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Thermodynamic evidence of non-muscle myosin II–lipid-membrane interaction

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Abstract

A unique feature of protein networks in living cells is that they can generate their own force. Proteins such as non-muscle myosin II are an integral part of the cytoskeleton and have the capacity to convert the energy of ATP hydrolysis into directional movement. Non-muscle myosin II can move actin filaments against each other, and depending on the orientation of the filaments and the way in which they are linked together, it can produce contraction, bending, extension, and stiffening. Our measurements with differential scanning calorimetry showed that non-muscle myosin II inserts into negatively charged phospholipid membranes. Using lipid vesicles made of DMPG/DMPC at a molar ratio of 1:1 at 10 mg/ml in the presence of different non-muscle myosin II concentrations showed a variation of the main phase transition of the lipid vesicle at around 23 °C. With increasing concentrations of non-muscle myosin II the thermotropic properties of the lipid vesicle changed, which is indicative of protein–lipid interaction/insertion. We hypothesize that myosin tail binds to acidic phospholipids through an electrostatic interaction using the basic side groups of positive residues; the flexible, amphipathic helix then may partially penetrate into the bilayer to form an anchor. Using the stopped-flow method, we determined the binding affinity of non-muscle myosin II when anchored to lipid vesicles with actin, which was similar to a pure actin-non-muscle myosin II system. Insertion of myosin tail into the hydrophobic region of lipid membranes, a model known as the lever arm mechanism, might explain how its interaction with actin generates cellular movement.

Keywords: Non-muscle myosin II; Lipid membranes; Insertion; Hydrophobic binding; F-Actin

Myosins play an enormously diversity of motile functions in cells: from muscle contraction to phagocytosis. To date more than 18 different myosins are recognized in nature, some are restricted to plants, others are known as acomplexans with some 40 genes in 12 classes of myosins in the human genome [1,2].

Despite the huge number of myosin family members, we are focusing on non-muscle myosin II, a two-headed myosin that is responsible for contractile functions such as cytokinesis in the vast majority of non-muscle cells. Non-muscle myosin II mini-filaments are found in many cells, where they perform contractile functions related to cell adhesion, spreading, and locomotion. They induce contractile tension between adhesions and as cells progress form focal contacts at the rear of cells. A combination of focal contact/adhesion, assembly/disassembly, and a build up of strain results in the release of the adhesion and elastic recoil of the material toward the cell body. These recoil events are often believed to correspond to episodes of increased protrusion at the leading edge. Cells locomoting on normal substrates develop adhesions through which the cell develops force [3–5].

Basically non-muscle myosin II is a group of motor proteins capable of transforming chemical energy in the form of ATP to movement *via* the amplification (by levers) of

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conformational changes within the ATP hydrolyzing the head group. Each catalytic head group is controlled by two light chains, a regulatory and an essential light chain. Light chains are calmodulin-like proteins which wrap around a helical neck region, whilst the heavy chain tail regions wrap round each other. Each non-muscle myosin II molecule self-associates in an anti-parallel manner regulated by phosphorylation at the C-terminus. It performs many functions in cells beside cell locomotion but probably its most important function is to constrict the waist of the cell and so allow it to divide. Phosphorylation regulates the assembly of the myosin mini-filaments that allow them to exert a pulling force on actin filaments. The assembly of myosin mini-filaments in cells is crucial to non-muscle myosin II's contractile function which is regulated by a large number of different types of kinases that are in turn are activated by a large number of signaling pathways to regulate their assembly [6–8].

Since the discovery of myosin II in non-muscle cells, it was hypothesized that myosin II plays a pivotal role in cell motility and locomotion that was based on the premise which involves myosin II and actin contractility. To execute these functions effectively in an intact cell system, however, it would be essential for non-muscle myosin II to attach to and/or insert into the lipid membrane [9–12].

Here we report that non-muscle myosin II tail has the potential to interact with, or embed into, lipid membrane regions consisting of phospholipid vesicles. Our experimental analyses using differential scanning calorimetry (DSC), stopped flow measurements and freeze/thaw/centrifugation assays indicate that non-muscle myosin II may play an essential role in providing a stable membrane anchor for actin-non-muscle myosin II interaction. First computational results that the C-terminal tail region could contribute to lipid interaction/insertion will be discussed.

