



Investigation of the phosphatidylserine binding properties of the lipid biosensor, Lactadherin C2 (LactC2), in different membrane environments

Kathryn Del Vecchio¹ · Robert V. Stahelin^{1,2}

Received: 15 January 2018 / Accepted: 31 January 2018
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Abstract

Lipid biosensors are robust tools used in both *in vitro* and *in vivo* applications of lipid imaging and lipid detection. Lactadherin C2 (LactC2) was described in 2000 as being a potent and specific sensor for phosphatidylserine (PS) (Andersen et al. Biochemistry 39:6200-6206, 2000). PS is an anionic phospholipid enriched in the inner leaflet of the plasma membrane and has paramount roles in apoptosis, cells signaling, and autophagy. The myriad roles PS plays in membrane dynamics make monitoring PS levels and function an important endeavor. LactC2 has functioned as a tantamount PS biosensor namely in the field of cellular imaging. While PS specificity and high affinity of LactC2 for PS containing membranes has been well established, much less is known regarding LactC2 selectivity for subcellular pools of PS or PS within different membrane environments (e.g., in the presence of cholesterol). Thus, there has been a lack of studies that have compared LactC2 PS sensitivity based upon the acyl chain length and saturation or the presence of other host lipids such as cholesterol. Here, we use surface plasmon resonance as a label-free method to quantitatively assess the apparent binding affinity of LactC2 for membranes containing PS with different acyl chains, different fluidity, as well as representative lipid vesicle mimetics of cellular membranes. Results demonstrate that LactC2 is an unbiased sensor for PS, and can sensitively interact with membranes containing PS with different acyl chain saturation and interact with PS species in a cholesterol-independent manner.

Keywords Biosensor · Cholesterol · Lactadherin · Lipid-protein interaction · Phosphatidylserine · Surface plasmon resonance

Introduction

As lipids gain recognition as both structural molecules as well as components of signaling processes, it is important to be able to monitor quantity, speciation, and cellular location of lipids. Lipid sensors are powerful and robust tools to monitor location and relative quantity of lipid species in various membranes. Many lipid species have associated biosensors that have greatly expanded the toolkit of lipid sensing: the ENTH domain (Yoon et al. 2011) and PLC δ PH (Varnai and Balla 2006) as proxy for PI(4,5)P₂; Spo20 as a phosphatidic

acid sensor (Ferraz-Nogueira et al. 2014); the phox homology (PX) domain of p40^{phox} as a detector for phosphatidylinositol 3-phosphate (PI(3)P) (Ellson et al. 2001); SidM for PI(4)P (Hammond et al. 2014), D4H for cholesterol (Maekawa and Fairn 2015), and many others summarized elsewhere (Stahelin et al. 2014; Stahelin 2009; Szentpetery et al. 2009; Lemmon 2008).

Protein biosensors as mentioned above are often used to identify specific organelles in the cell during imaging experiments, as each organelle has a fine-tuned lipid composition (described in detail in a 2008 review, van Meer et al.) (van Meer et al. 2008). The segregation of lipid species to select organelles can also provide the basis for organelle-selective labeling using native lipids as a proxy for each organelle (Behnia and Munro 2005). While the primary site of lipid synthesis is at the endoplasmic reticulum, lipids are trafficked by intracellular proteins as well as within vesicles to their final destination. Phosphatidylinositol and its subsequent phosphoinositol phosphates, for example, make up a very small percentage of total phospholipids within the cell, but

✉ Robert V. Stahelin
rstaheli@purdue.edu

¹ Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556, USA

² Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907, USA

pools of PI(4,5)P₂ are enriched in the plasma membrane, while PI(4)P resides within the plasma membrane and Golgi, and PI(3)P can be found in most enriched at endosomes.

Phosphatidylserine has been shown to be the most abundant anionic lipid at the plasma membrane, but is notably enriched in the cytosolic-facing leaflet, and bilayer asymmetry is maintained by ATP-dependent lipid flippases. It is this organelle-specific enrichment of phospholipids that prime the use of lipid biosensors as organelle markers. In 2000, Andersen et al. described the C2 domain of the Lactadherin protein as a selective and powerful biosensor for the anionic phospholipid phosphatidylserine (PS) (Andersen et al. 2000). Shortly after this discovery, the Grinstein lab developed a fluorescently labeled LactC2 for use in imaging experiments (Yeung et al. 2008; Leventis and Grinstein 2010). Many other groups have used LactC2 in cellular and *in vitro* experiments for PS detection (Baetz and Goldenring 2014; Faure et al. 2013; Yeung et al. 2008; Yoon et al. 2011; Adu-Gyamfi et al. 2015). While LactC2 has proven a robust tool especially in the realm of cellular imaging, no studies have been done to characterize the biophysical properties of LactC2 *in vitro* lipid binding: namely, the contributions of other phospholipids and sterols on membrane binding ability as well as the LactC2 selectivity for various phosphatidylserine species. If we are to use this robust biosensor in a variety of different *in vitro* and *in vivo* assays, we need to understand both the nuances and limitations of LactC2-PS binding.

