

Validating a Method for Measuring Protein Domain and Lipid Binding Interactions

Using Luciferase Fusion Proteins

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Abstract

One example of how proteins interact with lipids is through specific regions called Pleckstrin homology (PH) domains. These domains bind to phosphatidylinositol phospholipids on membranes and can activate changes in the protein's function or change where the protein localizes within a cell. Utilizing well-researched PH domains from AKT3, DAPP1 and PLEK2, a new technique was explored for identifying protein and lipid binding by fusing PH domain gene sequences to the luciferase gene within a bacterial expression plasmid. The luciferase-tagged PH domains were incubated with nitrocellulose membranes containing various phosphatidylinositol phospholipids. The bound fusion protein was detected using luminescence, allowing the relative strength of the interaction between the domains and the phospholipids to be assessed. The binding results were mostly consistent with previous findings, indicating this new method could be a biologically relevant tool for researching protein domain and lipid interactions.

Validating a Method for Measuring Protein Domain and Lipid Binding Interactions Using Luciferase Fusion Proteins

Cellular membranes are biological barriers that separate the interior of the cell from the outside environment (Singleton, 1999). The cell membrane protects the cell from its surroundings, but it is also important for biological functions such as cell signaling, adhesion, and ion conductivity. These processes occur due to the composition of the membrane, which is comprised of a phospholipid bilayer with embedded proteins. There are three classes of lipids within the bilayer: glycolipids, sterols, and phospholipids. Phospholipids are the most abundant in a majority of cellular membranes (Lodish, Berk, & Zipursky, 2004). A particularly interesting category of phospholipids are the phosphatidylinositols. While they constitute a smaller percent of the plasma membrane lipid composition, there are multiple unique forms that contribute to a variety of cellular functions.

Phosphatidylinositols (PtdIns) are a group of lipids that have a glycerol backbone, two fatty acid tails and a polar inositol head group that can be phosphorylated at five different hydroxyl groups, giving rise to the several different forms of PtdIns. The monophosphates include PI(3)P, PI(4)P, and PI(5)P and the bisphosphates are PI(3,4)P₂, PI(3,5)P₂, and PI(4,5)P₂. The only PtdIns trisphosphate is PI(3,4,5)P₃ (Muller-Roeber & Pical, 2002). These different phosphorylation sites are important in cellular functions. For example, if the 3-hydroxyl group is phosphorylated, as in PI(3)P, the lipid can be used for membrane trafficking and aid in cell division. PI(4)P is capable of binding to a protein on the cytoskeleton, as well as recruiting proteins to the Golgi apparatus (Payraastre, et al., 1992). PI(5)P is involved in signaling at the nucleus and cytoplasm, and modulating responses to stress, hormones, and growth factors. PI(4,5)P₂ contributes to the regulation mRNA processing (Barlow, Laishram, & Anderson,

2010) and PI(3,5)P₂ controls signaling responses to stress. PI(3,4)P₂ aids cell growth, pre-mRNA splicing, and survival (Yokogawa, et al., 2000). The PI(3,4,5)P₃ is implicated in cell growth, survival, and signaling in the nucleus (Barlow, Laishram, & Anderson, 2010).

Phosphates are added to the inositol head group of PtdIns by an enzyme called phosphoinositide 3-kinase (PI(3)K) (IUPAC-IUB, 1976). The PI(3)K family is divided into three classes in regard to the kinase's primary structure, substrate specificity, and regulation (Leevers, Vanhaesebroek, & Waterfield, 1999). PI(3)K elevates the intracellular levels of phosphorylated phosphatidylinositols (PIPs) within a cellular membrane (Klippel, Kavanaugh, Pot, & Williams, 1996). Class I are capable of phosphorylating PI(3)P, PI(3,4)P₂, and PI(3,4,5)P₃ and are activated by receptor tyrosine kinases and G-protein coupled receptors, Class II lack a critical asparagine amino acid residue that interferes with its ability to coordinate binding of calcium ions and only produce PI(3)P and PI(3,4)P₂, and Class III is distinctly involved with the trafficking of proteins and can only produce PI(3)P (Leevers, Vanhaesebroek, & Waterfield, 1999).

