



Optimization of flotation assay conditions for syndapin binding to phosphatidic acid containing liposomes

MAGDA PIAŚCIK, JOLANTA ZEGARLIŃSKA, ALEKSANDER F. SIKORSKI, ALEKSANDER CZOGALLA*

Department of Cytobiochemistry, Faculty of Biotechnology, University of Wrocław
ul. F. Joliot-Curie 14a, 50-383 Wrocław
E-mail: aleksander.czogalla@uwr.edu.pl

ABSTRACT

Flotation is one of the best method for preliminary identification of protein-lipid interactions. In most widely used approach it utilizes large unilamellar vesicles, that are excellent models of freestanding membranes and do not require any additional components, like solid supports or beads that are needed in other methods commonly used for protein-lipid binding studies. Here we present results obtained during our studies on phosphatidic acid - syndapin interactions and discuss some technical aspects of this method underlying how relatively small changes in the conditions can influence the results.

KEY WORDS: protein-lipid interactions, LUVs, density gradient, ultracentrifugation

Introduction

Phosphatidic acid (PA) is the simplest phospholipid that consists of phosphate group attached to glycerol backbone in sn-3 position and two acyl chains. PA has a very small anionic head group and under physiological conditions it has a molecular shape of a cone (Kooijman *et al.* 2005). Although the amount of PA in cells is very low, (1% of total phospholipids in plants) (Guo *et al.* 2011) it is involved in many processes like regulating membrane dynamics or serving as an intermediate in lipid biosynthesis. It is also a binding partner for many proteins, being often the key point in their signaling pathways. Syndapin belongs to the group of defined PA- binding proteins. It is a member of F-BAR superfamily of membrane binding proteins (Brian 2004), that plays significant role in membrane tubulation, and by that it is identified as a

regulator of synaptic vesicle endocytosis (SVE) (Quan & Robinson 2013). The presence of PA is essential for syndapin to fulfill its function. It was observed that the membrane tubulation activity of syndapin was decreased in membranes of lower concentrations of PA, what suggests that PA may be the membrane recruiting molecule. In fact in *in vitro* studies it appeared that syndapin binds to the model membranes by direct interactions with PA (Srinivas *et al.* 2013).

In this work we would like to present a method suitable for identification of protein-membrane interactions. There are many experimental approaches to establish the presence of protein-lipid interactions, such as lipid overlay assays (Castellana & Cremer 2006). Such methods use lipids deposited on solid supports, which are not present in

physiological context of lipid bilayer. Moreover, it is usually difficult to quantitate the bound fraction of a protein. Further issues are connected with the fact that the deposition of various classes of lipids may not be equally efficient and some lipids may be partially removed from the substrate during the experiment. Going further with experimental procedures, interaction of lipids with proteins may also be identified using lipid monolayers. Such approach is highly homogenous but the single lipid leaflet is far different from natural membrane state (Maget-Dana 1999).

Flotation is the method where large unilamellar vesicles (LUVs) are used. Significant advantage of this approach over other lipid protein interaction identification methods, like overlay assay is the use of lipid vesicles also known as liposomes. This method is based on the effect of the flotation of vesicles in density gradient of a biochemically inert sugar, where the highest density is at the bottom of a test tube. The liposomes float during

the ultracentrifugation until they reach the conditions where there is no difference between their own effective density and the density of the surrounding medium (Fig.1). Comparison of the protein concentration in the fractions collected after centrifugation allows to determine the presence of interactions with membranes, which is reflected by the presence of a protein in the upper fractions together with liposomes. If protein stays at the bottom and does not migrate together with liposomes it reveals that no interaction exists (Bigay & Antony 2006). Sedimentation is a similar method that utilize lipid vesicles and ultracentrifugation. In this approach protein concentration in the pellet (sedimented vesicles) is compared with the supernatant. However this method has some drawbacks, namely during the centrifugation process the unbound protein can precipitate and sediment together with the vesicle-bound fractions and thus give false positive results (Bigay & Antony 2006).