Materials and methods

Protein and lipid preparations. Complete (intact) non-muscle myosin II was isolated from mice brain according to Murakami and Elzinga [13] and the modified protocol by Isenberg [14]. Purified non-muscle myosin II was normally used within 10 days after purification. Unused non-muscle myosin II was stored at −20 °C by dissolving it in 0.5 M KCl, 1 mM DTT, 10 mM K_2HPO_4/KH_2PO_4, pH 6.5, and mixing it with an equal volume of glycerol. Prior to experimentation, non-muscle myosin II was first dialyzed against high ionic buffer (0.6 M KCl, 50 mM KH_2PO_4, and 2 mM DTT, pH 6.5) and then against low ionic buffer (15 mM NaCl, 1 mM EDTA, and 10 mM Hepes, pH 7.5) at 4 °C. The purity and viability of non-myosin II was routinely tested.

The phospholipids (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) were purchased from Avanti Polar Lipids (Birmingham, AL, USA) and used without further purification. Lipid stock solutions were prepared by dissolving these lipids in chloroform/methanol 2/1 (v/v). From aliquots of these solutions, a dry lipid film was formed on the walls of an extensively rinsed glass beaker by evaporating the solvent with a stream of nitrogen followed by vacuum desiccation for at least 3 h. The lipid film was dissolved in 15 mM NaCl, 1 mM EDTA, 10 mM Hepes, pH 7.5, for the preparation of multilamellar vesicles. The lipid dispersion was then pressed 10 times through 200-nm filters [15] and used in experiments at these buffer conditions.

Differential scanning calorimetry. A differential scanning calorimeter Q100 from TA Instruments was used. Briefly, the reservoirs for the sample and reference solution are made of stainless steel to hold a volume of around 100 μl each. Lipid-buffer solutions are placed in the reference cell and the lipid vesicle-myosin-II-buffer solutions in the sample cell. Under sealed conditions, both solutions are heated/cooled at a rate of 0.5 °C/min between +7 °C and +35 °C in six to eight cycles until the equilibrium of the phase transition enthalpy was reached, using a mixture of DMPC and DMPG at 10 mg/ml and molar ratio of 1:1. A phase transition was observed at around 23 °C. Traces from various cycles (at different protein/lipid ratios) were averaged and data analysis was performed using the software from *Universal Analysis 2000* (TA Instruments) and *Origin 7G* [16].

Stopped flow measurements. Rapid-mixing studies of non-muscle myosin II (bound to phospholipids) and non-muscle myosin II in the absence of phospholipids with F-actin were performed at ambient temperature on a stopped-flow spectrophotometer (Hi-Tech Ltd., Salisbury UK). In brief, a driving mechanism operates by compressed air at 4 bar, pushes the sample into the observation cell and a micro-switch is triggered by a front stop that initiates signal detection. The dead time of the apparatus is about 2 ms. Changes in light scatter signal at 355 nm and at a 90° angle were recorded with time [17].

Data analysis was performed according to Hiromi [18]. The plot of $[A] - \alpha[M]$ as a function of $[A]/\alpha$ was obtained from binding studies using the following equation: $[A]_0/\alpha = ([M]_0 + K_d) + ([A]_0 - \alpha[M]_0)$, where, $[A]_0$ = variable actin and $[M]_0$ = constant non-muscle myosin II concentration at $t = 0$ s; K_d = binding (dissociation) constant ($K_d = 1/K$), and α = the fractional saturation of non-muscle myosin II by actin. α is defined by the relationship, $\alpha = \frac{I_0 - I}{I_0 - I_\infty}$ where, I_0 = light scatter signal at 355 nm in the absence and I_∞ = at infinitely high actin concentration.

Freeze/thaw, centrifugation, and SDS–PAGE gel. The phospholipid vesicle dispersions in the presence and absence of increasing protein concentrations were subjected to six independent freeze/thaw cycles (from +7 °C to +35 °C). At the end of these cycles at various P/L molar ratios, the proteo-liposomes were centrifuged at 100,000*g* for 30 min at 4 °C to separate bound from unbound proteins to lipids followed by SDS/PAGE gel analysis [19]. The reconstitution of increasing non-muscle myosin II concentrations into mixed vesicles were densitometrically determined using the program ImageJ measuring the intensities, i.e. gray scales of the gel (http://rsb.info.nih.gov/ij/download.html).

