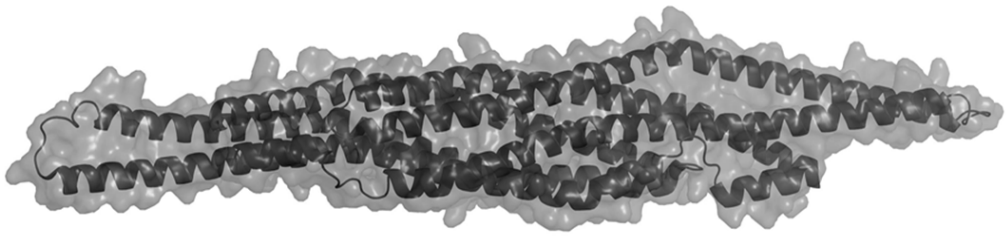
Ph.D. THESIS

The role of IRSp53 protein in the filopodia formation



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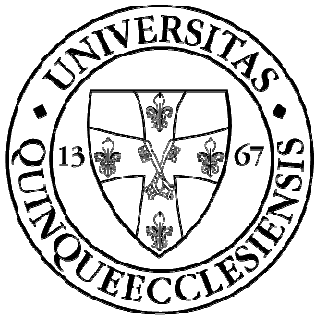

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Introduction

Actin contributes to biological processes such as sensing environmental forces, internalizing membrane vesicles, moving over surfaces and dividing the cell in two. These cellular activities are complex; they depend on interactions of actin monomers and filaments with numerous other proteins [1].

The 53-kDa insulin receptor substrate protein (IRSp53) is part of a regulatory network that organizes the actin cytoskeleton in response to stimulation by small GTPases, promoting formation of actin-rich cell protrusions such as filopodia and lamellipodia. Filopodia are membrane-based actin-rich finger-like protrusions that play roles in a number of cellular and developmental processes. IRSp53 is widely expressed in different tissues and cell-types in learning, memory and synaptic plasticity. Furthermore, certain IRSp53 alleles in humans have been linked to neurological problems such as attention-deficit/ hyperactivity disorder [2-4]. Importantly, lens-retina inter-epithelial filopodium formation is defective in IRSp53 knockout mice, providing evidence that this protein promotes filopodium formation also in vivo [5].

The Inverse BAR (I-BAR) domain (IRSp53/ MIM Homology Domain) [6] is a 250 amino acid long region at the the N-terminal of the IRSp53 protein that has been shown to induce negative membrane curvature both in vitro and in vivo. In the recent work that is way we concentrate on the N-terminal part of IRSp53 protein [7]. Purified recombinant I-BAR bundled actin filaments *in vitro*, and it was postulated that its actinbundling property was critical to its function [6]. I-BAR shows structural similarity to BAR domains, which bind to to membranes of a specific curvature [8-10]. Positively charged lysines are concentrated at the ends of the I-BAR and are important for binding the I-BAR to the membrane [7, 11-13]. These results suggest that the I-BAR can enahance filopodia formation by simply bundling actin filaments at the cell periphery. However, more recent has suggested that the I-BAR can bind to and deform membrane phospholipids and questioned the relevance of the actin interaction [5]. The membrane bending activity of the I-BAR proteins is also coupled to actin dynamics. Consequently, I-BAR shares its function between actin and membrane binding but the details of this interaction are highly unclear.

In my PhD work I would like to describe the molecular mechanisms of actin- and membrane binding properties of IRSp53 protein, because cell protrusions plays role in many cellular processes, however the dynamics of filopodia is not well known. Filopodia formation is necessary to understand the cell communication. We expect that the results of the work will greatly improve our understanding about filopodia formation and the role of IRSp53 and its I-BAR domain in it.

Aims

The aim of our project is to investigate the IRSp53/I-BAR–actin interaction *in vitro* and *in vivo* in response to filopodia formation. Our aims were:

1. to describe the membrane-binding propertise of IRSp53-I-BAR proteins and determine the equlibrium dissosiation constant, which is characteristic on it
2. to characterise the actin-binding properties of I-BAR domain at different ionic strength
3. to investigate the affect of monomeric and filamentous actin on the I-BAR-actin interaction
4. to study the effect of I-BAR on the actin filament polymerisation
5. to follow the IRSp53/I-BAR overespresson in COS-7 cells
6. to study the changing of the polymerisation state of actin in COS-7 cells.

