ProLIF – quantitative integrin protein–protein interactions and synergistic membrane effects on proteoliposomes

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ABSTRACT
Integrin transmembrane receptors control a wide range of biological interactions by triggering the assembly of large multiprotein complexes at their cytoplasmic interface. Diverse methods have been used to investigate interactions between integrins and intracellular proteins, and predominantly include peptide-based pulldowns and biochemical immuno-isolations from detergent-solubilised cell lysates. However, quantitative methods to probe integrin–protein interactions in a more biologically relevant context where the integrin is embedded within a lipid bilayer have been lacking. Here, we describe ‘protein–liposome interactions by flow cytometry’ (denoted ProLIF), a technique to reconstitute recombinant integrin transmembrane domains (TMDs) and cytoplasmic tail (CT) fragments in liposomes as individual subunits or as αβ heterodimers and, via flow cytometry, allow rapid and quantitative measurement of protein interactions with these membrane-embedded integrins. Importantly, the assay can analyse binding of fluorescent proteins directly from cell lysates without further purification steps. Moreover, the effect of membrane composition, such as PI(4,5)P₂ incorporation, on protein recruitment to the integrin CTs can be analysed. ProLIF requires no specific instrumentation and can be applied to measure a broad range of membrane-dependent protein–protein interactions with the potential for high-throughput/multiplex analyses.

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KEY WORDS: Integrins, Liposomes, Protein–protein interactions, Protein–lipid interactions

INTRODUCTION
Lipids provide an essential platform for protein interactions and biochemical reactions at biological membranes. Many techniques are available to assess protein–lipid binding and phosphoinositide (PI) specificity (Zhao and Lappalainen, 2012). Many of these assays and in particular those based on liposome generation – currently considered more representative of the in cellulo situation – need specialised equipment or employ complex protocols (e.g. surface plasmon resonance, isothermal titration calorimetry and lipid microarray) (Ananthanarayanan et al., 2003; Besenich et al., 2006; Lemmon et al., 1995; Saliba et al., 2014; Wu et al., 2012) that restrict their usage to specialised laboratories. Furthermore, these approaches require high lipid/protein concentrations that prevent large and systematic analyses and/or remain merely qualitative. Recently, several microscopy-based methods have been developed (Ceccato et al., 2016; Saliba et al., 2014) that provide quantitative data on protein interactions with liposomes and have the potential for high-throughput analyses. Flow cytometry has also been employed to quantify binding of purified recombinant proteins to liposomes (Temmerman and Nickel, 2009). However, none of these methodologies have been designed to incorporate transmembrane proteins within the lipid bilayer.

It is estimated that transmembrane proteins constitute up to one third of the human proteome (Ahram et al., 2006; Almén et al., 2009) and are essential components of biological membranes, constituting ~50% of the membrane volume (Müller et al., 2008). Transmembrane proteins regulate a plethora of essential cellular events, ranging from signal transduction to the flux of ions and metabolites across the membrane in response to a changing microenvironment. Owing to their functions and accessibility, they represent more than 60% of drug targets (Arimamipathy et al., 2009). In spite of their importance, versatile methodologies to explore protein–protein interactions of transmembrane proteins within an experimentally controlled lipid microenvironment remain underdeveloped.

Integrins, an essential family of heterodimeric transmembrane adhesion receptors, recruit and support the formation of cytoplasmic protein complexes, collectively known as the integrin adhesome, at the plasma membrane to generate the cell machinery responsible for cell adhesion and adhesion-induced signalling and migration (Winograd-Katz et al., 2014). Currently, molecular interactions between integrin and adhesome components are mainly studied by qualitative techniques such as pulldowns using synthetic peptides or soluble recombinant proteins mimicking the integrin cytoplasmic domains. Alternatively, endogenous integrins are immunoprecipitated and predominantly include peptide-based pulldowns and biochemical immuno-isolations from detergent-solubilised cell lysates. However, quantitative methods to probe integrin–protein interactions in a more biologically relevant context where the integrin is embedded within a lipid bilayer have been lacking. Here, we describe a simple, sensitive and quantitative technique called ‘protein–liposome interactions by flow cytometry’ (denoted ProLIF) to simultaneously detect and quantify protein–protein and protein–lipid interactions in reconstituted proteoliposomes. We reconstituted ‘artificial integrins’ into proteoliposomes and investigated talin binding, as it is the most studied protein interacting with both the integrin CT and the plasma membrane
a phosphatidylinositol phosphate (PIP)-dependent manner (Calderwood et al., 2013). We used this interaction to demonstrate the applicability of our method for probing integrin–cytoplasmic-protein interactions in the context of a lipid bilayer of defined composition. We optimised ProLIF towards a mammalian expression system to circumvent the requirement for protein purification, preserve post-translational modifications and to enable the presence of possible essential co-factors to provide a more realistic biological characterisation of protein–protein binding.

