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Immunodetection of Glycophosphatidylinositol-anchored Proteins Following Treatment with Phospholipase C

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Many important regulatory proteins are attached to the extracellular leaflet of the plasma membrane by a glycophosphatidylinositol (GPI) anchor [1]. One of the most common methods used to determine whether a protein contains a GPI anchor involves treating membranes with the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC), followed by detection of the “released” candidate protein into the supernatant fraction by traditional Western blotting [2]. Here, we report that the amounts of two representative GPI-anchored proteins, Thy-1 and the cellular prion protein (PrP^C) [3], detected by Western blotting decreased >95% following PI-PLC treatment. The diminished signals appear to be caused by failure of the PI-PLC treated proteins to adhere to PVDF or nitrocellulose membranes, and could lead to false negative results in PI-PLC release assays. Detection of PI-PLC treated PrP^C molecules was successfully achieved by (1) slot blotting in the absence of SDS onto negatively charged Nylon membranes and (2) in-gel immunostaining.

Crude hamster brain homogenates were incubated for 8 hours at 37°C either with or without purified PI-PLC enzyme (Molecular Probes, Eugene, OR), and analyzed by SDS-PAGE. Following semi-dry Western transfer onto a polyvinylidene difluoride (PVDF) membrane, the samples were probed with anti-Thy 1 monoclonal antibody (Abcam, Cambridge, MA) (Figure 1A, lanes 1–2). Unexpectedly, the intensity of the ~25 kDa immunoreactive Thy-1 band was >95% lower in PI-PLC treated samples than in untreated samples, as determined by densitometry (Image Gauge software, Fujifilm USA, Valhalla, NY). To study whether this effect was specific for Thy-1, we repeated the experiment using monoclonal antibody 3F4 to detect PrP^C, a different GPI-anchored neuronal protein (Signet, Dedham, MA). As in the case of Thy-1, the amount of PrP^C detected by Western blotting on PVDF was significantly lower in PI-PLC treated samples of crude brain homogenate than in untreated samples (Figure 1A, lanes 3–4), showing that PI-PLC treatment significantly reduces Western blot signals for two different GPI-anchored proteins.

We performed a series of experiments to test the possibility that the reduced Thy-1 and PrP^C Western blot signals might have been caused by proteolytic digestion during incubation with PI-PLC. First, we incubated samples of purified PrP^C [4] with either PI-PLC or control buffer, and compared the amount of PrP^C remaining after incubation by silver-stained SDS-PAGE.

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The results of this analysis show that PI-PLC treatment does not reduce the actual amount of PrP^C present in incubated samples (Figure 1B), whereas the Western blot signal for PI-PLC treated samples of purified PrP^C are reduced compared to untreated samples (Figure 1C, lanes 1–4). Second, we treated samples containing recombinant hamster PrP expressed in *E. coli* (and therefore lacking a GPI anchor) (InPro Biotechnology, South San Francisco, CA) with PI-PLC, and observed no reduction in Western blot signal (Figure 1C, lanes 5–6). This result shows that enzymatic treatment does not reduce Western signal intensity if the substrate protein lacks a GPI-anchor. Third, we added high concentrations of Complete™ protease inhibitor cocktail during enzyme incubation, and found that the presence of protease inhibitors did not increase the Western blot signals of samples treated with PI-PLC (Supplemental information, Figure S1). Finally, we found that purified, recombinant PI-PLC obtained from a different commercial source (Prozyme Glyko, San Leandro, CA) produces the same loss of Western blot signal as the purified *B. cereus* enzyme obtained from Molecular Probes (Figure 1C and 1D, and Supplemental information, Figures S1 and S2). Taken together, these results show that PI-PLC treatment does not reduce the Western blot signals of GPI-anchored proteins simply by protein degradation.

We next tested the possibility that PI-PLC treated proteins might either: (1) be retarded in migrating out of polyacrylamide gels during Western transfer, or (2) fail to denature completely during Western transfer, resulting in lack of epitope exposure. SDS-denatured samples containing purified PrP^C molecules were directly applied onto PVDF membranes using a modification of a well-characterized slot blot method [5], and the membranes were probed with two different anti-PrP antibodies that recognize spatially distinct epitopes [6]. The results of this experiment show that PI-PLC treatment reduced the slot blot signals with both antibodies (Figure 1D). Similar results were obtained using nitrocellulose membranes (data not shown), indicating that PI-PLC treated PrP^C molecules do not adhere well to either of the commonly used types of Western transfer membranes.