Methods

Proteins

α-actin from rabbit skeletal muscle was prepared according to the method of Spudich and Watt [14]. After the preparation the actin was stored in buffer A: 2 mM TRIS/HCl (pH8.0), 0,2 mM ATP, 0,1 mM $CaCl_2$, 0,005% NaN_3, 0,5 mM 2-merkaptoetanol or DTT.
IRSp53-I-BAR was encoded in pGEX4T2 plasmid and expressed in E. coli strain BL21 DE3) pLysS. [12]. The four lysine-lacking mutant I-BAR (K142E, K143E, K146E, K147E [12]) (K4E-I-BAR) was also cloned in the same vector and purified similarly. The recombinant proteins were loaded onto a GSH- column, eluted from the column after cleavage by thrombin and further purified by affinity cromatography (Pharmacia FPLC). After the preparation the proteins were kept in - 80 °C in storing buffer.

Preparation of vesicules and micells

Phospholipids from bovine brain were purchased from the Avanti Polar Lipids (Alabama, USA): phosphatidylcholine (PC), phosphatidylserine (PS), L-α-phosphatidylinositol-4,5-bisphosphate (PIP_2). Large unilamellar vesicules (LUVETs) of various compositions were made by an extrusion technique. Lipids were dissolved in chloroform and were dried in a glass tube under a stream of nitrogen. PIP_2, which had beeen resuspended in deionized water, was added to dried lipids, along with enough deionized water to produce the desired final concentration; all lipids were then resuspended by vortexing. After five cycles of freezing in liquid nitrogen and thawing in 37°C water bath, samples were passed 10 times through a polycarbonate filter (200 nm pore size) in an Extruder (Avestin, Ottawa, ON, Canada). LUVETs were prepared of 100% PS, or 100% PC or the combination of 70% PS and 30% PC (PS/PC (70/30) lipids. We also used $PI(4,5)P_2$ in 4 or 15% together with 96 or 85% PC liposomes (PIP_2 /PC (4/96) or PIP_2/PC (15/85)) in the studies, respectively. Homogen PIP_2 micells were made, 1 mg PIP_2 was solved in 0,5 ml buffer [15].

Fluorescence labelling of actin and IRSp53, spectrophotometry

Towards the fluorescence spectroscopic measurements the Cys374 residue of actin monomer and the Cys137, 195, 202, 230 residues of I-BAR were labelled with IAEDANS or IAF fluorophores.

The concentrations of the examined proteins and the fluorescence labels were determined on the basis of absorption of the samples. The absorption was measured with a Jasco V-660 spectrophotometer. The concentrations of the proteins were determined by measuring the absorption at 280 nm.

„Steady-state" fluorescence spectroscopy

Fluorescence was measured with Horiba JobinYvon (Longjumeau, France) fluorimeter equipped with a thermostated sampler holder. The appropiate values of the exication and emission wavelengths were set with monochromators. The exitacion and emission slits were 5 nm.

Förster-type Resonance Energy Transfer (FRET) measurements

Steady-state Förster Type Resonancy Energy Transfer (FRET) was measured between fluorescence-labeled I-BAR (with IAEDANS on cysteine as donor) and labeled LUVETs (with DiO as acceptor), and its efficiency was calculated. The efficiency of FRET (E) can be determined from the donor intensities by using the following equation:

$$E = 1 - (F_{DA}/F_D) \quad (1)$$

where F_{DA} and F_D are the fluorescence intensities of the donor in the presence and absence of the acceptor, respectively.

The binding characteristic was determined by increasing concentrations of lipids and fixed I-BAR concentration. We have used a quadratic equation to fit the binding data and calculate the Kd [16].

$$[PL]/[P] = (([P] + [L] + K_d) - (([P] + [L] + K_d)^2 - 4 * [P] * [L])^{0,5}) / (2 * [P]) \quad (2)$$

where [L] is the total concentration of the lipids, which is varied in the experiments. [PL] is the concentration of the I-BAR bound to the lipids and [P] is the total concentration of I-BAR. The ratio of these two parameters, [PL]/[P], measures the fraction of I-BAR domain bound to lipids.