RESULTS
Generation of streptavidin-bead-coupled liposomes for FACS detection
We first tested ProLIF by analysing the coupling of bare liposomes, containing a small fraction of biotinylated lipids, to streptavidin-coated carrier beads, according to steps 1, 2 and 4 outlined in the workflow in Fig. 1A. Liposomes are produced by lipid solubilisation in Triton X-100 and subsequent detergent removal by gradual addition of Bio-Beads™ (Rigaud et al., 1995). Although bare liposomes can also be produced through extrusion, giving control over the size of the resulting small unilamellar vesicles (SUWs) (Temmerman and Nickel, 2009), this technique does not allow for incorporation of transmembrane proteins. In contrast, detergent removal by Bio-Beads™ is a robust method that has been used to reconstitute many functional transmembrane proteins (Geertsmal et al., 2008; Kolena, 1989; Lacapère et al., 2001; Moriyama et al., 1984; Mouro-Chanteloup et al., 2010; Nesper et al., 2008; Neves et al., 2009; Richard et al., 1990; Smith and Morrissey, 2004; Young et al., 1997) resulting in unilamellar vesicles (Rigaud et al., 1995). Such vesicles are close to the detection limit of the scatter of laser light in FACS instruments (Temmerman and Nickel, 2009). In order to make these liposomes amenable to standard flow cytometry detection, we incorporated biotinylated lipids (2% of total lipid content) during liposome preparation to enable vesicle capture on Streptavidin–Sepharose (SA) beads that have an average diameter of 34 μm. The SA beads are easily detected in a flow cytometer with forward scatter (FSC) and side scatter (SSC) plots (Figs. S1A). Upon addition of biotinylated liposomes, a distinct population of small objects appears (Fig. S1B); however, this population was gated out during the analysis. Importantly, addition of biotinylated liposomes did not appear to promote bead aggregation, as the forward scatter area (FSC-A) versus forward scatter width (FSC-W) plot demonstrated a single population. To confirm that liposomes were captured by the SA beads, we produced liposomes encapsulating Cy5 dye (Fig. 1B). A strong signal was detected by flow cytometry when the Cy5-encapsulated liposomes were captured on SA beads. Importantly, interactions between SA beads and Cy5-encapsulated biotinylated liposomes could be effectively outcompeted by the addition of soluble biotin (Fig. 1B), confirming specific biotin-mediated binding of liposomes to the carrier beads.

Optimal detection of lipid interactions with proteins isolated from mammalian cell lysates
Protein purification can be time consuming and, depending on the protein production source, critical post-translational modifications regulating protein binding to cell membrane components may be lacking. To overcome this limitation, we tested the suitability of ProLIF to detect membrane interactions of phosphatidylinositol (PI)-binding proteins generated in human embryonic kidney cells (HEK293 cell line). Cells expressing EGFP-tagged PI-binding domains, known to interact with specific PIPs in membranes, were lysed in a detergent-free extraction buffer, and fractions enriched in cytoplasmic proteins and devoid of transmembrane and membrane-associated molecules were isolated by ultracentrifugation (Fig. S1C). To overcome experimental variability due to changes in protein expression levels and to allow comparison between different experimental conditions, the fluorescence intensity of the cytoplasmic fractions were measured in relation to an external fluorescein standard and equalised before the binding assay.

Detergent-free cell lysates were subsequently incubated with liposomes followed by SA beads and then liposome-bound SA beads were analysed by flow cytometry, according to the steps indicated in Fig. 1A. All the cytometer settings (count rate, gates, voltages and trigger strategy) and the sample preparation conditions were kept constant for all samples. Beads were gated based on forward and side scattering, and the fluorescence intensity of the gated population was visualised using a histogram (fluorescence intensity versus particle count) (Fig. 1C,D).

SA beads have a detectable level of auto-fluorescence (Fig. 1C); thus, in each experiment a sample containing beads only was also included and the auto-fluorescence was subtracted from all samples. Thus, the specific fluorescence signal corresponding to EGFP-protein-bound liposomes was obtained.

To determine the conditions providing the best signal-to-noise ratio, decreasing amounts of the phospholipase C-δ1 (PLCδ1) pleckstrin homology (PH) domain (PLC-PH–EGFP), which binds preferentially to phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P$_2$] (Lemmon and Ferguson, 2000) were incubated with a constant amount of bare biotinylated liposomes or PI(4,5)P$_2$-containing biotinylated liposomes, captured on SA-beads and analysed by flow cytometry. The resulting titration data indicated that a concentration close to 8 nM provided a good compromise between achieving an optimal signal:noise ratio and minimising the amount of biological material needed for the experiment (Fig. S1D, see below for the equation for calculating the protein concentration).

Detecting specific protein–lipid interactions
Having established optimal experimental conditions to detect binding of fluorescently tagged proteins to liposomes, we next investigated whether ProLIF could be used to detect well-documented protein–lipid interactions in a reproducible manner. PH domains are broadly expressed in cytosolic phospholipid signalling proteins and are known to promote protein binding to specific lipids in the membrane. We first compared binding of EGFP alone to binding of the EGFP-tagged Bruton tyrosine kinase (BTK) PH domain (BTK-PH–EGFP) to various liposomes. Beads alone were used as a control for autofluorescence (as described above). In addition, bare liposomes (no PI) were compared to liposomes containing 2.5% PI(4,5)P$_2$ or PI(3,4,5)P$_3$. As shown in Fig. 1D,E and Fig. S2A, EGFP alone demonstrated background level binding with the signal intensity remaining similar in all liposome conditions. In contrast, BTK-PH–EGFP bound efficiently to PI(3,4,5)P$_3$ liposomes, whereas binding to PI(4,5)P$_2$ was very low, in line with the previously reported PI specificity for this PH domain (Kojima et al., 1997; Rameh et al., 1997).

To explore the specificity of ProLIF further, we analysed binding of two additional biologically distinct lipid-binding domains to liposomes. The PLCδ1 PH domain binds to PI(4,5)P$_2$, serving as a specific tether that guides the protein to the plasma membrane (Garicia et al., 1995). In contrast, the zinc-finger FYVE domain, found in proteins such as the early endosomal antigen 1 (EEA1), binds phosphatidylinositol 3-phosphate [PI(3)P], which is specifically enriched on endosomal membranes, and fluorescently tagged fusions of tandem FYVE-domains (2xFYVE) serve as faithful reporters of PI(3)P-enriched
membranes in cells (Gillooly et al., 2000; Stenmark et al., 2002). Importantly, the PI specificity of both of these lipid-binding domains was recapitulated with ProLIF. We detected PLC-PH–EGFP binding specifically to liposomes containing 2.5% PI(4,5)P\(_2\) (Fig. 1F; Fig. S2B) and strong binding of a tandem FYVE zinc finger domain to PI(3)P (Fig. 1G).

