# Membrane effects on Talin mediated integrin activation

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**Abstract** Integrin is a cell surface receptor involved in cell migration, cell adhesion and cell-cell interactions. Activation of integrin is regulated by the protein called Talin. Talin integrin protein-protein interactions have been well studied but to understand the mechanism of Talin mediated integrin activation, it is necessary to further study the protein-lipid interactions. Here, I used DMPS (1,2-dimyristoyl-sn-glycero-3-phospho-L-serine) and natural brain PIP2 (L-α-phosphatidylinositol-4,5-bisphosphate) to investigate the role of lipids in interaction of Talin with the membrane surface. Furthermore, I performed studies on the Talin mutants K322E and K324E to determine their roles in membrane interactions. Thus, I demonstrated that PIP2 could be a potential regulator and important membrane component for Talin mediated integrin activation, and I supported the hypothesis that K322 and K324 are key residues for Talin membrane interaction.

## Introduction

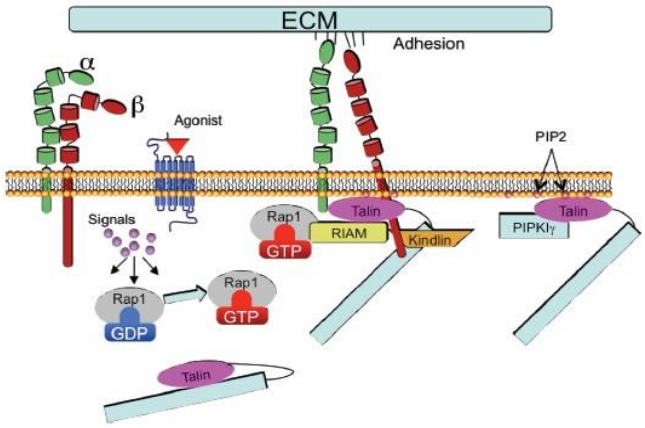


**Figure 1.** Inside out pathway of integrin activation

Activation of integrin is critical to a wide range of biological processes including embryonic development, hemostasis, cell migration, wound healing, and the immune response (1). Integrins normally exist in an inactive resting state, characterized by a bent extracellular domain (2-5). Talin is widely believed as the main regulator and final step of integrin activation (6).

Talin is a ~270 kDa protein consisting of a head domain (THD; 45kDa), a small linker region (80 aa), and a rod domain (~250 kDa) (7, 8). The interactions of Talin and integrin β-tail induce the separation of the integrin cytoplasmic tails and a subsequent conformational change that extends the extracellular domain and allows ligand binding (9). Talin normally exists with auto-inhibited conformation in cytosol with C-terminal rod domain masking N-terminal FERM domain. Such interdomain interaction would impede Talin mediate integrin activation. Several mechanisms have been identified for Talin activation: (1) interaction with Rap1 Interacting Adapter Molecule (RIAM); (2) cleavage of the linker region by calpain; (3) binding PIPK1γ; (4) PIP2 is also known as one of the Talin activators (10) (Fig. 1).

The protein-protein interactions in Talin mediated integrin activation have been well studied. The protein-lipid interactions require further investigation. One of the obstacles is that many existing experimental techniques have little control of the local lipid composition of the membrane. Traditionally, protein targets are inserted into synthetic liposomes, or solubilized in detergent and these heterogeneous systems have several drawbacks.

To overcome these limitations, we utilized the Nanodisc system. The Nanodisc membrane mimetic has been applied to a wide variety of membrane bound receptors and enzymes such as the cytochromes P450, the seven trans-membrane helical GPCRs, enzymes and cofactors of the coagulation cascade, chemotaxis receptors, and our recent work with integrins (11-19). Preparation of Nanodiscs is achieved through a self–assembly reaction consisting of Membrane Scaffold Protein (MSP), which is derived from human apolipoprotein A1, and detergent solubilized phospholipids.

Through this Nanodisc system, we are able to control the local concentration of anionic phospholipids. Beyond this advantage, there are several other benefits of Nanodiscs. When a target receptor is incorporated into a Nanodisc, the researcher has access to both sides of the membrane. Thus, for a receptor such as integrin, which can undergo both extracellular and intracellular signaling pathways, careful experimental design will allow analysis of both pathways.

**Aim 1 – Understanding the role of lipids in membrane interaction of Talin**

Based on my previous proposal, the main purpose was to understand how phospholipid bilayer modulates Talin activation and localization to focal adhesion, which in turns lead to efficient integrin activation. I first focused on the Talin-membrane interaction in inside-out signaling to investigate the role of the lipid head groups. Here, I used two different types of major anionic phospholipids: DMPS (1,2-dimyristoyl-sn-glycero-3-phospho-L-serine) and natural brain PIP2 (L-α-phosphatidylinositol-4,5-bisphosphate).