Fluorescence quenching experiments

Fluorescence quenching experiments were carried out on IAEDANS-I-BAR which was titrated with a neutral quencher (acrylamide). We analyzed the data collected in steady-state quenching measurements by using the classical Stern-Volmer equation [17]:

$$F_0/F = 1 + K_{SV} * [Q] \quad (3a)$$

where F_0 is the fluorescence intensity of the sample in the absence of the quencher molecule whereas F is the fluorescence intensity at different quencher concentrations [Q].
The Stern-Volmer constant (K_{SV}) was determined with the bimolecular quenching constant (k_+) and the fluorescence lifetime (τ_0):

$$K_{SV} = k_+ * \tau_0 \quad (3b)$$

„Steady-state" fluorescence anisotropy

Fluorescence anisotropy of IAEDANS-labelled actin monomers in the absence and in increasing concentration of IRSp53-I-BAR. The equilibrium dissociation constant (K_d) was determind by the following formula [16]:

$$[PL]/[P] = (([P] + [L] + K_d) - (([P] + [L] + K_d)^2 - 4 * [P] * [L])^{0,5}) / (2 * [P]) \quad (4)$$

where [L] the total concentration of I-BAR (increasing concentration during the experiment), [PL] is the concentration of the I-BAR bound to the IAEDANS-actin, [P] the total concentration of IAEDANS-labelled actin monomers.

TNS fluorescence assay

TNS fluorescence intensity was followed during the experiment. TNS can be sensitive to the disorder of the aliphatic core of lipid micelles or vesicles [29, 30]. The measurements were performed with Horiba Jobin Yvon (Longjumeau, France) spectrofluorometer equipped with a thermostable cuvette holder. The excitation wavelegth was 360 nm, the flurescence emission was recorded between 360-560 nm.

Investigation of actin polymerisation

The polymerisation of actin was followed by pyrene intensity. In monomeric form pyrene-actin has a relatively low fluorescence signal but as it incorporates into filaments its fluorescence emission increasis. The fluorescence intensity was measured as a function of time, the excitation wavelength was 365 nm and the emission wavelength was 405 nm. The polymerisation curves were normalised. The elongation rate was determined from the slope of the linear fit to the pyrene fluorescence curves at half-maximum polymerisation. We calculated the rate of polymerisation from these slopes to s^{-1} units and these were plotted as a function of I-BAR concentration.

Co-sedimentation assay

To characterise the binding of IRSp53-I-BAR to actin filaments co-sedimentation assays, were carried out. For the experiment pyrene-labelled actin was used. The excitation and emission wavelengths were 365 nm and 407 nm, respectively. After the fluorescence spectroscopic measurement the samples were ultracentrifuged (Beckman: TLA 100 rotor, 80000 rpm, 20^{o}C, 40 min). The protein content of the pellet and the supernatants were analysed by SDS-polyacrylamide gel electrophoresis (12%) and the gels were stained with coomassie blue.

Statistic

During the data analysis standard error of the mean (SEM) was given biside the average values. In some cases the average values were compered with two-sampled t-test using 0,05 significance level.

Fluorescence microscopy

COS-7 cells were transfected with EGFP-actin, mCherry-I-BAR and mCherry-IRSp53 plasmids. After the transfection samples were fixed.

The transfection efficiency was tested with Olympus IX81 inverted fluorescence and LSM710 confocal microscope.

The length of filopodia were analysed using Fiji [18]. For the calculation magnification of objective (63x) was used, then the pixel values were translated to length (0,106 µm / pixel, or 9,434 pixel / 1 unit (µm)). The measured filopodia values were given in µm unit.

Results

Investigation of I-BAR-phospholipid interaction with FRET

Previously we have expressed and purified the I-BAR and labeled it with IAEDANS fluorescent probe on its surface cysteines [19]. LUVETs were prepared from different lipid constructs and labeled with DiO fluorescence dye that penetrates into the lipid bilayer. IAEDANS-labeled I-BAR and DiO-labeled LUVETs were used in *in vitro* FRET assays to study their binding interactions. We followed the fluorescence intensity of the IAEDANS-I-BAR during its interaction with increasing concentration of DiO-labeled PC, PS/PC (70/30), PS, PIP2/PC (4/96) or PIP2/PC (15/85) containing LUVETs and the FRET efficiency was calculated. FRET efficiency increases with increasing concentration of the LUVETs indicating that more acceptors were located in the close vicinity of the donors. As LUVETs in solution are far from being close enough to produce efficient FRET, the increase of the FRET efficiency must have been due to the formation of binding complexes between LUVETs and I-BAR. The maximum FRET efficiency was estimated from quadratic fits in case of the PS, PS/PC (70/30) or PC liposomes. The IAEDANS-I-BAR and PIP2/PC (4/96) or PIP2/PC (15/85) LUVETs show the highest FRET efficiency at saturating concentration.