Fig. 1. See next page for legend.
Fig. 1. ProLIF is a flow cytometry-based assay for detection of specific protein-lipid interactions. (A) Outline of ProLIF workflow. Step 1: Bio-Beads™ are added to lipids solubilised in Triton X-100 to remove the detergent and obtain liposomes. Step 2: Liposomes are incubated with membrane-free cell extract containing the EGFP-tagged protein of interest. Step 3: Strepavidin–Sepharose (SA) beads are added in order to capture the liposomes via interaction with biotinylated lipids present in the lysosome membrane. Step 4: SA beads are analysed by flow cytometry (FACS). Red dots and blue dots represent biotinylated lipids and PIs, respectively. Green fragments represent EGFP-tagged proteins from the cell lysate.

(B) Biotinylated-lipid-containing liposomes were generated with and without encapsulated Cy5 dye, captured on SA beads in the presence or absence of increasing amounts of free biotin and analysed via FACS. The molar ratio between biotinylated lipids and soluble biotin added in each sample is indicated (n=1). (C) Scatter plot and fluorescence histogram from SA beads alone incubated with cell lysate from EGFP-transfected cells and analysed by FACS. (D) Biotinylated-lipid-containing liposomes, with the indicated PI content, were incubated with cell lysates from EGFP alone- or BTK-PH–EGFP-transfected cells (equal EGFP concentrations) and then captured by SA beads and analysed by FACS. Shown are representative dot blots, and size gating in FACS, and histograms depicting EGFP fluorescence intensity (FL1) profiles (note that the axis labels are as in C). The red population in the scatter plot was gated for quantification. Data shown represent three individual experiments.

(E) Binding of the BTK-PH–EGFP domain (from cell lysate) to biotinylated-lipid-containing liposomes, with the indicated PI content, were incubated with cell lysates from EGFP alone- or BTK-PH–EGFP-transfected cells (equal EGFP concentrations) and then captured by SA beads and analysed by FACS. Showed are representative dot blots, and size gating in FACS, and histograms depicting EGFP fluorescence intensity (FL1) profiles (note that the axis labels are as in C). The red population in the scatter plot was gated for quantification. Data shown represent three individual experiments.

(F) Binding of EGFP-tagged PLC-PH domain (from cell lysate) to biotinylated-lipid-containing liposomes, with the indicated PI content, relative to control PI-free liposomes (data are normalised median fluorescence intensities shown as the mean±s.e.m.; n=5 independent experiments).

(G) Binding of tandem FYVE-EGFP domains (from cell lysate) to biotinylated-lipid-containing liposomes, with the indicated PI content, relative to PI-free liposomes (data are normalised median fluorescence intensities shown as the mean±s.e.m.; n=6 independent experiments). **P<0.01, ***P<0.001

Quantitative analyses of protein-lipid interactions

To take the system a step further towards quantitative measurement of protein–lipid interactions, we first devised a way to calculate the concentrations of the EGFP-tagged proteins in the input mammalian cell lysates by using an external fluorescein standard. Based on the measured lysate fluorescence, a mathematical equation (Eqn 1) was derived (see Materials and Methods) to calculate EGFP-tagged protein concentration as follows:

\[
c_{\text{EGFP}} = \frac{F_{\text{EGFP}}C_{\text{EGFP}}\phi_{\text{EGFP}}}{e_{\text{EGFP}}\phi_{\text{EGFP}}F_{\text{Ext}}},
\]

where \(C_{\text{EGFP}}\) and \(C_{\text{EGFP}}\) are the concentrations of external standard (fluorescein) and the EGFP-tagged protein, \(\phi_{\text{EGFP}}\) and \(\phi_{\text{EGFP}}\) are the extinction coefficients of external standard and the EGFP-tagged protein, respectively. To validate this equation, the fluorescence of a recombinant GFP protein of known concentration was measured at serial dilutions and a standard curve was generated. These experimentally derived fluorescence values were inputted into Eqn 1, together with variables and extinction coefficients from the fluorescein standard curve, and GFP concentrations were reverse calculated. Using this approach, a GFP standard curve closely matching the original experimental data was reproduced (Fig. 2A). Mathematically derived standard curves for EGFP-tagged proteins were generated using predicted extinction coefficients (see Materials and Methods) and quantum yields, and the fluorescence intensity of cell lysates expressing EGFP-tagged proteins of interest. Taking advantage of the calculated standard curve for the BTK-PH–EGFP, we incubated predetermined increasing concentrations of BTK-PH–EGFP with liposomes containing 2.5% PI(3,4,5)P3. As expected, and as demonstrated earlier with a similar approach for a recombinant protein (Temmerman and Nickel, 2009), saturation of binding was achieved with increasing protein concentrations. Based on these data, we calculated a \(K_d\) of 174 nM±15.2 (R²=0.95) for BTK-PH–EGFP binding to PI(3,4,5)P3 (Fig. 2B), which is within range of previously reported values (Kojima et al., 1997; Rameh et al., 1997). We performed similar experiments for tandem FYVE domain binding to liposomes containing 2.5% PI(3)P and obtained a \(K_d\) of 33.3 nM (R²=0.81) (Fig. 2C), compared to the reported \(K_d\) of 50 nM for a single FYVE domain (Gillooly et al., 2000; Gaullier et al., 2000). However, while ProLIF is extremely sensitive and can detect protein–lipid interactions at low protein concentrations, we found that unlike approaches that use recombinant proteins (Temmerman and Nickel, 2009), the amount of GFP-fused protein (e.g. PLC-PH–EGFP) extracted from mammalian cell lysates in our approach, is not always sufficient for determining \(K_d\) (data not shown). With this limitation in mind, ProLIF is applicable for specific qualitative and quantitative analysis of biologically distinct protein–lipid interactions of proteins isolated from mammalian cell lysates.

