Protein-protein interactions on membrane surfaces analysed using SUPER template pull-downs

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Abstract

Discovery-based proteomics workflows that identify novel interactors rely on immunoprecipitations or pull-downs with genetically-tagged bait proteins displayed on sepharose matrices. But strategies to analyse protein interactions on a diffusible membrane surface combined with the practical ease of pull-downs remain unavailable. Such strategies are important to analyse protein complexes that mature in composition and stability because of diffusion-based interactions between participant proteins. Here, we describe a generic pull-down strategy to analyse such complexes using supported membranes with excess reservoir templates displaying His-tagged bait proteins. Using clathrin-mediated endocytosis as a paradigm, we find that the clathrin-binding adaptor protein epsin1 displayed as bait on these templates pulls down significantly higher amounts of clathrin from brain lysates than conventional sepharose matrices. Together, our results establish the potential of SUPER templates as superior matrices for analysing protein-protein interactions and resultant complexes formed on membrane surfaces.

Keywords

Protein-protein interactions, pulldowns, clathrin, epsin1, clathrin-mediated endocytosis, membrane

Introduction

Biochemical analyses of novel protein interactions generally rely on immunoprecipitation methods or pull-downs with genetically-tagged proteins displayed on appropriate sepharose matrices (Brymora et al. 2004; Schmidt and Skerra 2007; Pollard 2010; Lin and Lai 2017). Such strategies are however not ideal for analysing protein complexes that mature and strengthen from diffusion-controlled encounter between participant proteins. Formation of such protein complexes is apparent in several membrane-localized processes such as coated vesicular transport, formation of focal adhesion complexes and viral budding (Schmid and McMahon 2007; Shaw et al. 2008; Schumacher et al. 2021). During

vesicular transport, coated bud formation is initiated by low-affinity interactions between receptors and specific lipids with their cognate adaptors, which in turn recruit coat proteins and accessory endocytic proteins. The resultant protein complexes achieve stability by high avidity interactions formed between polymerizing coat proteins (Traub 2005; Schmid and McMahon 2007; Kaksonen and Roux 2018). Previous reports describe liposome-based templates for identifying such interactors but involve tedious and elaborate strategies, both at the level of template preparation and subsequent separation of liposome-bound proteins (Zhao and Lappalainen 2012; Saliba et al. 2016; Senju et al. 2021). To circumvent these issues, we focussed on a simplified pull-down strategy using SUPER templates, which are supported membranes formed on silica beads by the fusion of negatively-charged liposomes in presence of high salt (Pucadyil and Schmid 2008; Pucadyil and Schmid 2010; Neumann et al. 2013). These templates can be sedimented at low speeds, which is a property useful in removing excess unbound proteins in reactions carried out with native tissue lysates. Here, we describe results using SUPER templates in pull-down assays, focussing on the ability of His-tagged clathrin adaptors to recruit clathrin from native tissue lysates.

Materials and Methods

Protein Expression and Purification

Human epsin1 and its mutants cloned with an N-terminal 6xHis tag and a C-terminal StrepII tag in pET15b have been described earlier (Holkar et al. 2015). mEGFP was cloned with an N-terminal 6xHis tag in pET15b. Proteins were expressed in BL21(DE3) grown in autoinduction medium (Formedium, UK) at 18°C for 36 hours. Bacterial cells were pelleted and stored at -40 °C. The frozen bacterial pellet was thawed and resuspended in 20 mM HEPES pH 7.4, 150 mM NaCl (HBS) with a protease inhibitor cocktail tablet (Roche) and lysed by sonication in an ice water bath. Lysates were spun at 18,000x g for 20 mins. The supernatant was incubated with HisPur™ Cobalt Resin (Thermo Fisher Scientific) for 1 hr at 4 °C and poured into a PD-10 column. The resin was washed with 100 ml of HBS and bound protein was eluted with HBS containing 250 mM imidazole. For StrepII tagged constructs, elution from the HisPur™ Cobalt Resin was applied to a 5 ml streptactin column (GE Healthcare) and washed with HBS. Proteins were eluted in HBS containing 1 mM DTT and 2.5 mM desthiobiotin (Sigma-Aldrich). Proteins were spun at 100,000x g to remove aggregates before use in assays. Protein concentration was estimated using UV absorbance at 280 nm based on the predicted molar extinction coefficients from the ProtParam tool in Expasy.