While LactC2 has been used in mammalian cells, many other model organisms have drastically different lipid profiles, and this sensor will likely behave commensurably differently in terms of localization. In *S. cerevisiae*, for example, the primary lipid class was identified in 2009 to be ergosterols and ceramides, with only about 1.8% PS in the total membrane extracts (Ejsing et al. 2009). A 2012 analysis expanded on this work to enumerate the differences in phospholipid abundance among *S. cerevisiae* and four other strains of yeast (Hein and Hayen 2012). This work is important for outlining the possibility of stark lipidomic differences from organism to organism within the same family. Similar lipidomic analysis has been done for *Drosophila* (Niehoff et al. 2014; Parisi et al. 2011) and mammalian cell lines (Dennis et al. 2010; Nielsen et al. 2017; Sampaio et al. 2011). This work begs caution and attention when selecting lipid biomarkers for work within organisms whose nuanced lipid profiles are not enumerated. To assess LactC2 PS selectivity based upon PS acyl chain composition and the fluidity of the membrane environment, we employed lipid binding studies to determine the apparent affinity of LactC2 for PS containing membranes of different compositions. Results indicate LactC2 is a robust PS containing membrane biosensor regardless of PS acyl chain composition or membrane fluidity.

Materials and methods

Protein expression and purification

The LactC2 expression plasmid was a kind gift from Dr. Sergio Grinstein (University of Toronto). 6×-Histidine tagged LactC2 was expressed and purified as previously described (Yeung et al. 2008). Briefly, LactC2 in pET28a vector was transformed into BL21-DE3 *E. coli* and grown in liquid LB culture with shaking (200 rpm) at 37 °C, until OD₆₀₀ reached 1.0. Bacterial cultures were then induced with 10 μM IPTG for 3 h at 25 °C with shaking at 200 rpm. Cells were pelleted via centrifugation and then lysed in lysis Buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM Imidazole, pH 8.0) containing 1 mg/mL Lysozyme (Thermo Fisher) and protease Inhibitor (Thermo Fisher). Clarified lysates were applied to a Ni-NTA gravity flow column (Qiagen, Germantown, MD), washed successively with lysis buffer containing either 30 mM, 75 mM, or 130 mM imidazole, and finally eluted with lysis buffer containing 300 mM imidazole. Eluted LactC2 protein was dialyzed against 10 mM HEPES, 160 mM KCl, pH 7.4 overnight at 4 °C. Protein was then concentrated using a 10 kDa concentrator (Millipore) and analyzed for purity using 12% SDS-PAGE. Protein concentration was determined through the Micro-BCA assay (Pierce). Protein was then stored in aliquots at 4 °C until ready for use, and protein was used within 6 weeks of purification.

Liposome preparation

All of the following lipids were purchased from Avanti Polar Lipids (Alabaster, AL): 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE); 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS); 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DPPS); 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS); cholesterol (ovine wool, >98%) (CHOL); L-α-phosphatidylinositol (Soy) (sodium salt) (Soy PI); and N-lauroyl-D-erythro-sphingosylphosphorylcholine (Sphingomyelin).

Liposomes were prepared as described previously (Del Vecchio and Stahelin 2016). Briefly, stock lipids in chloroform were measured at a concentration of 0.5 mM with respect to phospholipid into amber glass vials and dried under nitrogen gas. The films were resuspended in 0.5 mL of 10 mM HEPES, 160 mM KCl pH 7.4 and incubated at 37 °C for 10 min. Liposomes were extruded through a 100 nm pore filter (Whatman, Boston, MA) 45 times using an Avanti Lipid Extruder and then used to coat their respective flow cell on an L1 surface sensor chip (GE Healthcare, Sweden). Lipid coatings of 2500 Response Units or better were used in SPR experiments. Lipid coatings between FC1 and FC2 agreed within 3% prior to collecting experimental data.

Surface plasmon resonance

SPR experiments were performed using a GE Biacore X instrument using the L1 surface sensor chip (GE Healthcare, Sweden). The instrument running buffer was sterilized and degassed prior to use and contained 10 mM HEPES, 160 mM KCl, pH 7.4.

Protein injections ranging from 25 nM up to 20 μ M were prepared by mixing stock purified recombinant hexahistidine-tagged LactC2 (see above) into buffer containing 10 mM HEPES, 160 mM KCl, pH 7.4. 80 μ L samples were injected at a flow rate of 30 μ L/min with an end-injection delay of 120 s to allow for monitoring of dissociation kinetics of protein from the lipid surface. Data were analyzed with BiaEval Software (GE Healthcare/Biacore, Pittsburgh, PA) and plotted with Kaleidagraph (Reading, PA). The apparent K_d of vesicle binding was determined using a non-linear least squares analysis:

$$R_{eq} = R_{max} / \left(1 + \frac{K_d}{C} \right)$$

Where R_{eq} (is measured in response units (RU)) is plotted versus protein concentration (C, for protein concentration injected in each experiment). R_{max} is the theoretical maximum RU response and K_d is the apparent membrane affinity (Del Vecchio and Stahelin 2016; Van Der Merwe 2001). Data were fit using the Kaleidagraph fit parameter of $(m_0 * m_1) / (m_0 + m_2)$; $m_1 = 1100$; $m_2 = 1$.

Results

LactC2 has a mid-nanomolar affinity to vesicles containing 20% POPS

Initial SPR experiments were performed with the simplest mixture of phospholipids to establish LactC2 affinity for vesicles containing 20% POPS. Figure 1a shows sensorgrams for injections ranging from 25 nM to 10 μ M LactC2. The binding to control lipid vesicles (100% POPC) was substantially lower than to POPS containing vesicles and was subtracted out from each respective LactC2 injection to yield the normalized SPR binding sensorgrams shown in Fig. 1a, b. The maximum RU response for each LactC2 concentration was then plotted versus LactC2 concentration to perform a non-linear least squares analysis of LactC2 binding and determine the apparent affinity (K_d) of LactC2 for different types of membranes as shown in Fig. 1a, b.