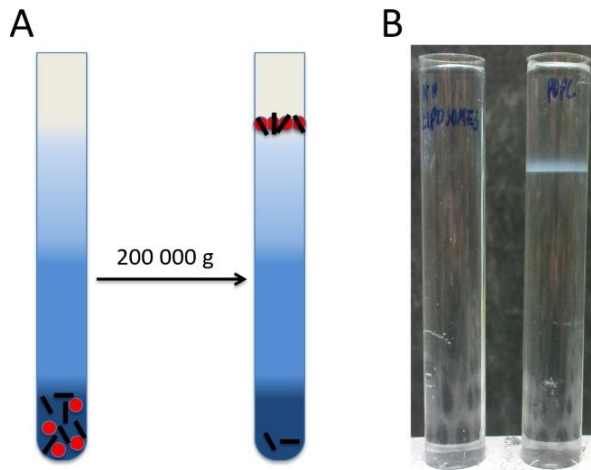


Figure 1. Flotation in the sucrose gradient. (A) Flotation scheme with sucrose gradient represented by blue shades of different intensities, protein as black sticks and liposomes as red dots. Left tube shows the situation before ultracentrifugation while right part shows the state after ultracentrifugation, directly prior to sample collection; floated liposomes with bound protein are at the interface between 10% and 0% of sucrose. (B) A representative image of test tubes after ultracentrifugation: without liposomes (left) and with POPC liposomes visible as a white cloudy fraction (right).

It is highly important that liposomes, used in the experiment are as homogenous as possible to obtain reliable results. Preparation of liposomes based on dry lipid film hydration leads to formation of a mixture heterogeneous vesicles with broad size distribution and lamellarity. This is why extrusion through polycarbonate filters with e.g. 200 nm pore size (diameter) results in obtaining vesicles smaller than 200 nm, and substantial fraction of very small liposomes passively go through the filter.

Materials and methods

Materials

POPA, POPC were bought from Avanti Polar Lipids (Alabaster, AL). ECLTM Prime Western Blotting Detection Reagent was bought from Amersham. Primary antibodies recognizing syndapin (pacsin) were bought from Santa Cruz and secondary antibodies Goat-Anty Rabbit were bought from Cell Signaling. Sucrose and NaCl was purchased from Chempur, HEPES from Roth, and Nitrocellulose from Amersham. The pGEX-6P-1 plasmid vector containing the sequence encoding mouse syndapin1 (Gen Bank accession number NM_011861) was a generous gift from Yvonne Groemping (Max Planck Institute for Developmental Biology, Tuebingen, Germany) and it was also deposited in Addgene repository (Plasmid #36859).

Protein purification

Protein was overexpressed in bacterial system in *E.coli* (BL21 (DE3) pLysS) (Promega). Bacteria was transformed with the plasmid vector and cultured on LB agar plates with ampicillin (100 µg/ml) overnight at 37°C. Then a single culture

Heterogeneous vesicle populations may hamper the analysis in membrane-protein interaction studies. It has been postulated that the use of heterogeneous liposome population would add the effect curvature-selective binding of amphipathic proteins, which may distort lipid-protein specificity (Czogalla *et al.* 2014). It was also shown that more homogeneous suspensions of the liposomes could be obtained by extrusion using membranes with 100 nm pore diameter (Kunding *et al.* 2008).

was transferred to test tube with LB medium with ampicillin (100 µg/ml). Overnight 37°C pre-culture was centrifuged (10 min, 10000 g, 4°C.) and the pellet was transferred to fresh LB medium with ampicillin (100 µg/ml), cultured at 37°C to reach OD₆₀₀ = 0.6 and induced with IPTG (final concentration 1mM) and cultured overnight at 18°C with constant shaking (200 rpm). Bacteria was then centrifuged (15 min., 4°C, 10000 g) and frozen with liquid nitrogen. Bacteria were thawed on ice and treated with lysis buffer (10 mM HEPES, 300 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 8.0), sonicated (Hielscher sonicator) 10 times for 15 seconds with 15 seconds intervals on ice and centrifuged (35000 g, 30 min., 4°C). The supernatant was loaded onto a 1 mL bed volume Glutathione Sepharose 4B (GE Healthcare) column which was pre-equilibrated with the lysis buffer. The resin was then washed with 50 mL of SLB buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) and 20 mL of prescission buffer (10 mM HEPES, 150 mM NaCl, 1mM EDTA, 1 mM DTT pH 7.0) until absorbance A₂₈₀ was lower than

0.05. 1 mL of Prescission buffer with protease 3C (Max Planck Institute for Cell Biology and Genetics, Dresden, Germany) (900 μ L + 100 μ L, respectively) was added to the resin and incubated overnight at 4°C. Elution was performed with prescission buffer. Concentration of Syndapin was determined using a Cary 1E spectrophotometer at $\lambda=280$ nm employing extinction coefficient parameters determined using ProtParam tool (www.expasy.com). Purity and concentration were also estimated from coomassie stained SDS-PAGE gels.