Preparation of Brain Lysates

Lysates from adult goat brains were prepared as described earlier (Wu et al. 2010; Kamerkar et al. 2018). Briefly, brain tissues were cleaned of meninges and blood vessels and homogenized in a Waring blender in breaking buffer (25 mM Tris pH 8.0, 500 mM KCl, 250 mM sucrose, 2 mM EGTA, and 1 mM DTT) with a protease inhibitor cocktail tablet (Roche). The lysate was spun at 160,000x g for 2 h. The supernatant was collected and desalted on a G-50 column into 20 mM HEPES pH 7.4 with 150 mM KCl. Lysates were flash-frozen with 10% glycerol in liquid nitrogen and stored at −80 °C.

Total protein content was estimated using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Supported Bilayer with Excess Reservoir (SUPER) Templates

SUPER templates were prepared as described previously (Pucadyil and Schmid 2010). All lipids were from Avanti Polar Lipids. Briefly, required aliquots of the chelating lipid 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (NTA lipid; 5 mol%) with or without 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS, 15 mol%) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were mixed in a clean glass tube and dried under high vacuum. Lipids were hydrated with de-ionized water to achieve a final concentration of 1 mM. The hydrated lipid suspension was probe sonicated in an ice-water bath and spun at 100,000x g at 25 °C for 20 min. The supernatant containing small unilamellar vesicles (SUVs) was collected and stored at 4 °C. To prepare SUPER templates, 10 µl of plain silica beads (5.3 µm diameter, Corpuscular Inc., cat. no.: 140226-10) was added to a premixed solution of 200 µM SUVs and 1 M NaCl in a 1.5 ml siliconized low-binding polypropylene tube (Star Labs, cat. no.: E1415-2600) and left with intermittent shaking for 30 min. SUPER templates formed on the silica beads were then washed 3 times by adding 1 ml of de-ionized water and pelleting at low speed (300x g) for 2 min in a swinging bucket rotor. 100 µl of water was left behind after each spin to prevent drying of templates. The excess membrane reservoir in the templates was checked by adding a small aliquot of the templates to a drop of HBS on a piranha-cleaned glass coverslip.

SUPER Template Stability

Template stability was assayed by monitoring the release of membrane reservoir. For this, liposomes contained an additional (1 mol%) of Texas Red-DHPE (Invitrogen). Briefly, 10 µl of templates was added to 90 µl of HBS containing increasing protein concentrations of brain lysate in 1.5 ml polypropylene centrifuge tubes. In a separate reaction, to estimate the total lipid concentration, 10 µl of templates was added to HBS containing 0.1% Triton X-100. Samples were left undisturbed for 30 min at room temperature and the beads were spun at 300x g for 2 min at room temperature. To estimate released membrane, 75 µl of the supernatant was mixed with 25 µl of 0.4 % Triton X-100 and added to a 96-well plate. To estimate total membrane, 100 µl of the supernatant from templates in 0.1% Triton X-100 was aliquoted into a 96 well plate. Samples were read for Texas Red fluorescence on a plate reader (Tecan Infinite M200 Pro).

SUPER Template Pull-downs

SUPER templates were incubated with 1 µM of purified bait proteins in a total volume of 500 µl of HBS for 30 mins at room temperature with gentle agitation and washed 3 times with 1 ml of HBS, making sure to always leave behind 100 µl of buffer. For pull-downs, templates displaying the desired bait were incubated with 500 µl of 1 mg/ml brain lysate. Templates were washed 3 times with 1 ml of HBS, making sure to leave behind 100 µl of buffer. After the last wash, beads were boiled in 1x Laemmli's buffer and bound proteins were resolved on 10% SDS-PAGE.