The apparent affinity (K_d) of LactC2 for POPC vesicles containing 20% POPS was 620 ± 60 nM. In contrast, LactC2 apparent affinity for lipid vesicles containing only

10% POPS as shown in Fig. 1b was reduced by ~2-fold weaker (1560 ± 20 nM). This data and the subsequent SPR affinity data are also summarized in Table 1. Notably, the K_d of LactC2 for vesicles containing 20% POPS established herein is in line with previous reports (Kay et al. 2012; Adu-Gyamfi et al. 2015). Further, the apparent affinity for either 10% or 20% POPS containing vesicles is in the range of other well-known lipid sensors that have been utilized to detect cellular lipids such as phosphoinositides (Ceccato et al. 2016).

LactC2 binding affinity to PS is unaffected by the addition of phosphatidylethanolamine (PE)

While LactC2 displays modest, mid-nanomolar affinity to vesicles containing 20% POPS versus vesicles containing only POPC, this is not an effective physiological comparison. In order to systematically determine the contributory effects of other phospholipids, we next compared LactC2 binding affinity to vesicles containing 20% POPS supplemented with 20% POPE since PE is a major phospholipid present in the plasma membrane inner leaflet where PS is enriched in healthy mammalian cells. Figure 2 displays sensorgrams of LactC2 (25 nM to 10 μ M) binding to POPC:POPE:POPS (60:20:20) containing membranes were the signal from POPC:POPE (80:20) was subtracted as a control. As shown in Table 1 and Fig. 2, LactC2 bound to PE containing PC:PS vesicles with a similar apparent affinity to PC:PS vesicles, suggesting the presence of PE doesn't have a significant effect on LactC2 association with PS containing membranes.

Cholesterol has modest effects on the apparent binding affinity of LactC2 to PS-containing membranes

Cholesterol is a major lipid found in mammalian cells and has been found in highest concentrations in the plasma membrane. Cholesterol can regulate binding of peripheral proteins through several mechanisms including 1) direct interactions, 2) formation of ordered lipid domains (e.g., lipid rafts), and 3) increasing the apparent anionic charge of membranes. Seeing as the addition of an abundant but neutral phospholipid, PE, did not contribute significantly to LactC2-dependent PS binding, we next assessed the effects of cholesterol (CHOL) addition on LactC2 membrane binding. Figure 3 and Table 1 illustrate sensorgrams and summarized data, respectively, indicating that supplementation of cholesterol increased LactC2 apparent affinity for POPS-containing membranes by as much as 20%.

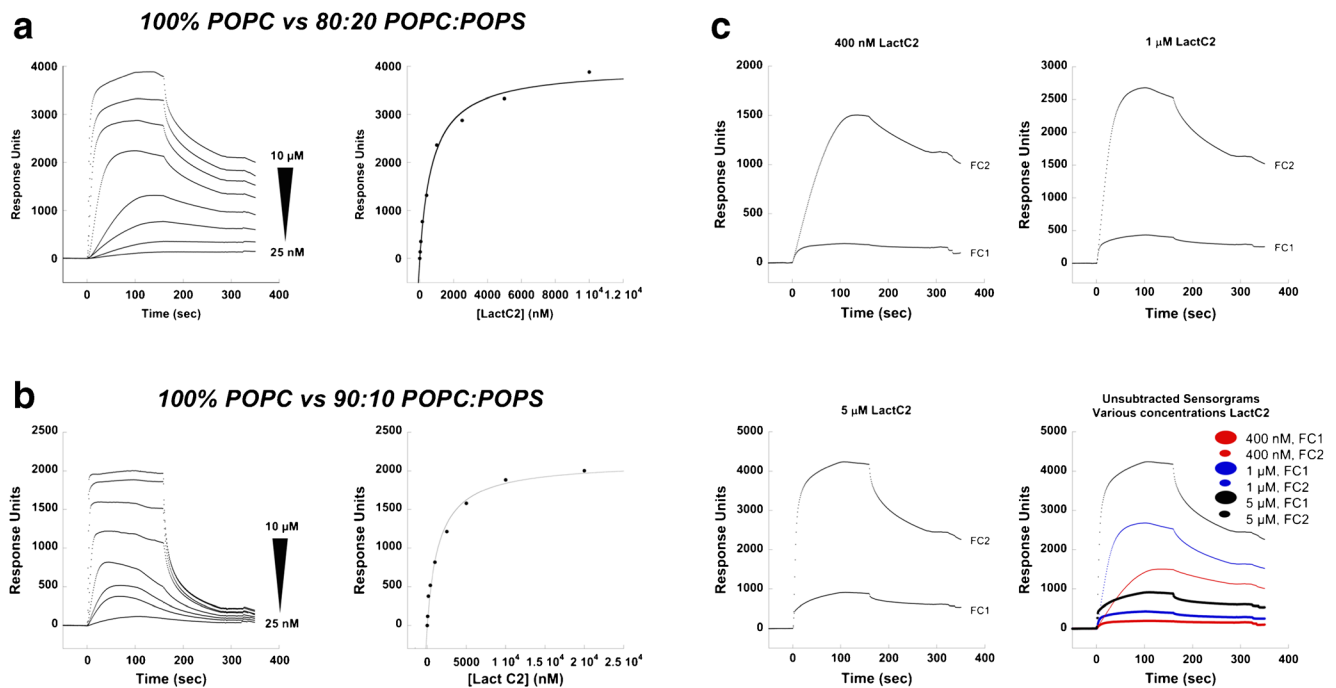


Fig. 1 LactC2 SPR binding sensorgrams and apparent affinity determination to vesicles containing 10% or 20% POPS. SPR was used to determine the binding response of increasing concentrations of LactC2 for POPC vesicles (flow cell 1) or POPC:POPS (90:x) vesicles (flow cell 2) for vesicles containing 20% POPS (**a**) or 10% POPS (**b**) where POPS is added at the expense of POPC. A & B left panels display the binding sensorgrams for normalized response units (RU) versus time (seconds). Normalized response units were determined by subtracting the RU for each respective control experiment with POPC and normalizing the RU to 0 before the point of injection of each experiment. LactC2 was injected at a flow rate of 30 $\mu\text{l}/\text{min}$ at