Liposome preparation

The required amounts of POPC and POPA in chloroform were dried under a stream of nitrogen and subsequently under vacuum in a glass tube and hydrated in SLB buffer (10 mM HEPES, 150 mM NaCl, pH=7.4). Liposomes were frozen/thawed five times and extruded through 0.2 μ m and 0,1 μ m filters (sequentially, 11 times each). Size and potential were determined using Malvern Zetasizer Nano ZS (Malvern). Liposomes were snap-frozen in liquid nitrogen and stored at -80°C.

Flotation assay

Protein concentration of 30nM and 0.4 mg/ml of liposomes were mixed and incubated at room temperature for 30

minutes. Samples were transferred to ultracentrifuge tubes and mixed with 250 μ L of 60% sucrose (in SLB buffer). This fraction was overlaid with 0.8 mL of 15% sucrose. Next two layers of the gradient consisted of 1.8 mL of 10% sucrose and 1 mL of SLB buffer. Ultracentrifuge tubes were placed in 60Ti rotor (Beckman Coulter). Samples were centrifuged (200 000 g for 2 hours at 4°C) using Beckman Coulter Optima™ L090K Ultracentrifuge). From each tube six fractions were gently collected. After addition of SDS (1% final concentration) fractions were analyzed by Dot Blot.

Dot Blot

Equal amounts of samples were loaded into the wells of Dot Blotter (Hoefer Scientific Instruments) and aspirated using vacuum pump. Membrane was blocked with milk for 1hour at room temperature after entire samples were filtered through the membrane. Incubation with primary antibodies (1:1000) was either for 3 hours at room temperature or overnight at 4°C. Membrane was washed three times in TBS-T (5 min. each). Secondary antibodies (1:10000) were added and incubated for 1 hour in room temperature and subsequently washed as previously. For detection commercial ECL was used. Images were made using the UVP (Biospectrum) in chemiluminescence mode.

Results

Thin layer chromatography (TLC)

TLC chromatography was performed to control lipid composition of liposomes used in the further

experiments. Extracted lipids correspond to lipids used for liposomes preparation (Fig.2).

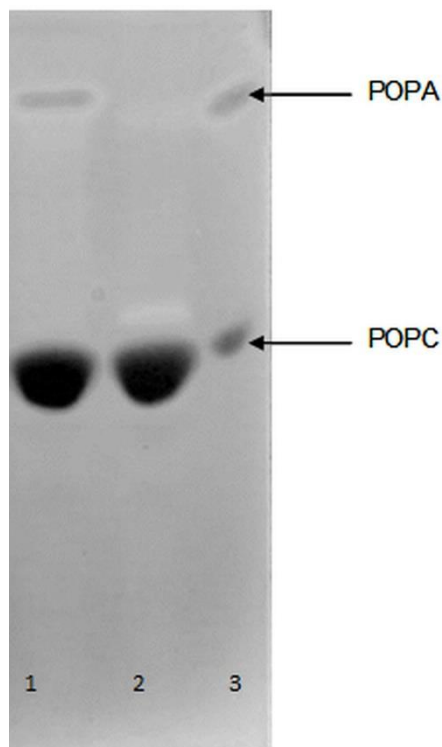


Figure 2. Liposome composition analysis using TLC. TLC analysis of: 1) liposomes with POPC/POPA in 9/1 molar ratio, 2) liposomes made of 100% POPC, 3) chloroform dissolved standards of POPC and POPA.

LUVs and the liposome-bound protein floated to the top fraction

As a result of vesicle flotation experiment, it is expected that after centrifugation liposomes will locate between two top fractions: 0% and 10% of sucrose concentration. Indeed in our experiments bound protein was detected within this liposomal fraction and any protein excess stayed at the bottom of test tube (30% sucrose). When no binding occurred, protein did not float and stayed at the bottom.