Fluorescence quenching on I-BAR domain protein

Steady-state fluorescence quenching experiments were carried out with IAEDANS-I-BAR in presence or absence of different LUVETs. At the starting time, I-BAR and a saturating concentration of LUVETs was added to the sample. The IAEDANS intensity was followed during the quenching and the Stern-Volmer quenching constants (K_{SV}) were determined in each case. The value of the K_{SV} was the greatest in the absence of LUVETs, while the quenching efficiency was reduced in the presence of the different LUVETs (PS or PC) The IAEDANS-I-BAR binds to the LUVETs, making the quenching less effective in both cases. The higher negatively charged PS LUVETs bind stronger to the I-BAR than the non-charged PC LUVETs providing lower value of its static quenching constant. We also tested the K4E-I-

BAR, this result is fairly similar to the results without LUVETs and indicates very weak or negligible interactions.

Effect of I-BAR on the structure of micells

We have tested whether the I-BAR disrupts the micellar structure when it binds to lipids. It was shown that TNS can be sensitive to the disorder of the aliphatic core of lipid micelles or vesicles [15]. In these experiments I-BAR was applied with micelles made form PIP_2/PC (4/96) lipids labeled with TNS fluorescent dye. $MgCl_2$ can aggregate the micelles into large multilamellar structures causing the TNS fluorescence to increase by 3-fold. We found that I-BAR bound to the micelles, but had minimal effect on the fluorescence of the TNSlabeled micelles. This shows that I-BAR binds to the head groups of the PIP_2 or PC lipids rather than penetrating into the core of the micelle affecting the environment of the TNS fluorophore. Although I-BAR does not increase TNS fluorescence, we cannot exclude the possibility of slight penetration into the hydrophobic regions of the membrane bilayers.

The role of actin in the I-BAR – phospholipid interaction

The I-BAR binds to membranes and filamentous actin simultaneously. We set up FRET experiments to study the effect of F- or G-actin on I-BAR-lipid interaction. Prior the experiment we incubated I-BAR with saturation concentration of PS liposomes. Then increasing concentration of Gactin or phalloidin stabilized F-actin was added to the sample and the change of the transfer efficiency was followed. The transfer efficiency decreased in the presence of G-actin reflecting the weakening of the I-BAR-lipid binding interaction. In the presence of F-actin the transfer efficiency increased, indicating that further I-BAR-lipid complexes were formed in the presence of actin filaments. This observation suggests that actin filaments tightened the affinity of the lipids for I-BAR. These results reveal the strong effects of actin on the I-BAR-lipid interactions that warrant further study.

Relation of IRSp53-I-BAR to the filamentous and monomer form of actin

Further investigations were needed to determine the realation of I-BAR to the monomer (G-actin) or polimer (F-actin) form of actin. To describe the I-BAR-actin interaction we set up FRET experiments, in case of F-actin at different ionic strength (25 mM and 100 mM KCl). IAEDANS-labeled I-BAR and IAF-labeled F-actin were used in *in vitro* FRET assays to study their binding interactions. We followed the fluorescence intensity of the IAEDANS-I-BAR during its interaction with increasing concentration of IAF-F-actin. With increasing F-actin concentration the FRET efficiency was increased at different ionic stregth.

The I-BAR- G-actin interaction was followed with „steady-state" fluorescence anisotropy. Fluorescence anisotropy of IAEDANS-actin was measured in the absence and the presence of I-BAR. With increasing concentration of I-BAR anisotropy of actin monomers were also increased, that suggest the binding of the proteins.

This result suggest that I-BAR can bind to the monomer and filament form of actin. They bind with similar affinity and the binding does not depend on the different KCl concentration.

The effect of IRSp53-I-BAR on the polymerisation of actin filament

In the polymerisation assay the actin/I-BAR ratio was between 1:0 and 1:10. In case of lower I-BAR concentration (1:1, 2,5:1) the actin polymerisation was hampered. However the process takes place, but needs longer time. We found an opposite mechanism in case of 1:10 actin-I-BAR ratio, where the polymerisation was faster than the control.

We conclude that the I-BAR has effect on the actin polymerisation and it depends on the concetration of it.

The effect of IRSp53-I-BAR on the nucleation of actin monomers

Because I-BAR binds to the monomer and filamentous form of actin our further question was weather I-BAR can nucleate the actin monomers. 1 μM pyrene-labeld actin was used in the experiment. In case of 1 μM or lower I-BAR concentration the pyrene signal was slightly higher. However, 2 μM I-BAR resulted a major changing in the pyrene intensity. If we increased the I-BAR concentration the signal did not increased significantly.