We experimented with three different approaches to improve the immunodetection of PI-PLC treated proteins. In the first approach, we dried the membrane in an autoclave after transfer in an attempt to stabilize protein binding. However, this method only improved the Western blot signal of the PI-PLC treated samples marginally (Supplemental information, Figure S2). In the second approach, we used an in-gel chemiluminescence detection protocol (UnBlot, Bio-Rad, Hercules, CA), and obtained approximately equal signals for PI-PLC treated and untreated samples (Figure 2A). This technique allows visualization of bands within SDS polyacrylamide gels, and therefore can be used to determine the molecular weight of immunoreactive bands. As previously reported, PI-PLC digestion causes PrP^C molecules to migrate more slowly on SDS-PAGE [7] (Figure 2A). In the third approach, we applied samples containing purified PrP^C molecules directly to negatively charged Biotodyne-C Nylon membranes (Pall, East Hills, NY) in the absence of SDS. This rapid technique also produced approximately equal signals for PI-PLC treated and untreated samples (Figure 2B), but cannot be used to determine molecular weight.

GPI anchors are complex structures composed of ethanolamine linked to an oligosaccharide, which in turn is joined to a phosphatidylinositol lipid containing two fatty acid chains [2]. PI-PLC hydrolyses the phosphodiester bond between inositol phosphate and diacylglycerol, thereby liberating the hydrophobic tail, while the rest of the anchor, including both phosphate groups, remains attached to the protein [2]. Based on our results, it seems likely that the retained, negatively charged portion of the anchor interferes with the ability of the PI-PLC treated protein to adhere to non-specific binding membranes such as PVDF and nitrocellulose. Therefore, we strongly recommend the use of alternative methods such as in-gel immunodetection, slot blotting onto positively charged Nylon membranes, or antibody capture

techniques to detect GPI-anchored proteins during diagnostic PI-PLC “release” studies and other assays that require PI-PLC digestion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1. Inefficient immunodetection of PI-PLC treated proteins on PVDF membranes

(A) Western blot of crude brain homogenate samples treated with 0.25 U PI-PLC or control buffer ($\emptyset$) for 8 h at 37°C, as indicated. Blots were probed with either B417 anti-Thy1 or 3F4 anti-PrP primary monoclonal antibodies, as indicated. (B) Silver stained 12.5% SDS polyacrylamide gel containing samples of purified PrP^{C} treated with 0.25 U PI-PLC or control buffer ($\emptyset$) for 16 h at 37°C, as indicated. (C) Western blot of samples containing purified PrP^{C} or bacterially expressed PrP molecules. Samples were either treated with 0.25 U PI-PLC or control buffer ($\emptyset$) for 16 h at 37°C, as indicated. Where indicated, some samples were also enzymatically deglycosylated with PNGase F, according to manufacturer's instructions (New England Biolabs, Beverly, MA). (D) Slot blots of samples containing purified PrP^{C} molecules, treated with either 0.25 U PI-PLC or control buffer ($\emptyset$) for 8 h at 37°C, as indicated. Blots were probed with either 3F4 or D13 anti-PrP primary antibodies, as indicated

Figure 2. Alternative detection methods for PI-PLC treated PrP^C molecules

(A) In-gel immunodetection of purified PrP^C treated with either 0.25 U PI-PLC or control buffer (Ø) for 8 h at 37°C, as indicated. Where indicated, samples were also enzymatically deglycosylated with PNGase F. The gel was probed with 3F4 antibody and developed using the UnBlot chemiluminescence detection kit, according to manufacturer's instructions (Pierce, Rockland, IL). (B) Samples containing purified PrP^C molecules, treated with either 0.25 U PI-PLC or control buffer (Ø) for 16 h at 37°C, as indicated, were either directly applied by slot blotting to Biodyne-C membranes or denatured, diluted into ethanol, and applied to PVDF membranes, as indicated. Both blots were probed with 3F4 antibody to detect PrP molecules.