Searching for an optimal protein concentration

Protein concentration can have a big impact for study protein-lipid interactions using flotation method. In our first approach the protein concentration was

overestimated and this led to some technical difficulties in the densitometry quantification of the dot blots. When too high amount of the protein was used the unbound fraction, that stayed at the bottom of the test tube largely exceeded the amount of protein present in liposomal fraction (bound to liposomes). This impeded the dot blot imaging due to fast overexposure of the parts of the image that correspond to bottom fractions and underexposure of the rest of the image, which makes the result unreliable and difficult to ascertain the presence of eventual interactions. Such situation can be avoided by changing the liposome-protein ratio in the sample. In accordance to that change of the protein concentration from 500 nM to 250 nM diametrically improved the obtained results.

Setting up the gradient and collecting the fractions

Appropriate approach to build the density gradient and collect the fractions after ultracentrifugation are the key steps for obtaining a clear results in a standard flotation assay. Even when all factors are optimized perfectly it does not guarantee legible result. Figure 3 presents result of an experiment when the two steps were conducted using inappropriate tools (i.e. automatic pipettes offering limited fluid

expel precision). Such misconduction leads to the presence of a protein in all fractions although this should not be expected for the control, where only protein (without liposomes) was tested (compare Fig. 3A with Fig. 4A). This example also clearly shows how important is to conduct the control experiment with protein not mixed with liposomes in parallel to the liposome-protein samples in order to achieve necessary reliability.

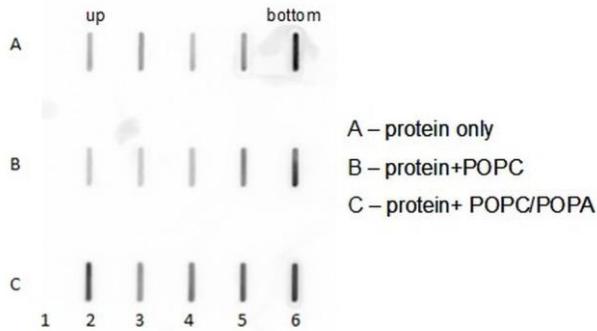


Figure 3. Dot blot analysis of fractions after flotation in non-optimized procedure. Fraction were collected from top (1) to the bottom (6) of the tube. (A) control sample containing syndapin without liposomes, (B) sample containing syndapin mixed with POPC liposomes, (C) sample containing syndapin mixed with liposomes composed of POPC/POPA 9/1 molar ratio.

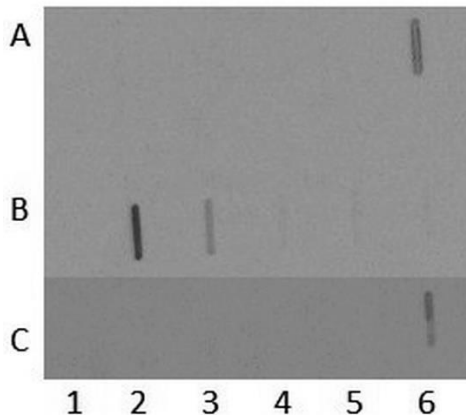


Figure 4. Dot blot analysis of fractions after flotation after optimization of the procedure. Fraction were collected from top (1) to the bottom (6) of the tube. (A) control sample containing syndapin I without liposomes, (B) sample containing syndapin I mixed with liposomes composed of POPC/POPA 9/1 molar ratio, (C) sample containing syndapin I mixed with POPC liposomes.

Figure 4 presents the experiment done properly, with optimized amount of the protein and under careful gradient building and fraction collection. First line is a control, only with protein. Protein without liposomes does not float, which means that 100% of protein stayed in the bottom fraction. As another control liposomes with POPC were used. No protein floating can be observed, which shows that syndapin I does not interact with membranes composed of pure phosphatidylcholine. On the other hand, a substantial fraction of the protein floated together with liposomes composed of POPC/POPA (90/10 mol%). Visible protein in second and third fractions provide evidence that almost all the syndapin I present in the sample was bound to the liposomes.