Results

Differential scanning calorimeter

Non-muscle myosin II insertion into artificial phospholipids membranes was demonstrated using calorimetric measurements. The measurements were performed with multilamellar vesicles (MLVs) at 10 mg/ml consisting of DMPC/DMPG and at molar ratio of 1:1. Adding increasing non-muscle myosin II concentration (traces b → e; 0.62 → 6.24 μM) to the lipid solution (a; no non-muscle myosin II), a widening and flattening of the peak curvature was observed (Fig. 1A). The relative widening between the start (T_S) and endpoint (T_L) of the phase transition for each non-muscle myosin II concentration indicated by the arrows was plotted in Fig. 1B. In control experiments, non-muscle myosin II alone showed no signs of degradation (data not shown).

Plotting the enthalpy changes $\Delta H/\Delta H_0$ against the molar ratios of non-muscle myosin II and lipids, an initial

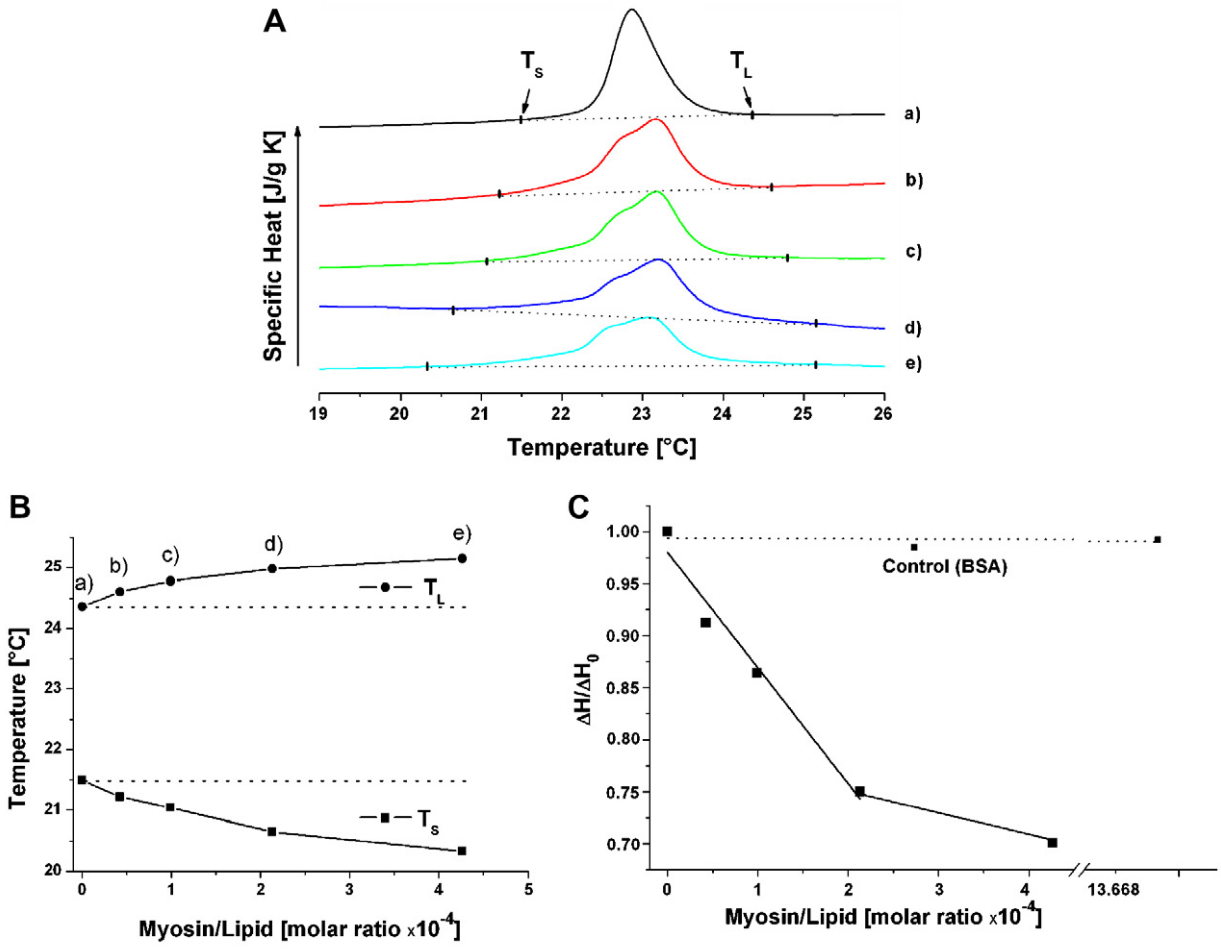

Fig. 1. (A) *DSC* thermograms of DMPC/DMPG without (a) non-muscle myosin II and with non-muscle myosin II (b → e). Conditions: lipid and non-muscle myosin II concentrations: 14.62 mM and (b) 0.62 μM (c) 1.45 μM (d) 3.12 μM, and (e) 6.24 μM, respectively. (B) A plot of T_S (solidus points) and T_L (liquidus points) taken from (a) as a function of non-muscle myosin II–lipid molar ratios. (C) Relative changes in enthalpy $\Delta H/\Delta H_0$ against non-muscle myosin II–lipid molar ratios. Bovine serum albumin (BSA) was used as a control protein.

linear relationship followed by a saturation behavior of the lipid vesicles for non-muscle myosin II was observed. The control protein bovine serum albumin (BSA) showed no changes (Fig. 1C). For a better comparison of the changes induced by the various non-muscle myosin II concentrations, the enthalpy changes, ΔH were normalized to pure lipids, against ΔH_0 (Table 1).