concentrations of 25 nM, 60 nM, 150 nM, 400 nM, 1 μM , 2.5 μM , 5 μM , and 10 μM . The normalized RU maxima for each LactC2 concentration was then determined and plotted versus LactC2 concentration in order to determine the apparent affinity by non-linear least squares analysis (A & B, right panels). (c) Raw sensorgrams for representative injections of LactC2 at 400 nM, 1 μM , and 5 μM with FC1 (POPC vesicles) and FC2 (POPC:POPS vesicles). Sensorgrams were not subtracted but were normalized to an average RU value of zero prior to LactC2 injection so as to show the low relative binding of LactC2 to FC1 (100% POPC) vesicles. In all cases, FC2 binding is increased from FC1 binding by >130%

Table 1 SPR binding studies were used to determine the apparent affinity (K_d) of LactC2 for lipid vesicles containing different types of PS or different membrane environments. Various mixtures of lipids were investigated to probe the effects of the addition of other lipids (POPE, CHOL); the effects of membrane fluidity (DOPS, DPPS); and the characterization of LactC2 affinity to lipid mixtures representative of physiological endosomes and the plasma membrane

	Flow Cell 1	Flow Cell 2	Apparent K_d (nM)
	100% POPC	80:20 POPC:POPS	620 $\pm$ 60
	100% POPC	90:10 POPC:POPS	1560 $\pm$ 20
	80:20 POPC:POPE	60:20:20 POPC:POPE:POPS	600 $\pm$ 20
	70:30 POPC:CHOL	50:30:20 POPC:CHOL:POPS	570 $\pm$ 30
	90:10 POPC:CHOL	70:10:20 POPC:CHOL:POPS	430 $\pm$ 10
$\uparrow$ fluidity – CHOL	100% POPC	80:20 POPC:DOPS	850 $\pm$ 20
$\uparrow$ fluidity + CHOL	70:30 POPC:CHOL	50:30:20 POPC:CHOL:DOPS	540 $\pm$ 50
$\downarrow$ fluidity – CHOL	100% POPC	80:20 POPC:DPPS	1500 $\pm$ 300
$\downarrow$ fluidity + CHOL	70:30 POPC:CHOL	50:30:20 POPC:CHOL:DPPS	1900 $\pm$ 600
Endosomes vs PM	44% POPC	15.5% POPC	See Fig. 5
	25% POPE	29.5% POPE	
	4.5% Soy PI	4.5% Soy PI	
	10% POPS	21% POPS	
	4.5% Sphingomyelin	4.5% Sphingomyelin	
	12% CHOL	25% CHOL	

80:20 POPC:POPE vs 60:20:20 POPC:POPE:POPS

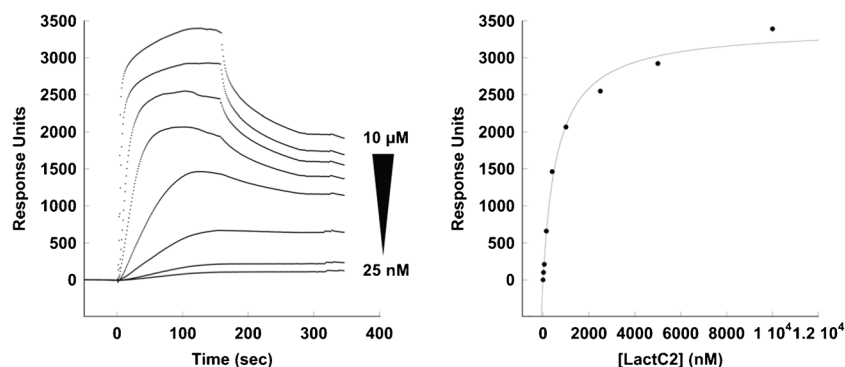


Fig. 2 LactC2 SPR binding sensorgrams and apparent affinity determination to vesicles containing POPC, POPE, and POPS. SPR data showing injections of LactC2 over liposomes containing 60:20:20 POPC:POPE:POPS vesicles (flow cell 2) compared to binding of 80:20 POPC:POPE vesicles (flow cell 1). Left panels display the binding sensorgrams for normalized response units (RU) versus time (seconds). Normalized response units were determined by subtracting the RU for