The phosphorylation of PtdIns introduces an opportunity for proteins to bind to the cellular membrane, and proteins interact with membrane PtdIns through specific lipid-binding domains. Two domains that are known to bind to phosphorylated PtdIns are Pleckstrin homology (PH) and FYVE domains. PH domains, approximately 100 to 120 amino acids in length, bind to inositol lipids through the inositol head groups (Lemmon, Ferguson, O'Brien, Sigler, & Schlessinger, 1997). They function to aid intracellular signaling and are constituents of the cytoskeleton (Ferguson, Lemmon, Schlessinger, & Sigler, 1995). PH domains can be found on proteins such as AKT3. Its activation is dependent on PI(3)K, producing PtdIns on the inner plasma membrane for the AKT3 PH domain (Klippel, Kavanaugh, Pot, & Williams, 1996).

FYVE domains are also implicated with the binding of phosphatidylinositols, such as in the case of the FYVE domain within the EEA1 protein, which binds PI(3)P located on endosomal vesicle membranes (Simonsen, et al., 1998). Although most FYVE domains bind PI(3)P, not all PH domains bind to the same lipid, due to differences in PH domain structures. (Yu, et al., 2004). Specific protein PH domains interact with different lipids and it is important to be able to determine to what lipid a given PH domain will interact.

Earlier research analyzed PH and FYVE domains from previously unstudied proteins. This was accomplished by utilizing luciferase fusion proteins to monitor binding between specific FYVE and PH domains and a panel of lipids (Balduc & Vande Kamp, 2013). Luciferase is a term for a class of enzymes that emit light in the form of bioluminescence. It can be found in fireflies such as *Photinus pyralis* (Gould & Subramani, 1988). By using polymerase chain reaction and restriction enzymes, a fusion protein is created through introducing the genetic codes for each PH domain from the selected proteins into a plasmid that contains the DNA sequence for the luciferase enzyme. The plasmid containing the fusion protein is cloned through bacterial transformation and extracted. The goal of this project was to research the binding affinity of known PH domains with a variety of PtdIns. By using ATK3, PLEK2, and DAPP1 PH domains, the method can be validated if expected results are achieved. AKT3 is known to bind with a high affinity to PI(3)P and PI(3,4)P₂. PLEK2 is known to have strong binding specificity towards PI(4,5)P₂ and DAPP1 an affinity for PI(3)P, PI(3,4)P₂, and PI(3,4,5)P₃ (Park et al., 2008). Previous research also found that PH domains in general have a strong affinity for PI(3)P and PI(4)P lipids (Balduc & VandeKamp, 2013). The binding affinities can be detected via luminescence by utilizing fusion proteins consisting of fused cDNA for PH domains from the selected proteins and luciferase enzyme DNA.

The fusion proteins will allow detection of whether or not the protein domain is binding to a lipid; incubation of the proteins with the lipids will permit them to bind and excess will be washed away. By adding a luciferin solution, the luciferase will emit light that can be detected in a luminometer; the amount of light emitted will infer the strength of the interaction.

Methods

Bacterial transformation

Bacterial transformation (heat-shock method) was used to transform ligated plasmids containing PH domain-luciferase fusion proteins into BL21 *E. coli*. The transformed bacteria were then incubated for one hour at 37°C. The bacteria were spun down and re-suspended, then plated onto nutrient agar plates containing kanamycin. The *E. coli* were allowed to grow overnight to allow for the formation of colonies.

Mini-prep

The mini-prep method was used to isolate the plasmid from the transformed *E. coli* to determine whether the proper DNA was present. Colonies from the kanamycin plates were selected and placed in tubes of LB broth and grown at 37°C overnight. Bacterial cells were spun down and a lysis solution was added to break the cells open. A neutralizing agent was added to bring the pH to a more neutral state, after which a mini-prep resin was added to bind the soluble plasmids. The resin was washed to rid of any unwanted cellular components, and then the DNA plasmid was eluted and collected in a catch tube. The plasmid was cut with HindIII and NotI (AKT3 and DAPP1), and KpnI and NotI (PLEK2), then run on an agarose gel to analyze whether it contained the fusion protein.

Protein purification

Bacterial cultures that contained the PH domain-luciferase fusion protein plasmids were used for purification. The cultures were grown overnight, centrifuged, the supernatant removed, and the spun down pellet was sonicated to break open the bacteria. The bacterial lysates were spun down and a nickel resin slurry was added. The tubes were rotated in a refrigerator for 30 minutes, centrifuged, the supernatant removed, and washed with a buffer twice. The fusion protein was eluted by adding a 300 mM imidazole wash and the solution was removed and placed in a tube to be snap-frozen and stored in the -80°C freezer. Presence of the domains was determined by running the proteins on an SDS-PAGE gel and performing a Western Blot.