Reconstituting integrin TMDs and CT domains on liposomes

To apply ProLIF to the study of transmembrane protein interactions, we chose integrins as model proteins. Integrin purification requires complex protocols that are not easy to scale up, precluding high-throughput application. For this reason, most of the studies involving purified full-length integrin are restricted to αIIbβ3, given the availability of platelets as a raw source. However, different integrin heterodimers can differ significantly in terms of physiological function and composition of their interactome (Rossier et al., 2012). In order to overcome this limitation, we designed two artificial genes encoding the TMD and CT of the extracellular receptors α5 and β1 integrins and fused these to enhanced N-terminal Jun and Fos heterodimerisation modules (cJun[R]–FosW[E]) (Worrall and Mason, 2011), respectively (Fig. 3A) to promote α5 and β1 integrin pairing (integrins exist as heterodimers on the plasma membrane) in the same orientation. Such modular organisation allows the study of different integrin heterodimers by simply modifying the TMD and cytoplasmic domains. Both Jun–α5 and Fos–β1 integrin chimeras could be purified from membrane fractions when expressed in Escherichia coli by taking advantage of their purification tags, maltose-binding protein (MBP) and glutathione S-transferase (GST), respectively (Fig. S3A,B). When analysed by SDS-PAGE, both Jun–α5 (molecular mass 52.8 kDa) and Fos–β1 (molecular mass 40.7 kDa) protein bands, recognised by specific antibodies raised against the α5 and β1 integrin cytoplasmic domains, appeared at the correct size (Fig. 3B). Moreover, Jun–α5 and Fos–β1 integrins were able to heterodimerise, as demonstrated by reciprocal co-immunoprecipitation (co-IP) assays with antibodies against either the α5 or β1 integrin cytoplasmic domains (Fig. 3B,C).

Next, we reconstituted the Jun–α5 and/or Fos–β1 integrin chimeras in liposomes using the same protocol as described above. The purified proteins, solubilised in mild detergent (see Materials and Methods), were added to the Triton X-100-solubilised lipids, and incorporated into the lipid bilayer during detergent removal via Bio-Beads™ (Fig. 3D). In this system, we lack the means to restrict the orientation of the fusion proteins on the liposomes resulting in ~50% of the reconstituted proteins having their cytoplasmic tails facing outwards. Given the strong affinity of the Jun–Fos dimer, in heterodimer-containing liposomes both α- and β-integrin tails are also expected to face the same way resulting
Talin did not bind to liposomes containing the Jun aggregation, as only a single main population was apparent in the containing the Fos (Fig. S3C), we observed significant talin binding to liposomes (3 nM) that was determined to provide a good signal-to-noise ratio system. Using a concentration of EGFP-tagged talin FERM domain as such, offers an excellent candidate for validating the ProLIF binding surface within its FERM domain (Elliott et al., 2010) and, receptor and recruitment of other proteins. Talin also contains a PI-tails and the subsequent change to the activated conformation of the subunits, an event that is linked to separation of the membrane overcrowding, as reducing the transmembrane protein: lipid molar ratio by 50% (1:7000 instead of 1:3500) preserved the binding pattern (Fig. S4B). The interaction of the talin FERM domain with liposomes was modestly, but significantly, increased when PI(4,5)P2 was included in the liposomes, in line with the affinity of the talin FERM domain for plasma membrane acidic phospholipids (Calderwood et al., 2013). Notably, the presence of PI(4,5)P2 and Fos–β1 integrin in the same liposomes substantially enhanced talin binding far beyond levels observed for each individual component, suggesting an additive and possibly synergistic binding effect, revealed by the ability of the ProLIF system to incorporate membrane-embedded integrins and membrane lipids in the same binding assay. In PI(4,5)P2-containing vesicles, talin FERM binding was reduced when both Fos–β1 and Jun–α5 were present (Fig. 4B). Binding of talin FERM domain to Jun–α5 and PI(4,5)P2 was similar to that in conditions containing PI(4,5)P2 alone, suggesting that the talin FERM–PI(4,5)P2 interaction is preserved despite loss of interaction with the β1 integrin receptor (Fig. 4B). Incubation with an excess of soluble biotin, which outcompetes liposome binding to the beads, resulted in the complete loss of the fluorescence signal (Fig. S4C), serving as an important control and confirming that the signal is only due to binding events occurring at the membrane rather than unspecific binding to the beads.

With ProLIF, we could also observe talin binding to PI(3,4,5)P3 alone, and detected a substantial enhancement in talin binding to PI(3,4,5)P3 and Fos–β1-containing liposomes that was equivalent

in 50% of dimers having the correct orientation. To verify whether the purified proteins were indeed being incorporated into liposomes, we performed a sucrose gradient flotation assay. In the presence of liposomes, the integrin chimeras, as single entities or as components of a heterodimer, were retrieved from the upper sucrose fractions indicating association between the integrin proteins and the lipid bilayer (Fig. 3E). In contrast, in the absence of lipids, protein aggregation was observed, and Fos–β1 was present in the bottom fraction (Fig. 3E). Importantly, when using the Bio-Bead reconstitution method, all protein is incorporated into liposomes, which makes a subsequent purification step unnecessary and helps to streamline the protocol.