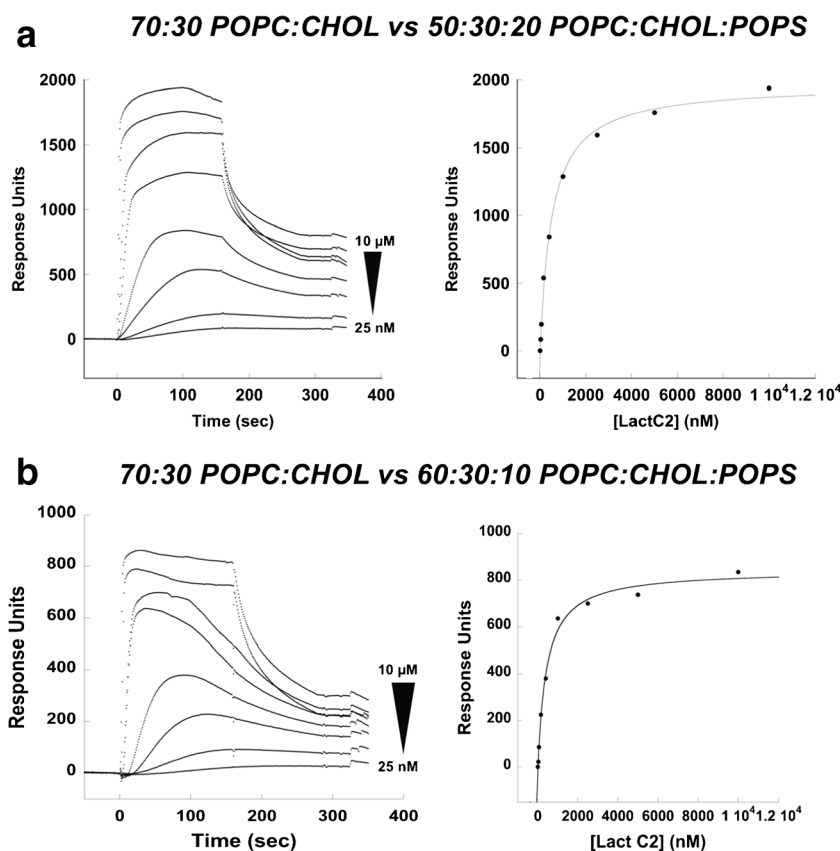
each respective control experiment with POPC:POPE and normalizing the RU to 0 before the point of injection of each experiment. Injections of LactC2 at a flow rate of 30 μ l/min at concentrations of 25 nM, 60 nM, 150 nM, 400 nM, 1 μ M, 2.5 μ M, 5 μ M, and 10 μ M are shown. The normalized RU maxima for each LactC2 concentration was then determined and plotted versus LactC2 concentration in order to determine the apparent affinity by non-linear least squares analysis

LactC2 membrane affinity is reduced for membrane containing saturated PS

Since we characterized the binding affinity of LactC2 to lipid membranes containing POPS, we were next curious about the implications of membrane fluidity and the acyl chain composition of PS. PS in mammals can harbor two acyl chains that

are saturated, diunsaturated, or monounsaturated (i.e., one acyl chain saturated and the other is unsaturated). To determine if LactC2 was able to bind selectively to membranes containing different PS species, we compared binding using SPR and lipid vesicles containing either 20% POPS, 20% DOPS, or 20% DPPS. In these experiments, flow cell 1 (control flow cell) contained vesicles consisting of 100% POPC while flow

Fig. 3 LactC2 binding sensorgrams and apparent affinity determination to vesicles containing POPC, CHOL, and POPS. SPR data showing injections of LactC2 over liposomes containing either (a) 50:30:20 POPC:CHOL:POPS (flow cell 2) or (b) 60:30:10 POPC:CHOL:POPS flow cell 2), compared to binding of 70:30 POPC:CHOL vesicles (flow cell 1). Normalized response units were determined by subtracting the RU for each respective control experiment with POPC:CHOL and normalizing the RU to 0 before the point of injection of each experiment. Injections of LactC2 at a flow rate of 30 μ l/min at concentrations of 25 nM, 60 nM, 150 nM, 400 nM, 1 μ M, 2.5 μ M, 5 μ M, and 10 μ M are shown. The normalized RU maxima for each LactC2 concentration was then determined and plotted versus LactC2 concentration in order to determine the apparent affinity by non-linear least squares analysis



cell 2 consisted of vesicles with POPC:xxPS (80:20). In a similar fashion to the experiments described for POPS and cholesterol above, we also measured the binding of LactC2 to vesicles containing 20% DOPS and 20% DPPS in both the absence and presence of 30% cholesterol.

Figure 4 and Table 1 highlight the binding of LactC2 to vesicles containing diunsaturated or saturated acyl chains. Notably, binding of LactC2 to DOPS containing membranes was similar to POPS containing membranes. However, the starkest difference was a 2-fold reduction in the affinity of LactC2 for DPPS containing membranes compared to membranes with either POPS or DOPS. Thus, LactC2 binds with some selectivity to vesicles containing a double bound on either one or both acyl chains of PS. As with POPS measurements, addition of cholesterol didn't have a major effect on LactC2 membrane binding, with cholesterol increasing LactC2 affinity by ~30% for DOPS containing membranes.

LactC2 binding to membranes with physiologically relevant lipid compositions

While the systematic validation of LactC2 behavior to membranes containing various species of PS yielded interesting and important results with respect to fundamental biophysics, it is imperative that we perform binding assays in a more physiologically-relevant context. We next looked at the binding profile of LactC2 to lipid mixtures mimicking the cytoplasmic leaflet of endosomes versus lipid mixtures mimicking the plasma membrane cytoplasmic leaflet. Several previous studies were able to determine the relative compositions of common intracellular membranes, and it is from this work where we derive the compositions for our experiments (Zachowski 1993; Stahelin et al. 2003; Corbin et al. 2007; Blatner et al. 2004). In this type of experiment, the compositions of flow cell 1 and flow cell 2 in the SPR instrument are very different. Table 1 delineates the percent contributions of the lipids in each mixture. Notably, the plasma membrane mimetic is enriched in PS and cholesterol in comparison to the endosomal membrane.

