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Determining selectivity of phosphoinositide-binding domains

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Abstract

The burgeoning of phosphoinositide-binding domains and proteins in cellular signaling and trafficking has drawn laboratories from a wide variety of fields into the study of lipid interactions with peripheral membrane proteins. Many different approaches have been developed to assess phosphoinositide binding, some of which are more problematic than others, and some of which can be quantitated more readily than others. With a focus on the methods used in our laboratory, we describe here the considerations that need to be taken into account when establishing—and quantitating—the specific binding of a protein or domain to phosphoinositides in membranes. We also discuss briefly a few examples in which no clear consensus has yet been reached as to the specificity of a given domain or protein because of discrepancies between different commonly used approaches.

Keywords: Phosphoinositide; PH domain; Surface plasmon resonance; Centrifugation; Lipid binding; Vesicle; Membrane; Inositol

1. Introduction

The number of proteins and small domains known to bind membrane phosphoinositides has increased dramatically over the past decade [1,2]. Of the 13 most populous classes of signaling interaction domain found in the human proteome [3], members of more than half have been reported to drive reversible membrane association by such interactions [1,2,4,5]. Domains that have been implicated in headgroup-specific recognition of phosphoinositides include pleckstrin homology (PH) domains [6,7]; phagocyte oxidase (phox) homology (PX) domains [8]; FYVE domains (for *F*ab1, *Y*OTB, *V*ac1 and *E*EA1) [9]; epsin or AP180 N-terminal homology (ENTH/ANTH) domains [10,11]; and plant homeodomain (PHD) zinc fingers [12]. In addition, phosphoinositide binding has been reported for PDZ (for *P*ostsynaptic density protein, *D*isc large, *Z*ona occludens) domains [13], FERM (for band *F*our-point-one, *E*zrin, *R*adixin, *M*oesin) domains [14], Tubby [15], and MARCKS [16] proteins.

It has not always been straightforward to reach a consensus as to whether a given domain is capable of specific and high affinity phosphoinositide binding. It is likely that the literature harbors several examples of reported phosphoinositide interactions that are not functionally important. Moreover, there are several cases in which the precise headgroup specificity of a particular domain is not agreed upon. With the burgeoning use of purportedly specific phosphoinositide-binding domains as cellular probes for analyzing distribution of the respective lipids [5,17], defining these specificities is especially critical. Active laboratories in this field differ significantly in their preferred methods for assessing the affinity and specificity of the phosphoinositide binding domains listed in the previous paragraph. The lack of 'standardization' of methods has advantages and disadvantages. An important benefit is that well-established phosphoinositide-binding domains have been studied in many different ways, so that affinities and specificities have been compared and reassessed under a plethora of conditions. A negative, however, is that proposals of new specificities have often been based on application of a single commonly used method that has known drawbacks. In any case, it should be insisted upon that any report of phosphoinositide-binding specificity must utilize

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two or more of the commonly used approaches, with at least one being applied to determine an apparent dissociation constant for the binding event. In the following we discuss some of the most commonly used methods, describing the approaches used in the Lemmon laboratory. We also cite examples in which there is a consensus among different laboratories as to the specificity of a particular phosphoinositide-binding domain or protein, as well as cases in which there is disagreement between groups.