Incubation with PIP strips

A panel of 15 different lipids appearing as separate dots was ordered on PIP strips from Echelon Inc. Each lipid was removed from the strip via a hole-punch and placed in a tube. The lipid tubes were washed with a buffer consisting of Tris (pH 8), Tris (pH 7), BSA, and dionized water. The fusion protein solution, consisting of the protein domain and buffer, was added to the lipid at 20 second intervals. The protein solution was removed at the same 20 second intervals after 20 minutes of incubation to ensure equal exposure among each lipid/protein combination. Luciferin solution was added to the tube to induce glowing and the light emittance was read in a luminometer.

Results

Production of the fusion proteins was determined using SDS-PAGE gel and a Western Blot. The SDS-PAGE gel contained multiple bands and the process of a Western Blot was used to ensure that the desired PH domains were present. The Western-Blot process is capable of selectively transferring PH domains to a nitrocellulose strip. Figure 1 shows the fusion protein

bands corresponding to the PH domains of AKT3, PLEK2, and DAPP1 in each of the lanes. The first lane is luciferase without a PH domain fused to it and it was used as a control. Luciferase is shown to have a molecular weight of 62 kD; the actual molecular weight of luciferase is 62 kD. The molecular weights of AKT3, PLEK2, and DAPP1 are 68 kD, 68 kD, and 65 kD, respectively. Since the bands corresponding to the luciferase/PH domain fusion proteins are shifted up from the luciferase control, it indicates the luciferase is fused to the PH domains. Each of the bands are at roughly the same intensity, indicating that the fusion proteins eluted at equivalent levels.

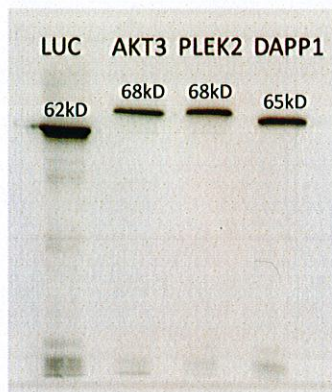


Fig. 1: Western Blot of Luciferase and AKT3, PLEK3, and DAPP1 PH Domain Luciferase Fusion Proteins. Fusionproteins were detected using an anti-His tag antibody by standard Western Blot techniques.

Nitrocellulose PIP strips that contained phosphatidylinositol lipids were used for incubation with the fusion proteins. The PIP Strips were purchased from Echelon Inc, and appeared as shown in Figure 2. Each lipid was removed from the strip with a hole punch to be incubated with the fusion proteins, allowing for maximum exposure of the PH domain to the lipid.

Lysophosphatidic Acid (LPA)	○	○	Sphingosine-1-phosphate (SIP)
Lysophosphocholine (LPC)	○	○	PtdIns(3,4)P ₂
PtdIns	○	○	PtdIns(3,5)P ₂
PtdIns(3)P	○	○	PtdIns(4,5)P ₂
PtdIns(4)P	○	○	PtdIns(3,4,5)P ₃
PtdIns(5)P	○	○	Phosphatidic Acid (PA)
Phosphatidylethanolamine (PE)	○	○	Phosphatidylserine (PS)
Phosphatidylcholine (PC)	○	●	Blue Blank

Fig. 2: Nitrocellulose PIP Strip Key.
 Each PIP Strip contained 15 lipids and a blue blank to be used as a control when incubating the fusion proteins.

The binding affinities from each PH domain fusion protein are illustrated in Figure 3. Light emittance from the blue blank is considered background and used as a control, and data from all three sample sets was normalized against the blue blank. AKT3 bound with a high affinity to PI(3)P and had a 5.6 fold higher luminescence in comparison to the blue blank. PI(3,4)P₂ and phosphatidic acid had a 5.1 fold high luminescence. DAPP1 bound to phosphatidylinositol with a 5.9 fold luminescence, PI(3)P with a 4.0 fold and PI(3,5)P₂ with a 4.2 fold higher. PLEK2 interacted strongly with phosphatidic acid and PI(4,5)P₂, which had a relative luminescence that was 4.2 fold higher than the blue blank. PI(3,5)P₂ had a luminescence that was 4.0 fold higher and PI(3)P had an luminescence that was 3.0 fold higher.