The thermodynamic measurements (DSC) proved sufficient to determine the insertion behavior of non-muscle myosin II into lipid membranes composed of DMPG/DMPC *in vitro*. The binding affinity of non-muscle myosin II associated with and without lipids and actin using the stopped flow method was of similar order of magnitude (Fig. 2), confirming (i) its viability when attached/inserted into the lipid membrane and (ii) previous observations of other membrane interacting proteins [20].

Table 1
Normalizing non-muscle myosin II concentration against constant lipid concentration and enthalpy changes ΔH against ΔH_0 (lipids only)

[Lipid]	[Myosin] in μM	[Myosin]/[lipid]	$\Delta H/\Delta H_0$	Trace
10 mg/ml ≅ 14.62 mM	0	0	1	a
	0.62	1/23.580	0.91251	b
	1.45	1/10.080	0.86383	c
	3.12	1/4.690	0.74985	d
	6.24	1/2.345	0.70055	e

Note: enthalpy, ΔH is here the integrated form of specific heat, which is the measure of the heat energy required to increase the temperature of a unit quantity of a substance by a certain temperature interval, see Fig. 1A.

Freeze/thaw, centrifugation, and SDS–PAGE analysis

To further investigate whether membrane-bound non-muscle myosin II was reconstituted into mixed DMPG/DMPC vesicles, unbound and stably incorporated proteins were separated into supernatant (S) and vesicular pellet fractions (P) after centrifugation, respectively. Fig. 3 confirms results from DSC measurements quantitatively and demonstrates (a) that non-muscle myosin II at concentrations between 0.5 and 5 μM are with increasing protein concentration almost completely incorporated into lipid vesicles and (b) that around 5 μM non-muscle myosin II

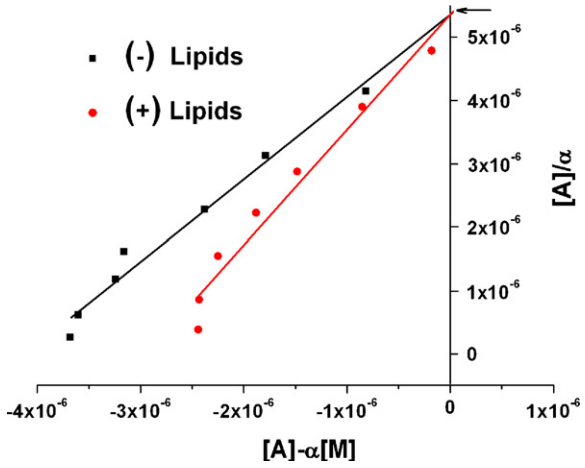

Fig. 2. A plot $[A] - \alpha[M]$ (x-axis) against $[A]/\alpha$ (y-axis) gives a linear relationship where, the intercept is the dissociation constant, K_d. The linear fit shows an intercept with the y-axis that equals $([M] + K_d)$. K_d for non-muscle myosin II (bound to lipids, $[M] = 5\ \mu M$) and F-actin $= 0.362\ \mu M$; K_d for non-muscle myosin II and F-actin $= 0.357\ \mu M$ [20].