The integrin β1 CT and PIPs synergise to recruit the talin head to liposomes

The integrin cytoplasmic domains have no enzymatic activity, and function by recruiting, and binding to, cytoplasmic adaptors and signalling proteins that link the receptor to the actin cytoskeleton (Bouvard et al., 2013). Talin is a classical integrin activator and one of the first proteins recruited to integrin heterodimers at the plasma membrane. The talin FERM domain binds directly to β-integrin subunits, an event that is linked to separation of the α- and β-integrin tails and the subsequent change to the activated conformation of the receptor and recruitment of other proteins. Talin also contains a PI-binding surface within its FERM domain (Elliott et al., 2010) and, as such, offers an excellent candidate for validating the ProLIF system. Using a concentration of EGFP-tagged talin FERM domain (3 nM) that was determined to provide a good signal-to-noise ratio (Fig. S3C), we observed significant talin binding to liposomes containing the Fos–β1 integrin protein (Fig. 4A,B). As expected, talin did not bind to liposomes containing the Jun–α5 integrin subunit alone. Importantly, none of the conditions caused bead aggregation, as only a single main population was apparent in the FSC-A versus FSC-W plots (Fig. S4A). Interestingly, talin binding to the Fos–β1 integrin protein was completely lost when the β1 integrin tail was embedded as part of the integrin heterodimer [(Jun–α5)–(Fos–β1)] within the liposome (Fig. 4B), suggesting that this construct may represent a ‘tails-together’ conformation of the integrin cytoplasmic face. This inhibitory effect was not due to membrane overcrowding, as reducing the transmembrane protein: lipid molar ratio by 50% (1:7000 instead of 1:3500) preserved the binding pattern (Fig. S4B). The interaction of the talin FERM domain with liposomes was modestly, but significantly, increased when PI(4,5)P2 was included in the liposomes, in line with the affinity of the talin FERM domain for plasma membrane acidic phospholipids (Calderwood et al., 2013). Notably, the presence of PI(4,5)P2 and Fos–β1 integrin in the same liposomes substantially enhanced talin binding far beyond levels observed for each individual component, suggesting an additive and possibly synergistic binding effect, revealed by the ability of the ProLIF system to incorporate membrane-embedded integrins and membrane lipids in the same binding assay. In PI(4,5)P2-containing vesicles, talin FERM binding was reduced when both Fos–β1 and Jun–α5 were present (Fig. 4B). Binding of talin FERM domain to Jun–α5 and PI(4,5)P2 was similar to that in conditions containing PI(4,5)P2 alone, suggesting that the talin FERM–PI(4,5)P2 interaction is preserved despite loss of interaction with the β1 integrin receptor (Fig. 4B). Incubation with an excess of soluble biotin, which outcompetes liposome binding to the beads, resulted in the complete loss of the fluorescence signal (Fig. S4C), serving as an important control and confirming that the signal is only due to binding events occurring at the membrane rather than unspecific binding to the beads.

With ProLIF, we could also observe talin binding to PI(3,4,5)P3 alone, and detected a substantial enhancement in talin binding to PI(3,4,5)P3 and Fos–β1-containing liposomes that was equivalent
to that for PI(4,5)P2- and Fos–β1-containing liposomes (Fig. 4C,D). The ability of talin to tether to the β1 integrin CT in conjunction with both PI(4,5)P2 and PI(3,4,5)P3 has not been carefully studied before and may be linked to interesting biological functions warranting further investigation in the future.

Next, we set out to determine the $K_d$ for talin FERM binding to integrins in our system but were unable to isolate enough of the protein from mammalian cell lysates to perform the experiment. However, we took advantage of ProLIF as a versatile system that can be tailored towards recombinant proteins, to monitor binding of a recombinant His-tagged talin FERM protein to β1-integrin-containing liposomes in vitro. Using this approach, we were able to determine a $K_d$ of 0.77 µM ($R^2=0.65$) for talin FERM (Fig. 4E). β1 integrin peptides binding to talin head fragments in solution have been reported by multiple groups to be significantly weaker (i.e. $K_d$ 490 µM for β1A binding to talin1 F3; Anthis et al., 2009) demonstrating the central role of the membrane in mediating these interactions, and illustrating why studying these interactions in their native environment, as is possible using the ProLIF assay, is imperative.
DISCUSSION

We demonstrate here that ProLIF is a sensitive, versatile and quantitative system to study protein interactions at the cytoplasmic interface of transmembrane proteins, taking into account the individual or synergistic contribution of protein–protein and protein–membrane lipid interactions.

The benefits and sensitivity of ProLIF are particularly exemplified with the integrin chimeras. Many individual protein–protein interactions in the integrin adhesome are characteristically of low affinity and much of the biology is based on synergistic binding events, clustering and multivalent interactions. Thus, studying the integrin cytoplasmic interactions with biochemical assays such as...
pulldowns with integrin tail peptides in detergent can be challenging and does not represent the situation in cells. This is highlighted by the ProLIF data, which demonstrates that the talin–β1 integrin interaction is strongly enhanced by the presence of specific PI species. Thus, it is important to investigate how protein–protein interactions are regulated in the context of changing membrane lipid composition, an aspect that is potentially underestimated in the current integrin cell adhesion literature. Indeed, a number of lipid-binding domains have been identified and characterised (Lemmon, 2008) and the domain architecture of many proteins, including trafficking proteins, kinases and scaffold proteins, combines lipid- and protein-binding modules (Cullen, 2008; Pearce et al., 2010). Thus, the synergistic effect observed for talin is likely to be a widespread phenomenon that could be addressed using ProLIF.