Initially, we see very little difference in the binding profile of LactC2 for either membrane at low nanomolar concentrations of LactC2 (as per Fig. 5, top-left). However, as LactC2 concentrations increase (i.e., 400 nM and above), we see that there is a similar shape to the association phase of the binding curve, but large differences in RU bound for plasma membrane versus endosomal membranes. For instance, at 400 nM LactC2 there is ~100 RU more binding to the plasma membrane than there is to the endosomal membrane mimetic. Similarly, as LactC2 concentrations increase, a larger RU difference is observed for LactC2 binding to the plasma membrane versus the endosomal membrane mimetic. While the association phase of the binding curves behaves similarly throughout the LactC2 concentration range employed, the

dissociation phase differed above 400 nM LactC2. This suggests the higher concentrations of PS and/or cholesterol in membranes mediate a slower dissociation of LactC2 from the membrane surface or promote rebinding and/or scooting of LactC2 as it dissociates from the membrane surface.

Discussion

PS has many cellular roles as both a membrane lipid and as a component in signaling. PS is enriched in the inner leaflet of the plasma membrane; the bilayer asymmetry of PS and other lipids is maintained by lipid-transporting transmembrane proteins. While the main pool of PS exists in the inner leaflet of the plasma membrane (van Meer et al. 2008), the site of PS synthesis is the endoplasmic reticulum (Vance 2015), where it is shuttled to various parts of the cell; PS is transported to the plasma membrane by proteins Osh6 and Osh7 (Maeda et al. 2013). PS is also found on the cytoplasmic face of endocytic vesicles (Fairn et al. 2011; Kay and Grinstein 2011) with a high enough PS content for LactC2 to label these vesicles. Whereas the majority of PS is localized to the inner leaflet of the plasma membrane, imaging studies performed with LactC2 overwhelmingly implicate specificity for the PM, deeming this protein as an unbiased biosensor.

Because of the differential PS localization in mammalian cells and the presence of PS with different acyl chain compositions, it is important to understand the specificity of lipid probes we use to study PS localization.

The specificity of LactC2 for PS membranes—and the plasma membrane in particular—is relatively dependent on a minimum threshold concentration of PS. Our results indicate that while LactC2 can detect PS in membranes containing only 10% POPS, it is not with as high affinity as LactC2 binding to membranes containing 20% POPS, a concentration more reflective of the environment of the plasma membrane inner leaflet. The sensorgrams in Fig. 1b indicate a much more dramatic off-rate for LactC2 with membranes containing only 10% POPS, thereby displaying a weaker apparent K_d . The addition of 20% phosphoethanolamine (POPE), a neutral phospholipid found in the plasma membrane, did not seem to have an effect on LactC2 membrane binding ability.

Our initial SPR characterization of the LactC2-PS membrane interaction did not include cholesterol, another enriched plasma membrane lipid that has shown to have clustering effects on pools of phosphatidylserine (Bach et al. 1992), and that PS is necessary for proper cholesterol bilayer distribution (Maekawa and Fairn 2015). The concentration of cholesterol in the membrane has a direct effect on the packing formation of PS in membranes (Epand et al. 2001), which could provide some reasoning as to why LactC2 has a higher affinity for membranes containing only 10% Cholesterol (CHOL) as opposed to 30% CHOL.

