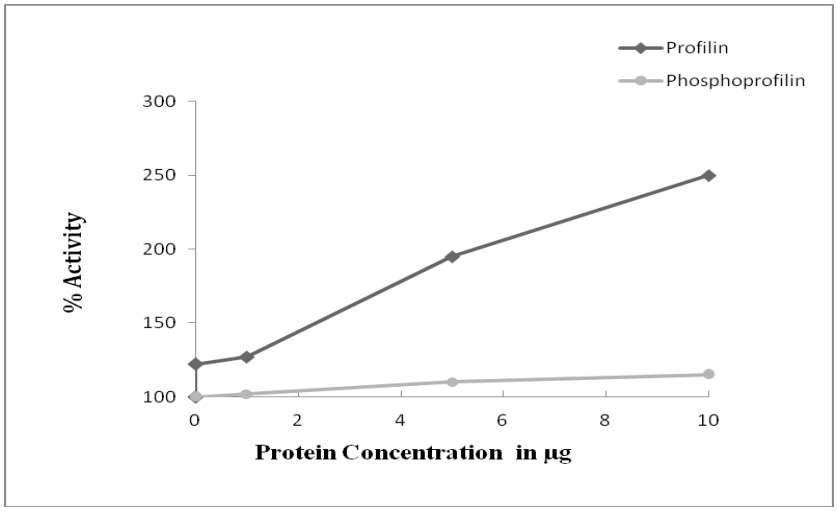

Figure 3: Activation of *Bt*PI-PLC activity in the presence of profilin and phosphoprofilin

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DISCUSSION

Profilin is involved in many signaling pathways, and its regulation may well be an important link between growth factor signals from outside and the internal state of the actin cytoskeleton. A central regulatory role for profilin in both the cytoskeleton and phosphotidylinositol pathway explains why deletion of the profilin gene can be lethal in yeast (Lu and Pollard, 2001, Theriot et al.; 1993).

Our results indicate that profilin activated the PI hydrolysis by bacterial PI-PLC in a concentration dependent manner. Since bacterial PI-PLC's are known to cleave PI and PI linkages in GPI linked membrane proteins (Heinz et al, 1998), we made an attempt to study whether profilin triggers GPI linkage hydrolysis using RBC ghosts. The results indicated that in presence of profilin more proteins are released in soluble fraction. These results further confirmed our data that profilin activates bacterial PLC activity. This discovery is significant as PI is absent in many bacterial membranes, suggesting that most bacteria do not require PI-PLC for their own survival. It is also interesting to note that GPI anchored proteins are found only in eukayotes. In the case of animal and human pathogens, PI-PLC's secreted by bacteria are considered potential virulence factors that facilitate infection of their respective hosts (Notermans et al., 1991). Using PI-PLC's, GPI cleaving ability, proteins tethered to the cell membrane by GPI anchors can be released, leading to regulation of different signaling pathways. As PI-PLC's secreted by bacteria play a role as virulence factors in pathogenic bacteria, we speculate that profilin could regulate these host- pathogen interactions.

These results are quite contrasting when compared to the eukaryotic system. The complexity and diversity of phosphoinositide metabolism in prokaryotes and eukaryotes makes it difficult to correlate such distinct and contrasting influence of profilin on PLC activity. This discovery is interesting and it raises many questions. The possible significance of the above results is that during intracellular pathogenesis some pathogens regulate the host cytoskeletal organization by recruiting profilin onto their surface through PLP interactions. Further, activation of pathogen may be mediated by profilin, triggering cleavage of specific GPI-linked protein and regulating signaling events of host in their favour. These hypothetical views need to be experimentally confirmed. The data presented here signals possibility of such regulation. Further studies in this direction are needed to identify specific proteins, whose interactions with profilin are regulated by phosphorylation and their role in signaling events.

The final purified fraction of PI-PLC from *B.thuringiensis* was loaded onto 10 % SDS-PAGE and electrophoresced. The gel was then stained using Coomassie Brilliant blue G-250 and the image was taken. Lane 1- protein molecular weight marker, lane 2- 1 µg of purified PI-PLC.

Pure PI-PLC (20 µg) obtained from *Bt* was incubated with PI (2 mM) in 100 mM sodium borate buffer (pH 7.5) and 0.8% Triton-X 100 at 37 °C. At different time intervals, the reaction was stopped by the addition of 2.5ml chlororform-methanol-Hcl (66:33:1) and the amount of phosphate released was calculated as described in methods. The data represents the average of three independent experiments.

Different concentrations of profilin and phosphoprofilin were incubated with PI (2 mM) for 15 minutes. The Profilin-PI mixture was then incubated with 20 µg of PLC at 37 °C for 20 minutes in 100 mM borate buffer (pH 7.5). Phosphate released was extracted with chloroform-methanol-HCL (66:33:1) and estimated as described in methods. The data represents the average of three independent experiments.

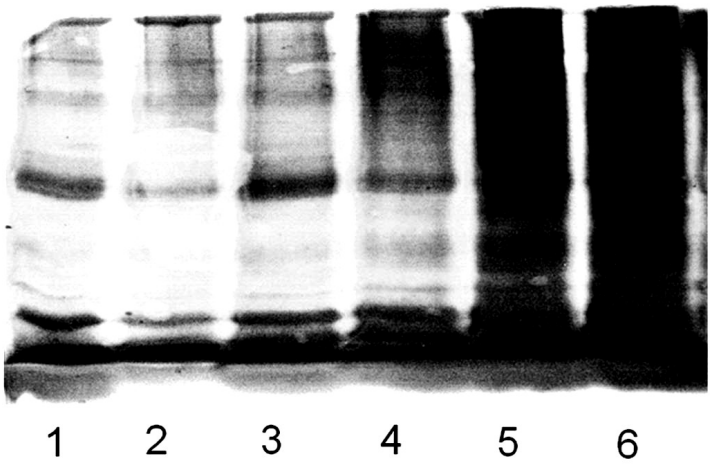

Figure 4: GPI linkage hydrolysis in RBC ghosts

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Erythrocyte membranes were incubated for 1 hr at 37°C with profilin and/or PLC, and supernatant was subjected to 10 % polyacrylamide gels. The protein bands on SDS-PAGE were visualized by silver staining. 40 µl of each sample was loaded in each well. Lane 1 : erythrocyte membranes; Lanes 2: erythrocyte membranes with 10 µg profilin; Lane 3: erythrocyte membranes with 18 µg of enzyme PI-PLC; Lane 4: erythrocyte membranes and enzyme with 1 µg profilin; Lane 5: erythrocyte membranes and enzyme with 5 µg; Lane 6: erythrocyte membranes and enzyme with 10 µg.

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