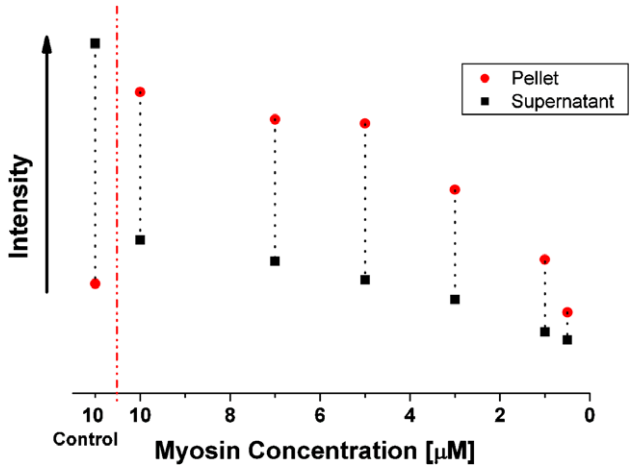

Fig. 3. Intensity score from densitometric measurements of a SDS–PAGE gel of bound and unbound non-muscle myosin II. As control, 10 μM non-muscle myosin II in the absence of lipids was used, which showed most protein in the supernatant and little in the pellet at 100,000g centrifugation.

concentration a saturation behavior of lipid incorporation occurs (see almost constant values for the pellet).

Discussion

The role of lipid interactions of actin binding proteins is of particular interest, since an increasing number of proteins are known to share this capability [21–24]. Lipid binding of actin-associated proteins is not an unspecific binding, but often enhanced by PIP_2. In general, the anchorage of actin cross-linking proteins to the plasma membrane is believed to be of crucial importance for stabilizing the cortical cytoplasmic actin filament network within cells [12]. Differential scanning calorimetry has proved to be a valuable method for measuring lipid phase transitions by giving information about the packing and order of lipids with respect to temperature. Hydrophobic and/or electrostatic changes due to protein–lipid interactions are detected accurately by this method.

To elucidate the potential interactions of non-muscle myosin II with lipid bilayers we have chosen a multilamellar vesicles system because it gives rise to very sharp main phase transitions, allowing the detection of subtle perturbations during the lipid melting process. The results obtained by differential scanning calorimetry clearly show that non-muscle myosin II interacts with phospholipids. The nature of non-muscle myosin II–lipid interactions depends on the type of phospholipids. For pure, uncharged DMPC vesicles (data not shown), we found that non-muscle myosin II suppresses the enthalpy. The addition of negatively charged phospholipids results in a significant decrease in the main phase transition enthalpy, and a marked shift of the solidus (T_S) and liquidus (T_L) lines [25]. These shifts are most readily explained by two effects: first, a hydrophobic interaction due to a partial penetration of amino acid side chains into the lipid bilayer core that expands, destabilizes, and rearranges the phospholipid structure will give rise to lipid phases with transition temperatures lower than those obtained for pure lipids. Second, an electrostatic, charge-neutralizing interaction occurring at the membrane surface, condensing, and stabilizing the gel phase of the phospholipid bilayer, will give rise to lipid phases with transition temperatures higher than those of the pure lipids. These effects can lead to an electrostatic attachment and hydrophobic insertion of non-muscle myosin II into charged phospholipid bilayers. Using freeze/thaw/centrifugation assays and SDS–PAGE analysis confirmed the non-muscle myosin II–lipid interaction and applying the stopped flow method showed that the binding kinetics of (non-muscle myosin II bound to lipids) with F-actin were of similar affinity, i.e. active, as non-muscle myosin II in the absence of lipids with F-actin.