The mammalian expression system, optimised for ProLIF, also adds novelty over other methods available for monitoring protein–lipid binding as it supports post-translational modifications of the soluble protein and the formation of protein complexes within cells. These events could be manipulated by biological reagents to gain further insight into mechanisms regulating protein binding to membrane components.

We believe that the simple strategy for lipid and protein reconstitution in liposomes and the use of a flow cytometer makes ProLIF a powerful, yet amenable, tool for the quantitative detection of binding events on membranes, which can be applied to other transmembrane proteins. Moreover, ProLIF can be further developed into multiplexed assays by taking advantage of the palette of fluorescent tags available.

MATERIALS AND METHODS

Plasmids and constructs

The Jun–α5 artificial gene (human α5 integrin, amino acids 989–1049) was synthesised by DNA2.0 (manufacturer) in pD441-HMBP. The Fos–β1 (human β1 integrin, amino acids 725 to 798) artificial gene was synthesised by DNA2.0 and cloned in the pGEX-4T vector using the

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Bio-Beads™ preparation and dosing

Bio-Beads™ (Bio-Rad) were sieved to exclude small beads and subsequently washed three times with methanol and five times with dH$_2$O. Beads were left to sediment and during liposome preparation (see below) added in volumes of 15 µl (reproductively corresponds to 3 mg of beads), collected from the bottom of the tube using a cut tip.

Liposome and proteoliposome reconstitution

The control lipid mix used throughout the study, unless otherwise indicated, was composed of 73% (w/w) Egg-PC, 10% (w/w) Egg-PA, 15% (w/w) cholesterol and 2% (w/w) biotinylated lipids. Where indicated, PIs were included at the expense of Egg-PA to preserve the percentage of negatively charged lipids at 10%.

In the case of BTK-PH–EGFP $K_0$ fitting and the BTK-PH–EGFP example histograms in Fig. 1D, the liposome composition used was 80.5% (+4°C and 1 mM EDTA, 5 mM AEBSF, GST) indicating total lipid solubilisation. Solubilised lipids were cooled to -20°C. For each liposome/proteoliposome reconstitution, 400 µg of total lipids were solubilised in Triton X-100 (Triton X-100/lipid ratio of 2.5, w/w) in a total volume of 400 µl of reconstitution buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl and 600 µM TCEP) at room temperature with constant stirring until the solution became clear indicating total lipid solubilisation. Solubilised lipids were cooled to +4°C and 1 mM EDTA, 5 mM AEBSF, GST–Fos–β1 and/or MBP–Jun–α5 were added to the solution and stirred at +4°C for 15 min. Prewashed Bio-Beads™ (total 48 mg) were gradually added to the solution at +4°C while constantly stirring; 3 mg of Bio-Beads were added and the solution was incubated for 90 min, followed by 3 mg of Bio-Beads and a 90 min incubation, followed by 12 mg of Bio-Beads and an overnight incubation. Finally, this was followed by 30 mg of Bio-Beads and 120 min of incubation.

Cell transfection

HEK 293 cells were seeded at a density of 25–35% confluence and transfected the next day at 50–70% confluence according to the following protocol for a 10 cm dish. The plasmid of interest (12 µg) was mixed with Opti-MEM for 5 min at room temperature) was then added and incubated for 5 min at room temperature. A premix of polyethylenimine, Linear, MW 25000 (PEI 25K) transfection reagent (stock in MQ H$_2$O; 1 mg/ml) (Polysciences Inc) (30 µl incubated with 250 µl of Opti-MEM for 5 min at room temperature) was then added and incubated for a further 30 min at room temperature. The transfection solution was placed on top of the 5 ml of culture medium present in the cell culture dish. Cells were harvested after overnight incubation.

Isolation of detergent-free cell lysate

Cells were washed twice with PBS and scraped in 400 µl of detergent-free lysis buffer (10 mM Tris-HCl pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM MgOAc, 20 µM ATP plus complete protease and PhosSTOP phosphatase inhibitor tablets, Roche) at +4°C. Cell extracts were passed through a syringe needle (0.5 mm) five times, sonicated at +4°C for 5 min and ultracentrifuged at 100,000 $g$ for 1 h at +4°C. The resulting supernatant, depleted of membrane and transmembrane fractions, was used for the experiment.

Co-immunoprecipitation

An equimolar mixture of Fos–β1 and Jun–α5 were subjected to immunoprecipitation using 1 µg of the indicated antibodies at +4°C for 2 h in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 600 µM TCEP plus 0.1% DDM. Immunoprecipitated complexes were isolated on protein-G beads (GE Healthcare) for 2 h at +4°C. Beads were then washed once with the same buffer and suspended into loading buffer. Samples were separated by SDS–PAGE and analysed by western blotting.

Flotation assay

Equimolar amounts of Fos–β1 and Jun–α5 were reconstituted in liposomes, mixed in a 1:1 ratio with a 60% sucrose solution and added to the bottom of an ultracentrifuge tube. Decreasing concentrations of sucrose were progressively layered on top to form a gradient and the sample was centrifuged overnight at 20,000 $g$ at +4°C. Fractions were retrieved and analysed by SDS-PAGE.