Western Blotting

Proteins were transferred onto PVDF membrane and blocked with 5% skimmed milk in TBST for 1 hr at room temperature. Anti-clathrin heavy chain (TD1, Abcam, cat. no.: Ab24578) was used at a dilution recommended by the manufacturer in blocking buffer. The membrane was incubated with the primary antibody in blocking buffer for 3 hrs at room temperature. Subsequently, the membrane was washed with TBST and incubated with suitable HRP-conjugated secondary antibody diluted in blocking buffer for 1 hr at room temperature. Blot were rinsed with TBST and developed with chemiluminescent substrate (WestPico, Thermo Fisher Scientific) and imaged on a G:Box (Syngene).

Fluorescence Imaging

Fluorescence imaging was carried using an Olympus IX83 inverted microscope equipped with a 100X, 1.4 NA oil-immersion objective. Fluorescent probes were excited with a stable LED light source (CoolLED) and fluorescence emission was collected through appropriate filters on an Evolve 512 EMCCD camera (Photometrics). Image acquisition was controlled by Micro-Manager and rendered using ImageJ.

Results and Discussion

SUPER templates are supported membranes with excess reservoir that are formed by the binding and fusion of anionic liposomes on silica beads (Pucadyil and Schmid 2010). They are formed by salt-induced increase in binding of liposomes to the silica surface before their fusion and contain membrane reservoir in excess of what is needed to cover the bead surface.

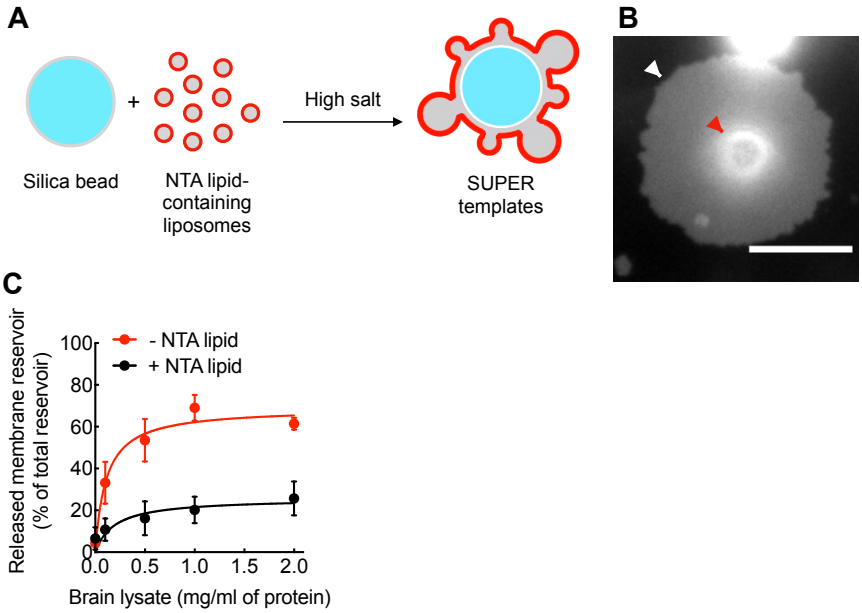

Fig. 1. Formation and stability of SUPER templates. (A) Schematic showing the process leading to the formation of SUPER templates. (B) Representative micrograph showing spillage of the excess membrane reservoir (white arrowhead) around a SUPER template (red arrowhead) on a glass coverslip. Scale bar = 10 μm.

(C) Stability of templates with and without NTA lipid in brain lysate analysed by estimating the release of fluorescently-labelled membrane. Data represent the mean ± SD of 3 independent experiments.

Anionic lipids are necessary for the excess reservoir and also contributes to the stability of SUPER templates. For the purpose of developing stable membrane matrices that can display His-tagged bait proteins, we first formed templates with liposomes containing the anionic chelator lipid 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (NTA lipid; 5 mol%) with the rest being dioleoylphosphatidylcholine (DOPC) on silica beads under high salt conditions (Fig. 1A). For imaging and testing for potential vesiculation of the membrane, liposomes contained trace amounts (1 mol%) of a fluorescent lipid Texas Red-DHPE. Excess unbound liposomes and salt were removed by washing the templates by sedimentation at low speed (300x g) and resuspension. When added onto a glass coverslip, these templates bind and spill their excess reservoir, which can be seen as a membrane patch (Fig. 1B, white arrowhead) around the silica bead (Fig. 1B, red arrowhead). We then tested template stability in presence of native tissue lysates. Templates were incubated with increasing concentrations of brain lysate for 30 min with intermittent mixing followed by sedimentation at low speed (300x g). The supernatant was then collected and checked for released vesicles by monitoring Texas Red-DHPE fluorescence. As seen in Fig. 1C, templates with the NTA lipid were quite stable with only 20% of the total membrane undergoing vesiculation at the highest concentration of lysate tested. In contrast, templates without the NTA lipid released 60% of their membrane reservoir. Thus, inclusion of the anionic NTA lipid, which would serve to display His-tagged baits, imparts stability to the templates. To prevent potential instability arising from shielding of the anionic charge on the NTA lipid upon binding to His-tagged baits, we included dioleoylphosphatidylserine (DOPS; 15 mol%) in the templates. For convenience, we hereafter refer to these as SUPER templates.