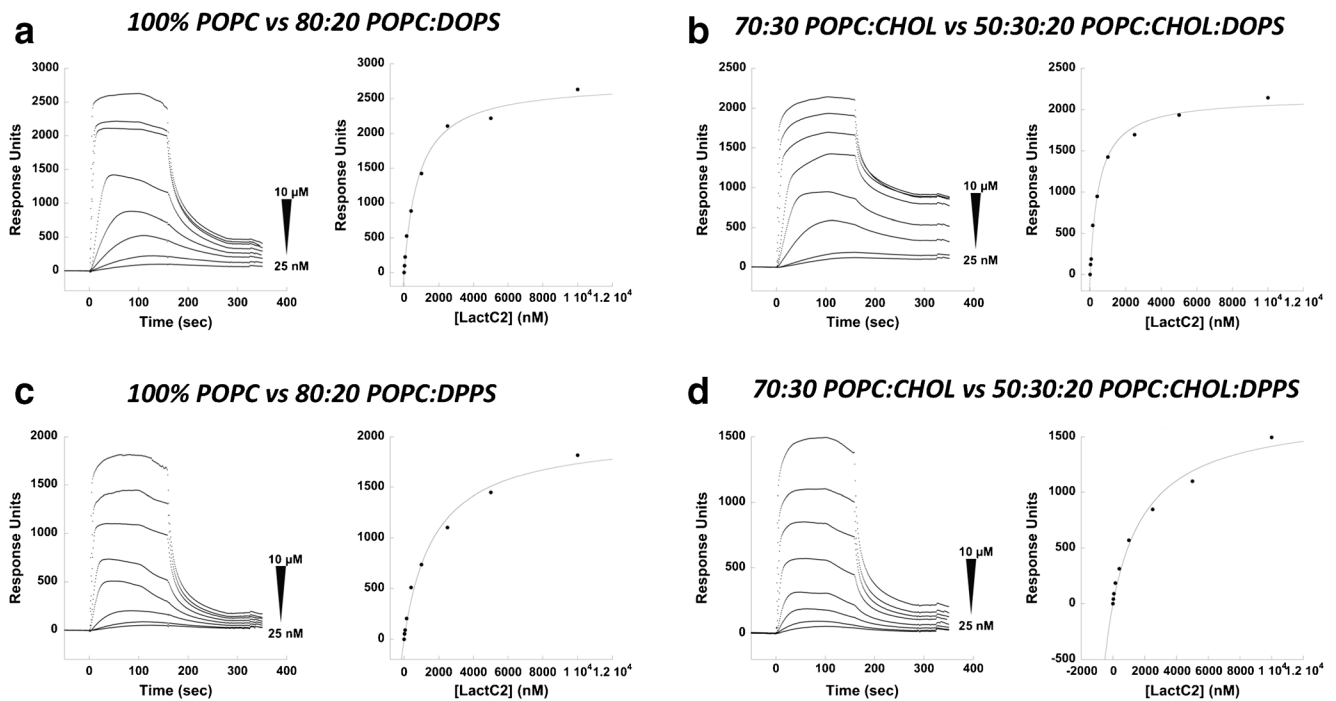


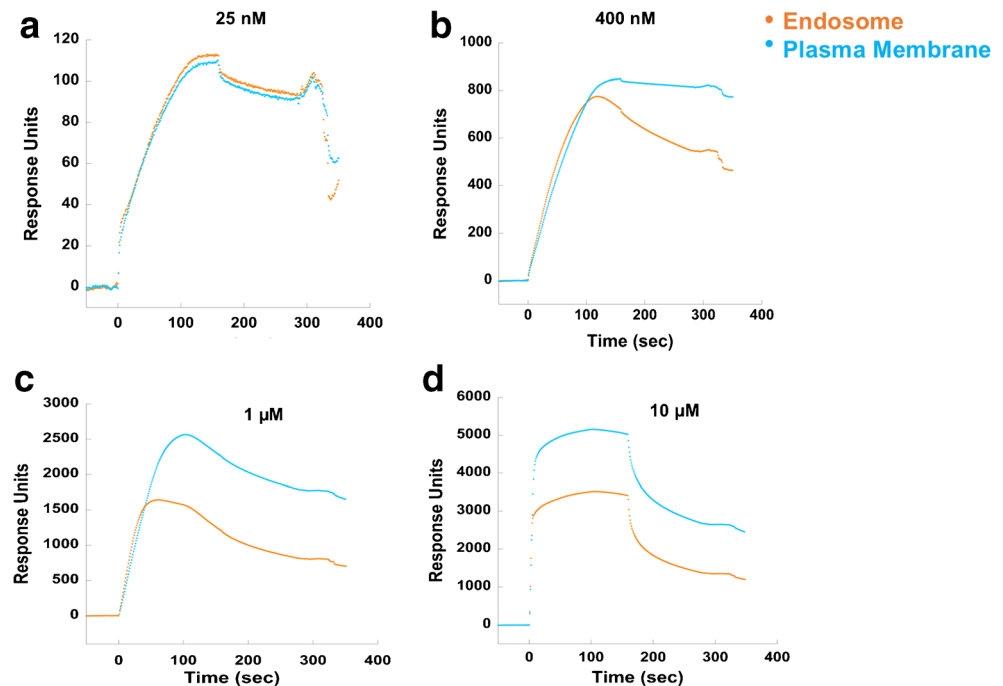
Fig. 4 Effects of PS acyl chain saturation on LactC2-membrane binding. **a & b:** SPR data showing LactC2 binding to “fluid” membranes containing DOPS, (A) without CHOL or (B) with CHOL. **c & d:** SPR data showing LactC2 binding to “rigid” membranes containing DPPS, (C) without CHOL or (D) with CHOL. Normalized response units were determined by subtracting the RU for each respective control experiment (100% POPC or POPC:CHOL (70:30)) and normalizing the RU to 0

before the point of injection of each experiment. Injections of LactC2 at a flow rate of 30 $\mu\text{l}/\text{min}$ at concentrations of 25 nM, 60 nM, 150 nM, 400 nM, 1 μM , 2.5 μM , 5 μM , and 10 μM are shown. The normalized RU maxima for each LactC2 concentration was then determined and plotted versus LactC2 concentration in order to determine the apparent affinity by non-linear least squares analysis

Regardless, addition of CHOL to liposomes modestly enhances LactC2 affinity under some conditions. This suggests that the differing cholesterol content of the plasma membrane

(high cholesterol) versus that of internal membranes and organelles (low cholesterol) would not be a driving factor in LactC2 organelle labeling selectivity.

Fig. 5 SPR binding sensorgrams of LactC2 at varying concentrations to vesicle mimetics of the cytoplasmic leaflet of the plasma membrane or endosomal membranes. SPR binding sensorgrams showing LactC2 binding to membranes mimicking endosomes (orange) or the plasma membrane (blue). Sensorgrams showing injections of 25 nM (**a**), 400 nM (**b**), 1 μM (**c**), and 10 μM (**d**) are shown. Both flow cell 1 (endosome) and flow cell 2 (plasma membrane) were normalized to 0 with respect to RU signal prior to LactC2 protein injection



