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A simple supported tubulated bilayer system for evaluating protein-mediated membrane remodeling

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ABSTRACT

Fusion and fission of cellular membranes involve dramatic, protein-mediated changes in membrane curvature. Many of the experimental methods useful for investigating curvature sensing or generation require specialized equipment. We have developed a system based on supported lipid bilayers (SLBs) in which lipid tubules are simple to produce and several types of membrane remodeling events can be readily imaged using widely available instrumentation (e.g., tubule fission and/or membrane budding). Briefly, high ionic strength during lipid bilayer deposition results in incorporation of excess lipids in the SLB. After sequentially washing with water and physiological ionic strength buffer solutions, lipid tubules form spontaneously. We find that tubule formation results from solution-dependent spreading of the SLB; washing from water into physiological ionic strength buffer solution leads to expansion of the bilayer and formation of tubules. Conversely, washing from physiological buffer into water results in contraction of the membrane and loss of tubules. We demonstrate the utility of these supported tubulated bilayers, termed "STuBs," with an investigation of Sar1B, a small Ras family G-protein known to influence membrane curvature. The addition of Sar1B to STuBs results in dramatic changes in tubule topology and eventual tubule fission. Overall, STuBs are a simple experimental system, useful for monitoring protein-mediated effects on membrane topology in real time, under physiologically relevant conditions.

1. Introduction

The execution of a variety of fundamental biological processes including vesicle budding, fission, and fusion, depends on extensive changes to the morphology of lipid membranes (Chernomordik et al., 2006; McMahon and Boucrot, 2015). Such dynamic remodeling likely requires the participation of proteins, which by scaffolding (Hinshaw and Schmid, 1995), active insertion (Peter et al., 2004; Frost et al., 2008), or modulating tension (Sheetz et al., 2006; Sheetz, 2001) exert their influence on membrane structure.

Because of the immense interest in these topics, a number of techniques have recently been developed to monitor curvature sensing or generation – but generally not both simultaneously – in controlled experimental systems. For example, a fluorescence-based microscopy assay allows quantification of curvature dependence of protein binding using differentially sized liposomes tethered to a glass surface (Hatzakis et al., 2009). Supported lipid bilayers (SLBs) formed on substrates patterned with nanoparticles of defined size have also been used to quantify curvature sensing (Alnaas et al., 2017). Generation of highly curved lipid tubules from pipette-immobilized giant unilamellar vesicles (GUVs) has been reported for a number of proteins, a technique which allows careful control of membrane tension but requires

Abbreviations: TIRF, total internal reflection fluorescence; pTIRF, polarized total internal reflection fluorescence; SLB, supported lipid bilayer; STuBs, supported tubulated bilayers; COPII, coat protein complex II; SMrT, supported membrane tubules; SUPER, supported membranes with extra reservoir; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycerol-3-phospho-L-serine (sodium salt); LRB-DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; DiD, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate; AF488, Alexa Fluor 488

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specialized instrumentation (Shi and Baumgart, 2015). Microbead-supported membranes with excess lipid reservoir (SUPER templates) can be used to detect curvature generation from flexible synthetic lipid bilayers based on formation of budded vesicles or tubules (Pucadyil and Schmid, 2010, 2008). With skill, the latter technique can be adapted to detect binding to pre-formed tubules. Another recently developed technique for quantitative, high-throughput analysis of membrane fission is supported membrane tubules (SMrT templates), which consist of arrays of long lipid tubules prepared using a flow cell and oriented parallel to a glass surface (Dar et al., 2017, 2015). Our goal was to develop a simple, broadly accessible assay system, based on SLBs and complementary to the techniques described above, for visualizing protein-mediated curvature sensing and generation. In this study, we describe such a system characterized by the presence of stable lipid tubules on a planar glass surface – a system we name supported tubulated bilayers ("STuBs").

In the context of intracellular trafficking, membrane remodeling results in the sequestration of membrane-enclosed spaces for the transport of proteins and lipids. Transport from the endoplasmic reticulum (ER) to the Golgi is mediated by coat protein complex II (COPII)-coated vesicles (Barlowe et al., 1994). Key components in their formation include the inner and outer coat complex proteins Sec23/24 and Sec13/31 and the small GTPase Sar1B (Barlowe and Schekman, 1993; Saito et al., 2017; Futai et al., 2004; Hanna et al., 2016). Recent studies in cell-based and cell-free systems suggest that Sar1B has a direct role in both sensing and driving membrane curvature (Hanna et al., 2016), can form tubules from GUVs (Hariri et al., 2014; Bacia et al., 2011), and localizes to vesicles with COPII (Kurokawa et al., 2016) – activities which have been characterized using a variety of experimental platforms. In this study, we demonstrate how the STuBs platform can be used to detect several membrane-dependent activities of Sar1B in a single experimental setup.

In the first part of the study, we describe the STuBs system and identify the key factors that lead to tubule formation. The formation of tubules is sensitive to the ionic conditions used during SLB deposition and subsequent washing. In particular, deposition of an SLB under high ionic strength followed by sequential washes with water and a buffer containing physiological salt concentrations promotes bilayer spreading and as a result, tubule formation. Inclusion of 0.5 mM Mg^{2+} in the physiological buffer, required for the study of GTPases, slightly decreases the extent of bilayer spreading, but nevertheless produces ample tubules for studying membrane remodeling by Mg^{2+}-dependent proteins such as Sar1B.