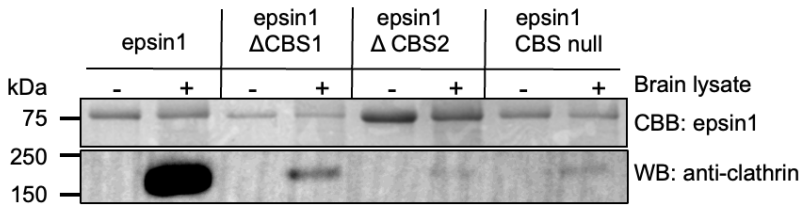

Fig. 2. SUPER template pull-downs with epsin1 as bait. Representative results of pull-downs from brain lysates using SUPER templates displaying purified epsin1 and its mutants lacking clathrin-binding sites (CBS). Coomassie Brilliant Blue (CBB)-staining shows bait proteins and Western Blot (WB) was performed using an anti-clathrin antibody.

Next, we tested if SUPER templates displaying 6xHis-tagged bait proteins can pull-down known interactors from native tissue lysates. Monomeric clathrin adaptors or clathrin-associated sorting proteins (CLASPs) contain an N-terminal structured domain that binds phosphoinositide lipids and sorting motifs on cargo receptors and a C-terminal unstructured region that displays multiple short linear motifs that engage with the adaptor protein 2 (AP2), endocytic accessory proteins and clathrin

(Traub 2009). We focussed on the monomeric clathrin adaptor epsin1 because of its well-characterised ability to pull down clathrin from brain lysates (Drake et al. 2000). SUPER templates were first incubated with purified 6xHis-tagged epsin1. Excess protein was washed off by sedimentation and resuspension. Templates were then incubated with brain lysate (1 mg/ml) for 30 min, washed with buffer by sedimentation and resuspension and the bound proteins were resolved using SDS-PAGE. Coomassie brilliant blue (CBB) staining revealed that the templates retained epsin1 in presence of cytosol while western blots (WB) using an anti-clathrin antibody revealed efficient pulldown of clathrin (Fig. 2). Templates displaying epsin1 mutants deleted in either one or both of its clathrin-binding sites (CBS) (Holkar et al. 2015) showed severely diminished or negligible pull-down of clathrin indicating that the epsin1-clathrin interaction was specific.