Calculation of EGFP concentration within cell lysates

The fluorescence intensity of serial dilutions of fluorescein (1–256 nM in dH$_2$O) was measured using the BioTek Synergy H1 hybrid reader to obtain a standard curve. The fluorescence intensity of cell lysates was measured in relation to this standard curve and EGFP-tagged protein concentration calculated using Eqn 1. The fluorescence quantum yield in dH$_2$O ($\phi_{\text{Ext}}$) and extinction coefficient ($\varepsilon_{\text{Ext}}$) are 0.76 and 76,900 M$^{-1}$ cm$^{-1}$, respectively (Song et al., 2000; Zhang et al., 2014); GFP quantum yield ($\phi_{\text{GFP}}$) and extinction coefficient ($\varepsilon_{\text{GFP}}$) are 0.53 and 70,000 M$^{-1}$ cm$^{-1}$ (for dimeric GFP) (Thermo Scientific), respectively. For EGFP-tagged proteins, extinction coefficients were calculated using the ExPaSy ProtParam tool at http://web.expasy.org/protparam/ to obtain the predicted coefficient for each EGFP-tagged construct.

The fluorescence intensity or number of excited molecules during passage of light through a sample can be derived from the Beer–Lambert law:

$$I = I_0 e^{-\ln(10) c Ext \lambda},$$

where $I$ corresponds to the transmitted light through the sample, $I_0$ is the incident radiation, $c$ is the concentration, $\lambda$ is the extinction coefficient at the excited wavelength, and $I$ is the light path length. For low absorbance values, this can be expanded to:

$$I = I_0 [1 - \ln(10) c Ext \lambda].$$

The emission intensity ($F_\lambda$) for one type of molecule at a given wavelength is a function of the quantum yield ($\phi_\lambda$), the fraction of emission that occurs at that wavelength ($f_\lambda$), and the fraction of the radiation that is actually collected by the detector ($j$):

$$F_\lambda = \frac{F_{\text{GFP}}}{\ln(10) \phi_{\text{GFP}} I_0 \phi_\lambda j},$$

Solving this equation for the concentration of our EGFP-labelled molecule, we obtain the following expression (sub-indices indicate the sample):

$$c_{\text{GFP}} = \frac{F_{\text{Ext}}}{\ln(10) \phi_{\text{GFP}} I_0 \phi_\lambda j \lambda},$$

Now, using the calibration curve obtained with external standard we can obtain the incident radiation ($I_0$):

$$I_0 = \frac{F_{\text{Ext}}}{\ln(10) \phi_{\text{Ext}} j \lambda};$$

that when combined with the previous equation results in Eqn (1) (see above) where the ratio $c_{\text{Ext}}/F_{\text{Ext}}$ is the inverse of the slope in the linear fit of $F_{\text{Ext}}$ as a function of $c_{\text{Ext}}$ in the calibration curve.

Flow cytometry-based binding assay

The fluorescence of cell lysate (excitation/emission, 485/528) was measured in relation to a fluorescein titration curve in dH$_2$O using the BioTek Synergy H1 hybrid reader. Eqn (1) was applied to calculate the EGFP-tagged protein concentration. The concentration of cell lysate was adjusted by dilution in detergent-free reconstitution buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl and 600 M TCEP). 150 µl cell lysate was transferred to an Eppendorf tube and incubated with 90 µl of reconstitution buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl and 600 µM TCEP) and 60 µl of the liposome/proteoliposome mix for 4 h with constant stirring at +4°C.

Samples were then incubated with SA beads (2 µl) for 30 min at +4°C. Samples were kept on ice and loaded one at a time on a BD LSFRFortessa™ cell analyzer (BD Bioscience).
Flow cytometry settings, data acquisition and analysis

Data acquisition was performed with a fluorescence-activated cell sorter (FACS) LSRFortessa™ flow cytometer (BD Biosciences) and the dedicated BD FACSDiva™ software.

To excite and detect liposome-bound EGFP fluorescence emission (excitation/emission, 488/509) a 488 nm laser line together with a filter set of a 505 nm long-pass filter and a 530/30 nm filter was used. To detect Cy5 (excitation/emission, 496, 656/670), a 532 nm laser line together with a filter set of a 635 nm long-pass filter and a 670/30 nm filter was used.

Before any measurements were made, voltages in the photomultiplier tube (PMT) were adjusted accordingly to make streptavidin bead population fit into the linear range of the instrument as visually evaluated by scatter plot (FSC-A versus SSC-A, Fig. 1C).

Subsequently, PMT was adjusted to accommodate both background fluorescence from the beads and sample fluorescence into the detection window. The typical count rate was below 200 events/second.

Raw data was analysed by using a non-commercial Flowing Software v. 2.5 (Mr Perttu Terho; Turku Centre for Biotechnology, Finland; www. flowingsoftware.com), where the appropriate population of beads was gated and analysed for their respective fluorescence intensities. Median fluorescence values were used for the subsequent data analysis as these are less sensitive for outliers than mean values.

\( \text{K}_d \) fitting for EGFP-tagged proteins isolated from cell lysates

To obtain minimal background, synthetic POPC was used for \( \text{K}_d \) measurement instead of EggPC. Liposomes containing synthetic POPC lipids (80.5% w/w), cholesterol (15% w/w), biotinylated lipid (2% w/w) and PIP(3,4,5)P, (2.5% w/w) (for BTK-PH-EGFP) or PIP(3)P (2.5% w/w) (for tandem FYVE-EGFP) were prepared as before. In control liposomes, used to measure background fluorescence resulting from non-specific binding events, POPC concentration was increased (83% w/w) to compensate for the absence of phosphoinositides. Cells expressing EGFP-tagged proteins were lysed and EGFP-tagged protein concentration was determined as described using Eqn 1. Serial dilutions of the EGFP-tagged proteins were then prepared and incubated with PI-containing or control liposomes. EGFP-tagged protein binding to liposomes was measured by flow cytometry, and background fluorescence was subtracted. The theoretical maximum fluorescence (\( F_{\text{max}} \)) value was estimated by curve fitting:

\[
F = \frac{F_{\text{max}}[P]}{[P] + K_d},
\]

where \( F \) is the raw background-subtracted fluorescence value and \([P]\) is protein concentration. Raw fluorescence values were then normalised to \( F_{\text{max}} \) to determine occupancy:

\[
\theta = \frac{F}{F_{\text{max}}} = \frac{[P_{\text{bound}}]}{[\text{PIP}_{\text{total}}]},
\]

where \([P_{\text{bound}}]\) is the concentration of the protein bound to PIP and \([\text{PIP}_{\text{total}}]\) is the total concentration of PIP at the vesicle. Finally, the \( K_d \) was calculated from the equation:

\[
\theta = \frac{[P]}{[P] + K_d}.
\]