We then use STuBs to capture a variety of functions of recombinant human Sar1B protein. We show that addition of Sar1B to STuBs results in the fission of pre-formed tubules and induction of stable Sar1B-dependent lipid structures. These Sar1B-induced structures are detected using a combination of epifluorescence and total internal reflection fluorescence (TIRF) microscopy. Dual-color imaging of fluorescent Sar1B and fluorescent lipid tracers provides evidence of protein localization to tubules and protein-driven fission in the presence of GTP. Overall, STuBs allow observation and quantification of protein effects on single tubules or many tubules in parallel, allowing imaging of tubule fission events and insight into processes of membrane remodeling.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycerol-3-phospho-L-serine (sodium salt) (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (LRB-DOPE), 1,2-dioleoyl-*sn*-glycero-3-[phosphoinositol-4,5-bisphosphate] (DOPIP2), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) in chloroform were from Avanti Polar Lipids (Alabaster, AL). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) was from Life Technologies (Eugene, OR). 2-mercaptoethanol was from Calbiochem (EMD Millipore, Billerica, MA). GTP, GDP, and GMP-PNP were from Sigma Aldrich (St. Louis, MO). Alexa Fluor 488 (AF488) TFP ester was from ThermoFisher Scientific (Waltham, MA). All other chemicals were ACS grade or higher.

2.2. Protein expression and purification

A GST-Sar1B fusion protein (Hanna et al., 2016) was expressed from a pGEX6P-1 plasmid in BL21 DE3 *E. coli* by induction with 50 μM IPTG for 4 h at 22 °C. The cells were isolated by centrifugation, resuspended in lysis buffer (phosphate-buffered saline with 1 mM EDTA, 10 μM GDP, and protease inhibitors), and lysed with chicken egg white lysozyme for 45 min. The lysate was clarified by centrifugation at 30,000 x g and loaded by gravity onto a glutathione Sepharose 4B resin column. Sar1B was released from the column by an overnight 4 °C digestion with PreScission protease (GE Healthcare). The Sar1B sample was concentrated in a 10,000 MWCO Amicon ultrafiltration device and resolved over a Superdex 200 10/300 GL column in Sar1B storage buffer (50 mM HEPES, 100 mM NaCl, 1 mM dithiothreitol, 10 μM GDP, pH 7.6) using an Akta FPLC (GE Healthcare). The monodisperse protein was collected and re-concentrated, prior to being flash frozen and stored at –80 °C (Fig. S1). For experiments, the protein was diluted > 100-fold from this storage buffer into the indicated experimental solution conditions. $Sar1B^{GTP}$ indicates Sar1B in the presence of 5 μM GTP in Buffer A + 0.5 mM $MgCl_2$, $Sar1B^{GDP}$ indicates Sar1B in the presence of 5 μM GDP in Buffer A + 0.5 mM $MgCl_2$, and $Sar1B^{GMP-PNP}$ indicates Sar1B in the presence of 5 μM GMP-PNP in Buffer A + 0.5 mM $MgCl_2$. A portion of the purified Sar1B was labeled with AF488 TFP ester following the manufacturer's protocol (ThermoFisher Scientific).

2.3. Liposome preparation

Chloroform suspensions of lipids were mixed in the indicated molar ratios along with either DiD or LRB-DOPE as a fluorescent tracer, evaporated under a stream of N_2 and kept under a vacuum for a minimum time of 1 h, and then resuspended in Buffer A (140 mM KCl, 15 mM NaCl, 20 mM HEPES, pH 7.4) to a total lipid concentration of 3.0 mM. The resuspended multilamellar vesicles were then converted to liposomes via either sonication (Sonics VibraCell) or extrusion (Avanti Mini Extruder) using a polycarbonate filter of 100 nm pore size. The use of sonicated versus extruded liposomes had no noticeable effect on tubule formation.

2.4. Preparation of SLBs and supported tubulated bilayers

Glass coverslips (#1.5 thickness; Fisher or VWR) were etched through immersion of each cover glass in a 3:1 mixture of H_2SO_4:H_2O_2 (30% solution) for 1 h. The coverslips were thoroughly rinsed and stored in deionized water for ≤ 24 h prior to use. Before depositing each SLB, the coverslips were dried with N_2 and fitted with a 9-mm diameter silicone-gasketed perfusion chamber with a height of 0.6 mm (CoverWell, Grace BioLabs).

SLBs and STuBs were then formed via the vesicle fusion method. Liposome suspensions were mixed with bilayer deposition buffer and pipetted into the perfusion chamber mounted on the coverslip. Several bilayer deposition buffers were tested. Our standard condition for formation of STuBs included a final concentration of 1 M NaCl in Buffer A. For some samples, fluorescently-labeled and unlabeled liposomes were prepared separately and mixed together during bilayer deposition to the desired molar ratio of fluorophore. After incubation at room temperature for 30–40 min, each SLB sample was washed extensively with deionized water, taking care not to introduce air bubbles. For samples not imaged under perfusion, buffer was introduced into the samples by 4 sequential additions of 1 chamber volume of the desired buffer