2. Description of methods

2.1. Lipid state and context: an important consideration

The first issue to consider when comparing methods for analyzing phosphoinositide specificity and affinity is the state and context of the phospholipid that is presented to the putative binding domain. Some commonly applied methods assess protein binding to pure phosphoinositide that has been immobilized in one way or another. For example, commonly used 'fat blots' [18,19] or dot-blots [20] employ pure dipalmitoyl phosphoinositide that has been dried on to a nitrocellulose support. Other immobilization procedures can be used [21]. For example, biotinylated phosphoinositides can be immobilized on solid streptavidin beads or plate supports, and protein binding to these substrates can be assessed [22]. An alternative approach, used by Rameh *et al.* [23] in one of the first assessments of PH domain specificity, is to employ labeled soluble short-chain (dioctanoyl or dibutanoyl) phosphoinositides and to assess their binding to immobilized protein. In each of these cases it should be appreciated that the phosphoinositide is presented to its potential binding partner in a clearly non-physiological context. First, the phosphoinositide is not present in a lipid bilayer as it would be in any cellular situation. Second, the effective local concentration of the phosphoinositide when used in the majority of these approaches will be substantially greater than is ever likely to be reached *in vivo*, where phosphoinositides are effectively 'diluted' by much more abundant components of cellular membranes including phosphatidylcholine and phosphatidylserine. Although it seems reasonable to expect that approaches utilizing pure phosphoinositide in this way will be prone to artifacts, these methods do lend themselves to a relatively high throughput, and so can nonetheless be of great value for first-pass assessments of phosphoinositide binding and potential specificity. It should be stressed, however, that their value is limited to this first pass.

The real biological question (in most cases) is whether or not a given domain or protein can be recruited specifically to an intracellular membrane in which a particular phosphoinositide accounts for perhaps 1% (at the very most) of all phospholipid molecules. The other >99% of 'background' phospholipid molecules (using values for the inner leaflet of the erythrocyte plasma membrane) are phosphatidylcholine (~35%), phosphatidylserine (~25%), phosphatidylethanolamine (~40%). A good argument can therefore be made for using physiologically 'representative' lipid mixtures in membrane bilayer form for assessing phosphoinositide binding by different domains and proteins. Several laboratories have taken this approach. We have often used 1–3% (mole/mole) of the phosphoinositide of interest in a background of pure phosphatidylcholine, to reduce background binding to negatively charged phosphatidylserine and for ease and reproducibility in vesicle preparations. Although the validity of any particular lipid mixture must always be questioned, a strong argument can be made that any vesicle-based assay is much more representative of the *in vivo* situation than any approach based on pure phosphoinositides. It is therefore essential to insist that *any* study that suggests specific phosphoinositide recognition and is based on studies with pure phosphoinositides be repeated with membrane mimetics. Both approaches have their place, but the key is to use multiple techniques.

2.2. Fat blots/dot-blots/lipid Westerns for analyzing binding to pure immobilized phosphoinositides

Perhaps the most commonly used (and arguably abused) method of assessing phosphoinositide binding specificity is to spot phosphoinositides onto nitrocellulose membranes, and to determine the extent to which a protein of interest will interact specifically with the phosphoinositide-bearing spots [18,20,24]. A similar approach was employed for immunological detection of glycosphingolipids over a decade previously [25]. Based on the studies of Dowler *et al.* [18], Echelon Inc. began in the late 1990s to market PIP Strips™, in which an array of phosphoinositides are pre-spotted (100 pmoles per spot) on nitrocellulose. We have assessed several different approaches for lipid dot-blots in our laboratory, focusing primarily on protein detection issues and minimizing the length of time for which the lipid-bearing nitrocellulose filters are incubated in aqueous buffer. We produce our own lipid-bearing nitrocellulose filters, and probe them with ^{32}P-labeled GST fusion proteins containing the domain of interest. Although this approach has the disadvantage of utilizing radioactivity, we have found the direct visualization method (with ^{32}P-labeled protein) to be more reproducible than alternatives involving indirect detection of the fusion protein with, for example, anti-GST antibodies.

As with all related approaches, this approach is very sensitive, but cannot be quantitated reliably. It should therefore only be used as a first-pass to determine whether binding to phosphoinositides (or other lipids) can be detected at all. As described in our genome-wide analyses of *Saccharomyces cerevisae* PH and PX domains [26,27], we have often detected phosphoinositide binding using this method that is too weak to be measured by any of the more quantitative approaches outlined later in this article. Other laboratories have reported similar findings [28]. A 'positive' result in this assay, while suggestive, must therefore be viewed with great skepticism. Such an outcome may simply