First, secondary structure analysis of the sequence of non-muscle myosin II molecule from the sequence data bank file (Q61879, mouse) revealed between residues 112–1976 hydrophobic and helical amphipathic segments in the tail region. On closer analysis using the hydrophobicity plot methods developed by Eisenberg [26] and expanded by Tempel [27], we observed a hydrophobic region near residues 786–799. According to Eisenberg [26] membrane penetration requires an average hydrophobicity of 0.68 or more. As shown in Fig. 4A at residue number 793, the average hydrophobicity value is ~0.85. Since hydrophobicity plots do not differentiate between alpha-helical and beta-strand structures nor whether a region is hydrophobic or amphipathic, we compared the computer predictions with published 3D structures for non-muscle myosin II for this species and revealed that the primary sequence residues 786–799 are alpha-helical (Fig. 4B). This behavior has been described for many other membrane-associated proteins (reviewed in [23]).

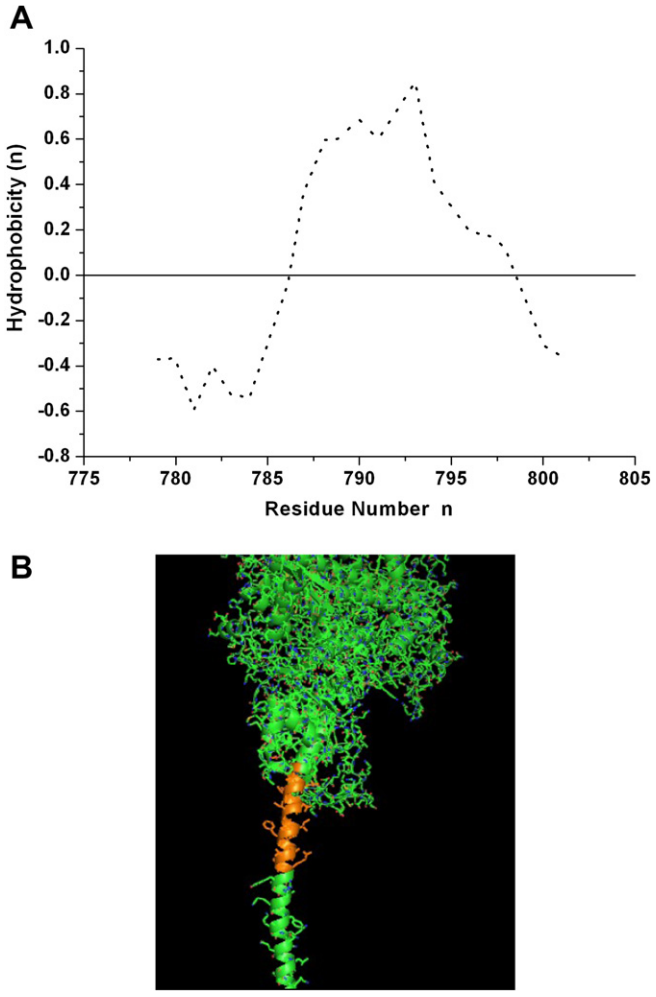

Fig. 4. (A) Structure prediction plot for the C-terminal end (residues 775–805) of myosin II (mouse) with 1976 total residues. The average hydrophobicity according to the method of Kyte and Doolittle [28] is displayed between residues 786–799. (B) The predicted lipid-binding site of mouse non-muscle myosin II from the sequence data bank (file Q61879) is shown on the tail domain (alpha-helical) in orange [23]. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

In summary, we present evidence by three independent approaches that non-muscle myosin II *in vitro* interacts with phospholipids (probably hydrophobically and/or electrostatically) and can be reconstituted into lipid vesicles. In light of the fact that non-muscle myosin II is regulated in its function by PIP_2, the described interaction with phospholipids membranes is of particular interest. We have therefore started molecular dynamics investigation of the non-muscle myosin II tail in different charged states under solvated conditions, approximating a range of conditions in the cytosol. Because of the size of the entire non-muscle myosin II tail, the simulations will be kept computationally manageable. The aim is to explore the conformational changes and identify the energetically favorable geometries. The favorable geometries could be used to *either* act as putative anchors in a future lipid membrane model *or* interact with conformations of the non-muscle myosin II tail and ensure there is a binding site for soluble phospholipids. Future elaborate work is now needed to verify these *in vitro* observations under physiological conditions in an *in vivo* system and their effect on cell spreading, motility and tractions.

Acknowledgments

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