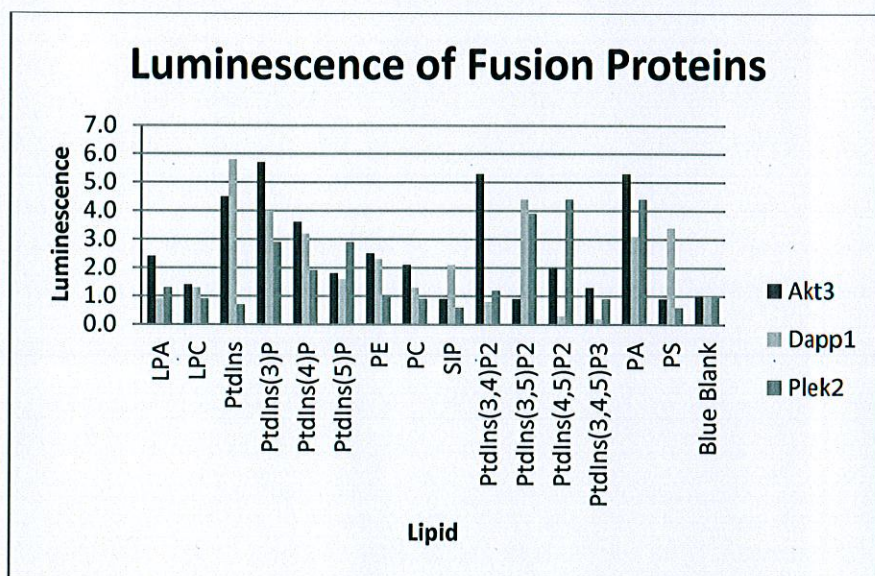


Fig. 3: Luminescence Detected from Fusion Proteins Bound to PIP Strips. The levels of light emittance from bound PH domains and lipids are compared against a blank.

Discussion

Exploring the interactions between lipids and protein domains is crucial because it is a key factor in understanding biological functions of a protein at a cellular level. Different proteins interact with different lipids and identifying interactions between unknown PH domains and PtdIns suggest biological implications for the interactions.

In comparison to previous methods using PIP strips, the method presented in this paper is faster at detecting interactions between protein domains and lipids. Typically, the PIP strips are used with primary and secondary antibodies to detect fusion protein binding. However, the required washing of the PIP strips while changing antibodies can remove the bound PH domains from the lipids. With the method utilized in this paper, no antibodies are used; only a solution to detect luminescence. The new method is faster and there is an improved chance of detecting fusion protein/lipid binding.

For attempting to validate this method, luciferase fusion proteins were generated for Pleckstrin homology domains from three proteins, AKT3, PLEK2, and DAPP1. These proteins were chosen because they are known to have high binding affinities for specific lipids. AKT3 was expected to bind with a high affinity for PI(3)P and PI(3,4)P₂; the results from this new method are consistent with the expected. The highest luminometer readings were for that of PI(3)P and PI(3,4)P₂. Previous research indicates that PLEK2 strongly interacts with PI(4,5)P₂; readings from the luminometer during this method suggest that PLEK2 binds with the highest affinity to phosphatidic acid and PI(4,5)P₂. PI(3,5)P₂ and PI(3)P also bound to PLEK2. DAPP1 was expected to interact with PI(3)P, PI(3,4)P₂, and PI(3,4,5)P₃. While DAPP1 did bind with PI(3)P, it did not interact strongly with PI(3,4)P₂ and PI(3,4,5)P₃. It did however demonstrate interactions with phosphatidylinositol and PI(3,5)P₂. The unexpected binding results achieved,

such as all three PH domains binding to phosphatidic acid, could be due to the fast completion time of this new method; there is a possibility that previously undetected interactions could now be identified. Other unexpected results, such as DAPP1 not binding strongly to PI(3,4)P2 and PI(3,4,5)P3 could be due to human error with the sample tubes.

The results of the binding interactions demonstrate the differences between the PH domains on each protein and with which lipid they prefer to interact. The results from this new method are mostly consistent with previous findings when considering AKT3 and PLEK2, however DAPP1 interacted with only one of the three expected lipids. Because of the comparisons between new and previous results, this luciferase fusion protein method should be tested with more trials in order to validate it as a biologically helpful tool for researching the interactions between lipids and protein domain binding.

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