Optimizing sucrose concentration

The concentration-volume configuration of a sucrose gradient prior to ultracentrifugation can obviously have an impact on experiment result. It is important to choose proper gradient to let all the liposomes in the sample float. It may occur that too small differences in sucrose concentrations between neighboring gradient steps cause blurred fractionation, where liposomes are not concentrated within a certain range of the density gradient. A good practice is to estimate not only the amount of protein but also concentration of liposomes (or lipids) in each of the fractions collected after centrifugation. One of the easiest approaches consists of fluorescent labeling of liposomes coupled with spectrofluorimetric analysis of the fractions. Another issue to consider is to use an adequate gradient generator. While utilizing OptiPrep™ or other similar compounds it should be considered that buffer conditions should be equalized throughout the whole density gradient. It

is because such compounds are available as water solutions of certain concentration (e.g. 60% in case of OptiPrep™) and includes neither salt nor buffering agents. Self-prepared sucrose solutions could be easily made by dissolving crystals directly with e.g. HEPES buffer. On the other hand, ready to use compounds, such as OptiPrep™ may exhibit superior properties such as relatively low viscosity and osmolarity.

Discussion

It is highly important to extend the existing knowledge about the protein-lipid interactions. Phosphatidic acid is essential for various signaling pathways. Its presence is often necessary to sustain activity of various proteins. Thus, fully understanding the nature of its interactions with proteins may be critical for understanding regulatory mechanisms of signaling pathways, thus may play a significant role in drug development.

Among the numerous methods of protein-lipid interaction identification, flotation is unique for several important aspects. First of all, flotation method is usually performed using calibrated LUVs, which helps to achieve lipid organization analogous to what can be found in cellular membranes. Other methods used for protein-lipid interactions identification (i.e. overlay assay) are proceeded with conditions far away from physiological. This fact together with the simplicity of the flotation gives it superiority over other methods. Moreover, LUV's used in flotation assay are composed with pure lipids and the composition can be fine-tuned in a broad range. Liposomes calibration to 100 nm allows obtaining required size and membrane curvature homogeneity which is not achievable in case of membrane-assisted calibration (extrusion) with filter pore size larger or equal to 200nm. This is also true in terms of multilamellarity, as during extrusion

through larger pores it is much more difficult to eliminate all multilamellar vesicles. It should be stressed that the latter are highly undesirable in lipid-protein studies, as the inner membranes of such liposomes are usually not accessible to the protein added to liposomal suspension.

Selection of appropriate conditions for the experiment allows to get clear and unequivocal results. Ultracentrifugation in density gradient is one of the best approaches for preliminary evaluation of lipid-protein interaction. However one should be aware that there are some minor drawbacks of this method. Namely, the whole procedure is based on a relatively long centrifugation time. The kinetics of protein-lipid interactions may be an issue, as some proteins that have a faster rate of dissociation may dissociate from the floating vesicles before centrifugation is finished. Nevertheless, it also gives us the

information about the reaction kinetics. In this case flotation can give us a hint about how stable in time the interaction is (in terms of the Koff value). However in some cases, when centrifugation time is extended, the protein will be on the bottom of the centrifuge tube and interaction will not be detected, even if it occurs. In such cases one should be aware that this result does not stay as a lack of interaction and the shortened centrifugation time may change the observations dramatically. Moreover, the described method does not provide fully quantitative analysis. Therefore, we would like to stress that each report on protein-lipid binding events should be underlined by results obtained using at least two fully independent experimental approaches, most preferentially based on various membrane model systems (Czogalla *et al.* 2014).

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Streszczenie

Flotacja jest jedną z najefektywniejszych metod wstępnej identyfikacji oddziaływań białko-błony lipidowe. W większości przypadków wykorzystuje się w niej małe jednowarstwowe pęcherzyki lipidowe, które służą jako modele błonowe i nie wymagają dodatkowych nośników, takich jak membrany czy nanocząstki polimerowe, które są często używane w innych metodach mających na celu identyfikację oddziaływań białko-lipid. W poniższej pracy prezentujemy wyniki uzyskane podczas badań oddziaływań kwasu fosfatydowego i syndapiny. Omawiamy także niektóre techniczne aspekty metody, kładąc nacisk na to jak małe zmiany w warunkach metody mogą wpłynąć na otrzymywane wyniki.