\( \text{K}_f \) fitting for recombinant His-tagged talin FERM

The His-tagged talin FERM construct and its purification have been described elsewhere (Elliott et al., 2010). For use in ProLIF, recombinant His-tagged talin FERM was first labelled with Alexa-Fluor-488-Maleimide (dye:protein ratio 1:10) overnight in 50 mM Tris pH 7.0, 150 mM NaCl, 600 µM TCEP and then dialysed overnight in 50 mM Tris pH 7.4, 150 mM NaCl, 600 µM TCEP. Binding to β1-integrin-containing proteoliposomes was measured after 2 h of incubation with the proteoliposomes at room temperature. For the fitting of the data, non-specific binding to control liposomes was first subtracted and the theoretical maximum fluorescence (\( F_{\text{max}} \)) value was estimated in order to determine occupancy.

Occupancy was then plotted as a function of concentration and this was fitted against Hill’s equation:

\[
\theta = \frac{[P]^h}{(K_h + [P]^h)}
\]

Where \([P]\) is protein concentration and \( h \) is Hill’s coefficient, which in the case of best fit was 1.368.

Statistical analysis

No statistical method was used to predetermine sample size. Unless stated otherwise all experiments were repeated three or more times for data where representative images are shown and for others sufficient sample size was chosen to reach statistical significance. Statistical significance was determined using the Student’s t-test (unpaired, two-tailed, unequal variance). n numbers are indicated in the figure legends. A P-value of 0.05 was considered as a cut-off for statistical significance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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Supplementary information

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.214270.supplemental

References


Supplementary Figure 1

a: Detection of SA-beads by flow cytometry. 1/1000 of the events typically detected in one sample are shown.

b: Scatter plots resulting from biotinylated-liposomes bound to SA-beads by flow cytometry. 1/1000 of the events typically detected in one sample are shown.

c: Western blot analysis of the isolated detergent-free cell lysate fraction used in liposome binding assays compared to the Triton X-100-solubilized fraction rich in transmembrane (β1 integrin) and membrane associated proteins (Rab21). Uncropped blots can be found in Figure S6.

d: Titration curve with decreasing amounts of PLC-PH-EGFP (n = 1). Cell lysates from PLC-PH-EGFP transfected cells were diluted to contain the indicated concentrations of the protein (calculated based on equation (1)), incubated with liposomes with and without PI(4,5)P2 and analyzed for protein-liposome binding using FACS.
Supplementary Figure 2

a: Overlay of fluorescence intensity histograms of EGFP and BTK-PH-EGFP bound to different PI species (individual histograms shown in Fig. 1d).

b: Representative scatter plots, fluorescence intensity histograms and histogram overlays (from experiments quantified in Fig. 1f) of PLC-PH-EGFP bound to different PI species. The red population in the scatter plot was gated for quantification.
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**Supplementary Figure 3**

a,b: Sequential steps in GST-Fos-β1 (a) and MBP-Jun-α5 (b) purification. Shown are coomassie-stained SDS-PAGE gels loaded with the following samples: #1, whole lysate after cell disruption; #2, supernatant from low-speed centrifugation; #3, supernatant from high-speed centrifugation; #4, resuspended membrane pellet from high-speed centrifugation; #5, resuspended membrane pellet after solubilisation in DDM; #6 supernatant from high-speed centrifugation; #7, flow-through from Ni²⁺ matrix; #8, eluted from Ni²⁺ matrix; #9, flow-through from glutathione or amylose matrix; #10, eluted from glutathione or amylose matrix; #11, protein stored after dialysis.

c: Titration curve with decreasing amount of Talin FERM-EGFP (n = 1). Cell lysates from Talin FERM-EGFP transfected cells were diluted to contain the indicated concentrations of the protein (calculated using equation (1)), incubated with liposomes with and without GST-Fos-β1 and analyzed for protein-liposome binding using flow cytometry.
Supplementary Figure 4

a: Representative flow cytometry FSC-A vs SSC-A and FSC-A vs FSC-W scatter plots of SA-beads for all Talin FERM-EGFP samples shown in Fig.4b. The red population was gated for quantification.

b: ProLIF assay monitoring Talin FERM-EGFP (3nM; cell lysate) binding to proteoliposomes containing GST-Fos-β1 and/or MBP-Jun-α5 at a lower (compared to Fig. 4b) protein:lipid molar ratio of 1:7000 (n = 1).

c: Competition of Talin FERM-EGFP binding to proteoliposomes by addition of soluble biotin. Biotinylated-lipid-containing liposomes (containing GST-Fos-β1 and PI(4,5)P₂ as indicated) were incubated with Talin FERM-EGFP-containing cell lysate and captured on SA-beads in the presence or absence of free biotin and analyzed using FACS. Molar ratio between biotinylated lipids and soluble biotin is 1:50 (n = 1).