Based on the changing of pyren fluorescence intensity the I-BAR/actin stochiometry could be calculated. We got 2:1 I-BAR/actin stochiometry. If we consider that the I-BAR is a dimer, than one I-BAR dimer binds to one actin protofilament.

After the fluorescence experiment the samples were examined with co-sedimentation and analysed with SDS-page gelelectrophoresis. In the pellet F-actin could be observed at higher concentration than 0,5 μM I-BAR. In the supernatant G-actin was remained.

Overexpression of IRSp53 and I-BAR proteins in COS-7 cells

Overexpression of IRSp53 and I-BAR proteins in COS-7 cells resulted morfologycal changing in the number and legth of filopodia. The state of the actin polymerisation was followed with Latrunculin B treatment. The Latrunculin B is a drug which can hampare the nucleation of actin monomers and results the degradation of actin filaments.

We found that I-BAR domain or full length IRSp53 improve the number of filopodia significantly. Decreased number of filopodia can be found after Latrunculin B treatment, without filamentous actin.

Effect of IRSp53, I-BAR and actin on the length of filopodia

The length of filopodia was investigated after overexpression of I-BAR, IRSp53 and actin proteins. Overexpression of actin resulted 4,4 µm filopodia length, in case of I-BAR 3,1 µm and in case of IRSp53 5,0 µm filopodia legth could be found. The cummulative relative frequency was calculated in all cases. The overexpression of actin and IRSp53 don't have effect on the length of the filopodia. I-BAR shifted the lenght to the shorter filopodia.

Overexpression of I-BAR improved the number of filopodia significantly and decreased the length of filopodia. Our results suggest that the I-BAR has a role the initializing of protrusions. I-BAR is necessary for filopodia formation while actin has a suportive role on it.

Synopsis

In the recent work we found, that

1. IRSp53-I-BAR binds to the different phospholipids with similar affinity, the binding depends on the polarity of lipids (negativ polarity stronger binding)
2. IRSp53-I-BAR shares its function between actin- and membrane-binding
3. IRSp53-I-BAR binds to the monomer (G) and filamentous (F) form of actin with similar affinity
4. F-actin can stabilize the I-BAR-membrane interaction, G-actin can weaken it.
5. IRSp53-I-BAR has an effect on the actin polymerisation
6. I-BAR domain or full length IRSp53 improve the number of filopodia
7. decreased number of filopodia can be observed without filamentous actin, after Latrunculin B treatment.

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Publications

The publication the thesis is based on:

Kinga Futó, Emőke Bódis, Laura M. Machesky, Miklós Nyitrai, Balázs Visegrády: Membrane binding properties of IRSp53-missing in metastasis domain (I-BAR) protein. *Biochimica et Biophysica Acta Molecular Biology of Lipids, 2013. **1831**(11):1651-5.*

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Other publications:

Xinzi Yu, Tobias Zech, Laura McDonald, Esther Garcia Gonzalez, Ang Li, Iain Macpherson, Juliana Schwarz, Heather Spence, **Kinga Futo**, Paul Timpson,Colin Nixon, Yafeng Ma, Ines M. Anton, Balazs Visegrady, Robert H. Insall, Karin Oien, Karen Blyth, Jim C. Norman, and Laura M. Machesky: N-WASP coordinates the delivery and F-actin mediatedcapture of MT1-MMP at invasive pseupodods to drive matrix remodeling and cancer cell invasion. *Journal of Cell Biology, 2012. **199**(3):527-44.*

IF: 10,26

Cummulative IF: 14, 394

Lectures:

Kinga Futó, Balázs Visegrády: The role of I-BAR in the filopodia formation. *43. Membrane-Transport Conference*, Sümeg, 2013 (Talk – Hungarian)

Kinga Futó: The role of actin in filopodia formation induced by IRSp53. *MÉT Conference*, Debrecen, Hungary, 2012 (Talk – Hungarian)

Balázs Visegrády, **Kinga Futó**, Laura M. Machesky: The role of the I-BAR domain in the filopodia formation. *8th European Biophysics Congress Satellite Conference*, Pécs, Hungary, 2011 (Talk – English)

Roland Kardos, Mónika Tóth, **Kinga Futó**, Elisa Nevalainen, Pekka Lappalainen, Miklós Nyitrai and Gábor Hild: The effect of actin-binding proteins on the dynamical properties of monomeric actin. *Hungarian Biophysical Society XXIII. Congress* Pécs, Hungary, 2009 (Talk – Hungarian)