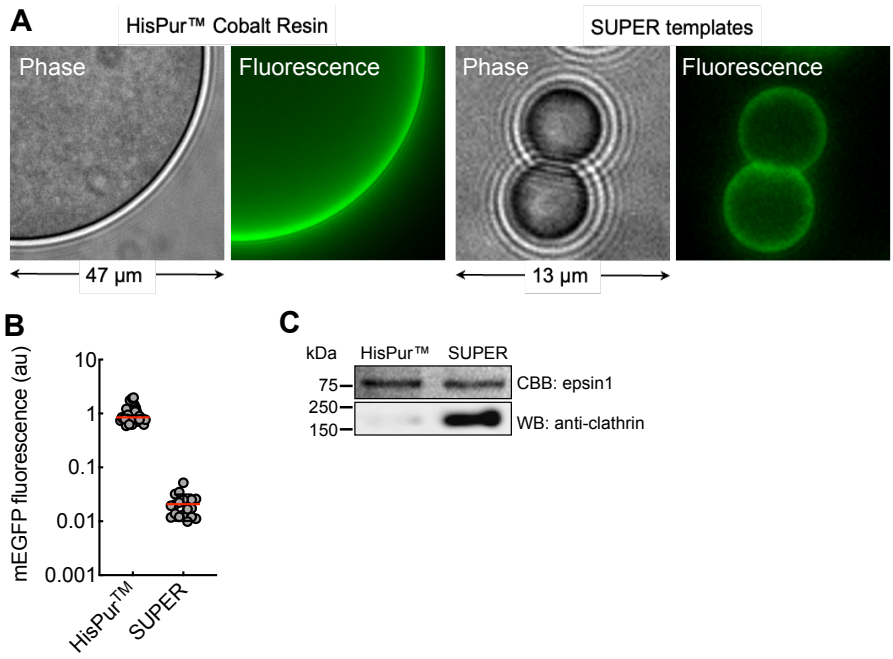

Fig. 3. Comparison between SUPER templates and sepharose matrices in pull-downs. (A) Representative phase contrast and fluorescence images of 6xHis-mEGFP bound to HisPur™ Cobalt Resin and SUPER templates. (B) Quantitation of fluorescence of 6xHis-mEGFP bound to HisPur™ resin and SUPER templates. Each point represents data from an individual template. (C) Representative results of pull-downs from brain lysates using epsin1-coated HisPur™ Cobalt Resin and SUPER templates. Coomassie Brilliant Blue (CBB)-staining shows the bait epsin1 and Western Blot (WB) was performed using an anti-clathrin antibody.

In comparison to previous reports showing clathrin pull-down using epsin1-coated sepharose beads (Drake et al. 2000), epsin1-displayed on SUPER templates appeared to recruit more clathrin for the amount of bait displayed on them. We tested if this was indeed the case by comparing the efficiency of clathrin pull-down with epsin1 displayed on SUPER templates and sepharose beads (HisPur™ Cobalt Resin). We first estimated the available NTA binding sites by incubating the sepharose beads and SUPER templates with saturating concentrations of 6xHis-mEGFP, followed by washing and measuring GFP fluorescence associated with the sepharose beads and SUPER templates. Such analysis revealed that HisPur™ Cobalt Resins displays 50-fold higher NTA-binding sites than SUPER

templates (Fig. 3A,B). So we titrated the amount of epsin1-saturated HisPur™ Cobalt Resins to match the concentration of epsin1 on SUPER templates and carried out clathrin pull-downs from brain lysates. Under conditions of equivalent amounts of epsin1 in pull-downs (see CBB staining of epsin1 in Fig. 3C), epsin1 displayed on SUPER templates recruited significantly higher amounts of clathrin than when displayed on HisPur™ Cobalt Resin (Fig. 3C). Previous reports of clathrin binding to purified adaptors have revealed that low-membrane tension and a pre-curved surface significantly facilitate clathrin binding and self-assembly to membrane-bound adaptors (Dannhauser et al. 2015; Saleem et al. 2015). Self-assembly would require diffusion of adaptors on the membrane and it is possible that better performance of SUPER templates over HisPur™ Cobalt Resin could reflect these facilitatory effects on clathrin self-assembly.

In sum, we describe here a novel pull down template that would benefit analysis of protein-protein interactions and the resultant complexes formed on membrane surfaces. While convenient, an obvious limitation of SUPER templates is the inability to incorporate transmembrane domain-containing membrane proteins. This restricts their use to analysing interactions between peripherally-associated membrane and soluble proteins. But a way around this limitation would be to display specific domains or motifs present on transmembrane domain-containing proteins on the template using His-tags. That aside, our results establish the versatility of SUPER templates in pull-downs since they are; (a) amenable to analysing interactions of any bait with a His-tag, (b) stable in complex mixtures of proteins such as native tissue lysates, (c) can be readily sedimented for the recovery and subsequent analysis of protein interactomes, and (d) can be further functionalized by incorporating desired lipids in the membrane.

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Author Contributions

The study was conceptualized by DSA and TJP. Experiments were performed by DSA. Data analysis and manuscript preparation was carried out by DSA and TJP.

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Declarations

Conflict of interest: The authors declare no conflict of interest.

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