Direct coupling of Ras to preformed maleimide-functionalized lipid membranes

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Method Article

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Abstract

Introduction

We developed an experimental system to study the kinetics of Ras activation by the Ras activator Son-of-Sevenless \(SOS) when Ras is tethered to lipid membranes. The procedure involves the covalent attachment of a C-terminal cysteine at position 181 in Ras to preformed maleimide-functionalized lipid membranes using thiol-maleimide crosslinking. Because the cysteine at position 181 in Ras undergoes posttranslational lipid modifications _in vivo_, the membrane anchorage of Ras at position 181 should result in the proper orientation of Ras at the membrane surface.

Reagents

Phospholipids and analogs including 1,2-dioleoyl-sn-glycero-3-phosphocholine \(DOPC), 1,2-dioleoyl-sn-glycero-3-\[phospho-L-serine] \(DOPS), 1,2-dioleoyl-sn-glycero-3-phosphate \(DOPA), phosphatidylinositol-4,5-bisphosphate \(PIP2) \(swine brain), 1,2-dioleoyl-sn-glycero-3-phosphoinositol-3,4,5-trisphosphate \(PIP3) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-\[4-\(p-maleimidomethyl)cyclohexane-carboxamide] \(MCC-PE) were purchased from Avanti Polar Lipids. Fluorescent lipid analogs Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine \(TR-DHPE) and Marina Blue 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine \(MB-DHPE) were purchased from Invitrogen. Ras is stored in 25 mM HEPES-NaOH pH 7.4, 100 mM NaCl, 10% glycerol and 2 mM TCEP at approximately 7–10 mg/ml.

Equipment

A glass-bottomed 96-well plate is needed for the preparation of supported lipid bilayers.

Procedure

A. Preparation of maleimide-functionalized lipid vesicles. 1) A particular maleimide lipid-containing phospholipid mix is prepared in chloroform in a 10 x 75-mm glass tube \(or a round bottom flask). A typical mixture contains 10 mol% DOPS, 0–10 mol% MCC-PE, 0.3 mol% TR-DHPE or 2 mol% MB-DHPE, and the balance consisting of DOPC. So, for example, if the mixture contains 1 mol% MCC-PE and 0.3 mol% TR-DHPE, a balance of 88.7 mol% DOPC is included. 2) The lipids are dried to a thin film under a gentle stream of nitrogen or argon gas \(or dried on a rotary evaporator). Residual chloroform is removed by drying under vacuum for about 1 hour. 3) Dried films are hydrated \(typically to a total lipid concentration of 2–10 mg/ml) with degassed buffer \(25 mM HEPES-NaOH pH 7.4, 100 mM NaCl, 10% glycerol) and raised into suspension by approximately 6–8 cycles of freezing in a dry ice/isopropanol bath and thawing in a 37°C water bath. 4) Unilamellar vesicles are formed by passing the suspension through a high pressure extruder \(from Northern Lipids) with a 100 nm diameter pore filter at least ten times. \(Alternatively, the suspension can be passed at least 21 times through an Avestin hand-held extruder with a 100 nm diameter pore filter.) **B. Preparation of Ras-coupled lipid vesicles.** 1) Ras is

directly coupled to preformed maleimide-functionalized lipid vesicles in a reaction containing approximately 1 Ras: 4 maleimide-lipids \(~0.07 μmoles Ras: 0.25 μmoles maleimide-lipid). The thiol-maleimide conjugation reaction is carried out under argon at room temperature for two hours, followed by incubation at 4°C overnight. 2) Reactions are terminated and excess maleimide-lipid quenched by addition of 5 mM ß-mercaptoethanol and incubation at room temperature for at least 10 minutes. 3) Unmodified Ras proteins are separated from Ras-coupled lipid vesicles by size-exclusion chromatography using an XK-16 column \(GE Healthcare) containing about 30 ml \(packed bead volume) of Sepharose CL-4B \(Sigma) resin equilibrated in gel filtration buffer \(25 mM HEPES-NaOH pH 7.4, 100 mM NaCl, 10% \(w/v) glycerol and 1 mM DTT). 4) The lipid concentration after Ras-conjugation is determined using the TR-DHPE absorbance \(Abs590) of the sample. The absorbance \(Abs590) of both 'stock' maleimide-functionalized vesicles \(before Ras-conjugation) and Ras-coupled vesicles is determined and used to find the "dilution factor" of the vesicles after completing the conjugation reaction. 5) The bulk volume concentration of Ras is measured by performing a Bradford assay on both 'stock' maleimide-functionalized vesicles \(no Ras proteins present) and Ras-coupled vesicles. The contribution of lipids to the Bradford reagent signal is determined using the "dilution factor" calculated from the TR-DHPE absorbance \(Abs590), and the difference in Bradford reagent signal between these two samples is assumed to correspond to the signal from Ras. \(When performing the Bradford assay, it is important to use the same volume of 'stock' vesicles and Ras-coupled vesicles, and thus, the same ratio of sample volume to Bradford reagent volume.) 6) Finally, SDS-PAGE gel analysis is used to confirm bulk concentration measurements by creating solutions of equal Ras concentration by volume and comparing to Ras protein standards. **C. Preparation of Ras-coupled supported lipid bilayers.** Supported lipid bilayers are first formed in a glass-bottomed 96-well plate using vesicles prepared as described above. Ras is then directly coupled to the lipid membranes. 1) Wells of a glass bottomed 96-well plates \(Nalge-Nunc) are soaked with 6 M NaOH to clean the surface in preparation for supported bilayer formation. After 2 hours of exposure, the wells are washed with 10 ml distilled/deionized H_2O using a pipette and aspirator, and the volume in the well is brought to approximately 100 μl. This is most easily achieved by filling the well to its maximum capacity \(400 μl) and removing an appropriate volume. The bottom of the well is never exposed to air. It is important not to touch the glass surface with the pipette tip during this process and to use high quality water, since adsorbed surface contaminants will affect bilayer quality. 2) 50 μl of maleimide-functionalized vesicles diluted with an equal volume of 250 mM TRIS-HCl pH 7.4, 1 M NaCl is added to each well and incubated for 10 minutes. During this time, vesicles adsorb to the glass surface and rupture to form a continuous supported bilayer. 3) To remove unbound vesicles, each well is washed with at least 5 ml of 25 mM TRIS-HCl pH 7.4, 100 mM NaCl followed by a 10 minute incubation in 0.1% \(w/v) BSA in 25 mM TRIS-HCl pH 7.4, 100 mM NaCl. During this time it is usually desirable to inspect supported bilayer quality by microscopy and fluorescence recovery after photobleaching \(FRAP, see Supplementary Fig. 4). Bilayers should display high lateral fluidity, and should not have observable holes or large bright lipid aggregates. 4) The volume in each well is then brought to 50 μl, and 100 μl of Ras \(at about 8 mg/ml in 25 mM TRIS-HCl pH 7.4, 100 mM NaCl) is added to the well solution and incubated at room temperature for at least 20 minutes, followed by overnight incubation in the refrigerator. It is important to keep the plate covered during the conjugation reaction.