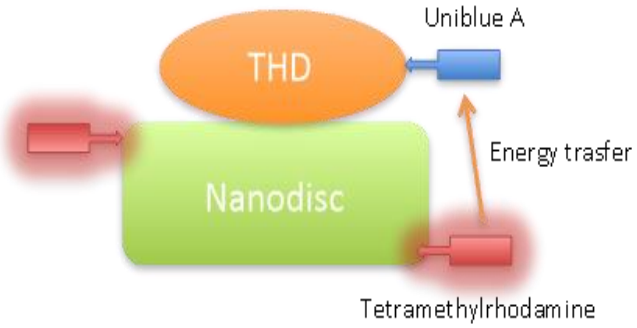


**Figure 2.** FRET in Nanodisc

To characterize the specificity of Talin head domain (THD) for DMPS and PIP2, we use a FRET based binding assay. A schematic is shown in figure 2. Nanodiscs are labeled with tetramethylrhodamine (TAMRA), and Talin is labeled with Uniblue A, a dark quencher. The

binding affinities of several different phospholipids and Talin are measured by monitoring the quenching of TAMRA upon Talin binding.

Wild type Talin head domain was engineered with a fast reacting accessible cysteine mutation for thiol-reactive labeling. Target protein was expressed in E.coli BL21 (gold) DE3 strain and then purified with Ni-NTA resin. To label the Talin head, 4 fold molar excess of TCEP was incubated with protein for 10 minutes at room temperature to reduce disulfide bonds between cysteine. Then 2 fold molar excess of Uniblue A, which contains a vinyl sulfone reactive group, was mixed with protein and incubated for 1 hour and 45 minutes at room temperature. MSP1D1 D73C was labeled by maleimide tetramethyrhodamine (TAMRA) with similar protocol as THD with 10 fold excess fluorophore dye and additional 4 degree overnight incubation.

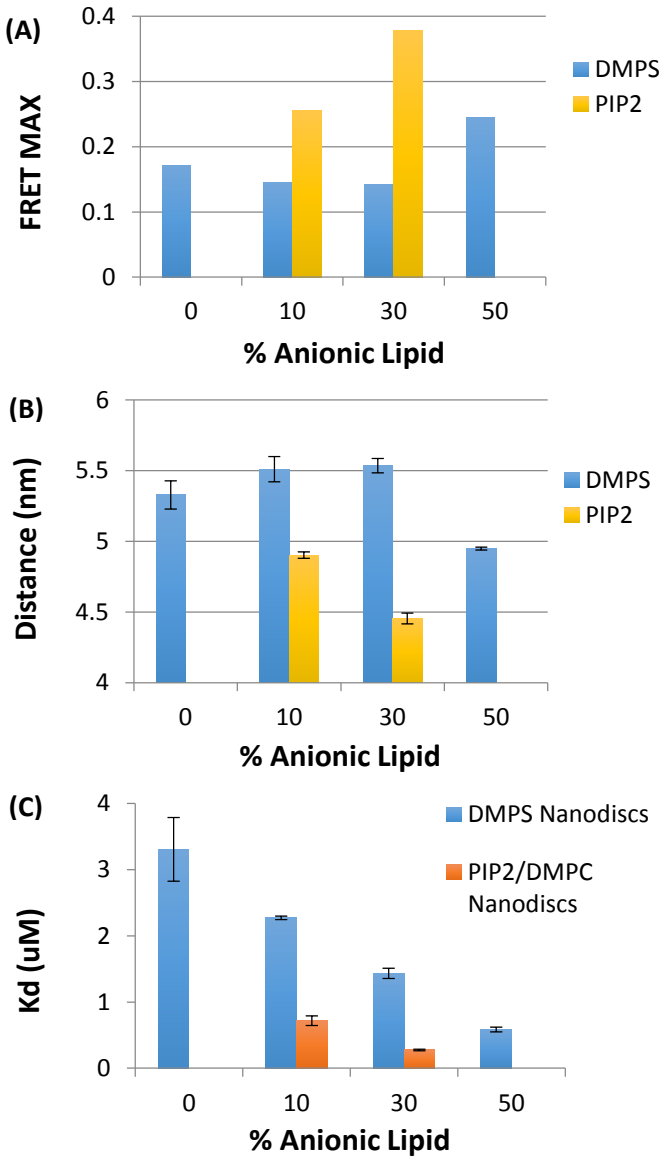


**Figure 3.** Binding of Talin to phospholipids

To make the Nanodiscs, DMPS, I prepared mixtures of 0%, 10%, 30% and 50% of DMPS, balanced with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), into glass tubes and dried the lipids under vacuum overnight. Then, a 2 fold molar excess of sodium cholate was added to each tube to solubilize the dried lipid. MSP1D1 TAMRA was mixed into each tube followed by the addition of bio beads. The mixture was incubated on a platform shaker for 4 hours to ensure complete removal of the detergent. Following that, the Nanodiscs were purified by sized exclusion column Superdex 200 increase, equilibrated with cytoplasmic mimic buffer, at flow rate 0.75mL/min. The FRET based binding assay was performed by titrating THD stepwise into 100nM Nanodiscs. By observing the change of Nanodiscs fluorescence signal, 557m excitation, 580nm emission, the FRET efficiencies were calculated as (F0-F)/F0 with after correction for dilution. Fitting was

performed using Matlab software suite modeled as a single binding site to obtain the dissociation constants and maximum FRET efficiencies.