After discerning the affects that CHOL addition had to LactC2-PS membrane affinity, we next were curious about the contributions of PS acyl chain saturation on membrane permeability and fluidity. It is known that organelles must maintain a delicate, fine-tuned balance of membrane fluidity for function, and dysregulation of membrane fluidity (in the ER, for example) is enough to induce a stress response triggering apoptosis (Deguil et al. 2011; Diakogiannaki et al. 2008). We found that more fluid membranes (i.e. membranes containing DOPS) had similar LactC2 membrane affinity to those containing POPS, and even more so upon addition of CHOL. Recent work by the Faim and Grinstein groups suggests that addition of CHOL to membranes limits charge repulsion and tendency for the membrane to curve, which is a characteristic of flexible, fluid membranes (Hirama et al. 2017). This work suggests that addition of CHOL on fluid membranes can help rigidify and pack lipids that otherwise would be more fluid, providing a binding interface more closely related to that of semi-fluid (i.e. POXX) lipids. However, there was a significant decrease in membrane affinity (albeit small, 2-3 fold) when we tested more rigid membranes containing DPPS, compared to membranes containing POPS. This is not the first noted instance of membrane fluidity playing a part in binding affinity: in 2005, Pande et al. described the necessity of fluid, unsaturated membranes for protein association and insertion into the membrane (Pande et al. 2005). Many other biosensors have not been as carefully characterized for their membrane affinities as they relate to acyl chain composition and the presence of cholesterol.

To further characterize LactC2-membrane interactions, we used SPR to qualitatively assess membrane affinity of a model membrane mimicking the cytoplasmic face of endosomes versus a model membrane mimicking the PM cytoplasmic leaflet. Figure 5 highlights that at low concentrations of LactC2, there is little difference between endosome or PM binding. However, at higher concentrations of LactC2 there is a clear preferential affinity for the PM mimic containing 21% POPS over the endosome mimic containing only 10% POPS. The major difference in binding was an increased off-rate of LactC2 for endosome like membranes suggesting a weaker affinity for LactC2 when PS makes up ~10% of the membrane composition. In addition to the increased PS, the PM mimetic harbors a higher concentration of cholesterol and PE compared to the endosomal mimetic. Thus, the two membrane mimetics likely have different distribution of cholesterol enriched domains some of which may cluster PS thereby increasing the binding of LactC2. The hypothesis that the PM mimetic would have increased regions/clusters of PS and cholesterol enriched domains is also supported by EM imaging of the plasma membrane (Fairn et al. 2011), cellular studies on cholesterol and PS distribution (Maekawa and Fairn 2015) and the rebinding/scooting observed in the dissociation phase of LactC2 from the PM mimetic.

Various groups have described methods using GFP expression as a proxy to quantitate GFP-fusion protein amounts in individual cells (Lo et al. 2015; Soboleski et al. 2005). Even semi-quantitative techniques such as Western Blots are able to provide an estimated idea of GFP-protein expression levels when compared to loading controls. Protein concentrations can vary wildly within the cell depending on protein function and the cell's metabolic state; for example, the concentration of cAMP-dependent protein kinase is 2 μM (Francis and Corbin 1994), and that of calmodulin is as high as 30 μM (Greengard 1984). In imaging experiments using GFP-LactC2, the system operates under LactC2 over-expression (Adu-Gyamfi et al. 2015; Baetz and Goldenring 2014), where minimum detected quantities of cytosolic GFP compared to autofluorescence are ~1 μM in HeLa cells although lower concentrations of GFP targeted to subcellular organelles can be effectively detected (Niswender et al. 1995). With LactC2 being employed with GFP or other fluorescent fusion tags in cellular expression systems, with 1-30 μM expression levels, LactC2 should be sufficient to detect cellular PS at cellular organelles as well as the plasma membrane based upon apparent affinity data collected and analyzed herein.

One unknown in lipid-protein interaction field is the effect of membrane proteins on PS clustering or masking and subsequent effects on lipid binding. Thus, the concentration of membrane proteins in a specific region of cellular membrane may limit the accessible PS binding sites of LactC2. In contrast, membrane proteins could also act as fences in corralling PS into smaller regions of cellular membranes thereby increasing the density of PS for LactC2 binding. While this has been a relatively unexplored area of research it does spark provocative questions that could be answered with high resolution imaging or *in vitro* mimetics of membrane protein crowding in PS containing membranes.

In summary, LactC2 is a potent and selective biosensor for PS in both *in vitro* and *in vivo* applications. The presence of phosphatidylethanolamine does not appear to effect LactC2-membrane binding. The impact of cholesterol within membranes seems to modestly enhance membrane binding only when there is a reduced proportion of PS, which could be due to cholesterol-induced PS clustering, increase in apparent membrane anionic charge, and/or raft domain formation. LactC2 did have a significant change in fluidity-dependent PS binding, where affinity of LactC2 for membranes containing saturated PS (DPPS) or saturated PS (DPPS) with cholesterol was reduced 2-3 fold compared to membranes containing mono- or diunsaturated PS (POPS or DOPS). Thus, it is possible under some cellular conditions that LactC2 may selectively label PS membrane with a more fluid environment as opposed to those enriched with saturated PS. In a direct-comparison, SPR studies with endosome vs plasma membrane mimetics, LactC2 selectively bound plasma membrane mimetics at protein concentrations >200 nM LactC2, implicating

LactC2 as both a potent and selective imaging tool for plasma membrane labeling.

Acknowledgements The authors would like to thank Dr. S. Grinstein for the generous gift of the LactC2 plasmid. K. Del Vecchio would like to thank K. Johnson for thoughtful discussion on LactC2 expression and purification.

This work was possible due to funding from the National Institutes of Health (NIH) Grant AI081077. Kathryn Del Vecchio was also supported by a NIH CDBI predoctoral fellowship (T32GM075762).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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