| Kd (uM) | 50% | 30% |
|---|---|---|
| DMPS | 0.583 | 1.431 |

| Kd (uM) | 10% | 5% |
|---|---|---|
| PIP2 | 0.276 | 0.716 |

**Table 1.** Kd (uM) of DMPS and PIP2

THD binds well to both DMPS and PIP2 lipid bilayers. THD has noticeable higher affinity for PIP2, with Kd of 0.72uM for 5% and 0.28uM for 10%, whereas 50% and 30% DMPS have Kds at 0.58uM and 1.43uM. (Table 1). The Results also show that PIP2 Nanodiscs has greater FRET maximum efficiency than that of DMPS Nanodiscs (Fig. 3A).This suggests that the distance between THD and PIP2 Nanodisc is much closer than that of THD and DMPS Nanodisc (Fig. 3B). These results indicates that THD has a preference for PIP2 over PS, which is consistent with earlier reports that PIP2 facilitates membrane localization of Talin. The difference in distances on PIP2 vs. DMPS bilayers between THD and Nanodiscs suggests that PIP2 induced a conformational change when it is presents in focal adhesion, which is in an agreement with previous studies that I plate PIP2 as a potential regulator and important membrane component for Talin mediated integrin activation.

**2) Aim 2 – Mutagenesis study of Talin membrane interaction**

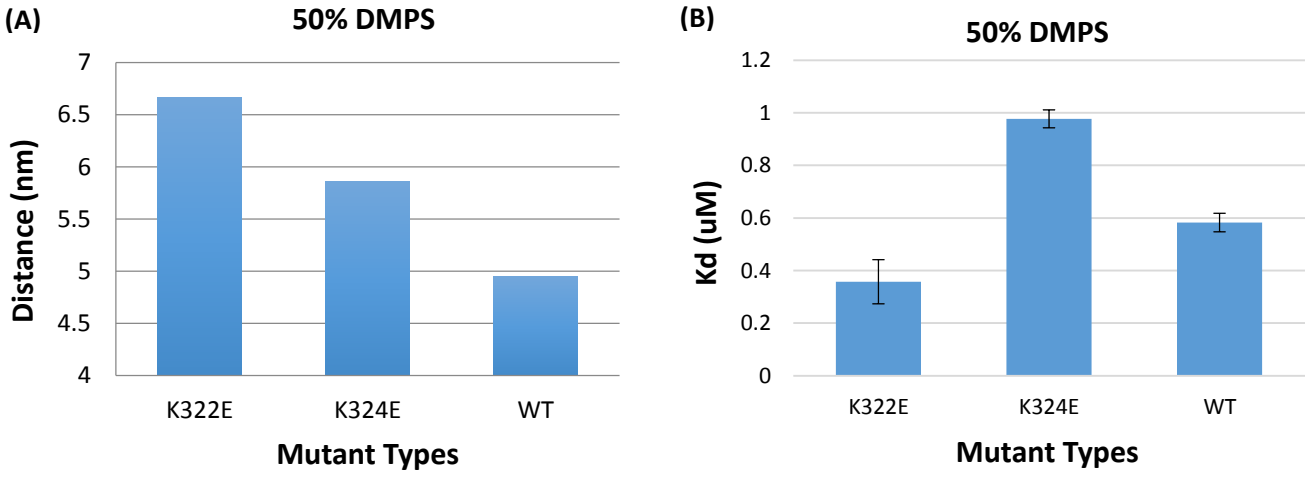


**Figure 4.** Binding of mutant Talin to phospholipids

MD simulations of the F2F3 subunit suggest a novel mechanism for membrane interactions, including a hydrophobic membrane anchor (F259 and F280) in the F2 domain and direct association (K322 and K324) of F3 with the bilayer triggered by binding of membrane orient patch (MOP). Many

other in vivo and vitro studies showed that mutations at K322 or K324 could lead to reduction of cell spreading and failure of activating integrins.

Here we propose that reversing the charge at K322 or K324 would alter the interaction between THD and membrane. To test this hypothesis, single site mutations K322E or K324E were introduced into THD by Quick change PCR. The FRET binding assay was used to determine the Kd and FRET max of K322E, K324E and WT (wild type) to 50% DMPS Nano discs. Here I observed K322E has the lowest FRET max and K324 is in between WT and K322E. In other words, changing charge in that area induced increased distances between THD F3 and membrane surface. This study supports the hypothesis that K322E and K324E mutation affects lipid binding and disrupts Talin mediated integrin activation by placing the F3 domain in an unfavorable position to integrin tail and membrane.

The experiments above shed a light on the role of Talin-membrane interactions in the activation of integrins. For the future plan, I will study the role of the FERM domain, which consists of F0, F1, F2, and F3. To investigate the function of each subdomains, I will clone each Talin subdomains (F0, F1, F2, and F3) and express them individually. Then, I will utilize the FRET Talin binding assay to probe the binding affinity of each subdomain with membrane.

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