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Dissection of Rho-kinase Signaling Using Biophysical and Cell Biological Tools

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ABSTRACT

ABSTRACT

The actin cytoskeleton is crucial for many cellular processes and is tightly controlled by small GTPases of the Rho family. A prominent member is RhoA which regulates such fundamental processes as cell migration and contraction. RhoA exerts its control on the actin cytoskeleton via different effectors with Rho-associated kinase (ROCK) being particularly important because of its direct and indirect effect on actomyosin. While the importance of ROCK substrates is known and their effect on the actin cytoskeleton is well described, the question of how ROCK activity is regulated is just poorly understood. In addition to being a downstream RhoA effector, ROCK can also bind to membranes and scaffolding proteins. The current view is that the kinase domain of ROCK is maintained in an inhibited state by the membrane and RhoA binding domains. However, the precise molecular details of this autoinhibitory mechanism are unknown.

This thesis addresses three different factors (oligomeric state, membrane binding and RhoA interaction) that are crucial for ROCK signaling and attempts to dissect to what extent they contribute to ROCK regulation by applying complementary biophysical and cell biological approaches.

This thesis reports a novel characterization method coupling fluorescence size exclusion chromatography (FSEC) with fluorescence correlation spectroscopy (FCS) to investigate the size/conformation and oligomeric state of native ROCK2 in mammalian cells. The obtained data suggest that ROCK2 is a constitutive, highly extended and exclusively dimeric species in mammalian cells. In vivo, ROCK2 does not translocate in response to PI(3,4,5)P₃ or arachidonic acid although both had been previously suggested to be specific membrane ligands for ROCK2. Finally, the recombinant RhoA was purified and a method was established to determine its nucleotide state. This is an essential prerequisite for further in vitro experiments to characterize the RhoA ROCK interaction.

On the basis of these findings and other experimental data from our group a structural model for ROCK in mammalian cells can be derived. Our model predicts that intrinsic ROCK kinase activity is not regulated by an autoinhibitory mechanism. As such our data demand a revision of the working mechanism by which ROCK controls essential cellular processes. We propose that ROCK is simply regulated by the discrete subcellular localization of itself and its substrates.

1 INTRODUCTION

1.1 Small GTPases control the actin cytoskeleton

The actin cytoskeleton is essential for many cellular processes such as cell migration, vesicle trafficking, endocytosis and cytokinesis. These different functions are carried out by different actin cytoskeletal structures which differ in actin filament organization and cellular localization (reviewed in Blanchoin et al., 2014). For instance, cell contraction depends on the cell cortex which is a thin shell found in close proximity to the plasma membrane that is comprised of a contractile actin-myosin network.

To a large extent, the actin cytoskeleton is regulated by small GTPases. Especially important are those that belong to the Rho family of small GTPases (reviewed by de Curtis and Meldolesi, 2012). In mammals there are 20 members of this family and they control the actin cytoskeleton in a tightly coordinated fashion. Rho GTPases like other small GTPases are proteins that have a low intrinsic GTPase activity and can exist in a GDP or GTP bound state. The pool of proteins that exist in the GDP or GTP bound state is controlled by several factors. Most importantly GTPases are regulated by enzymes that i) increase their intrinsic GTPase activity (GTPase activating protein, GAP) ii) catalyze their nucleotide exchange (guanine nucleotide exchange factor, GEF) or iii) inhibit their nucleotide exchange (guanine nucleotide dissociation inhibitor, GDI). When small GTPases are in their “active” GTP bound state they are primed to interact with downstream effector proteins. These effectors can be numerous and it is through these effectors that small GTPases control the actin cytoskeleton. It has been reported that different Rho GTPases recruit different effectors that can have similar or opposing functions. Therefore cytoskeletal rearrangements are associated with spatio-temporally controlled programs that involve different members of the Rho GTPase family to different extents (reviewed in Pertz, 2010).

One prominent member of the family of Rho GTPases is RhoA which is involved in the regulation of cell contraction and migration (reviewed in Pertz, 2010). An important effector of RhoA is Rho-associated kinase (ROCK). This serine/threonine kinase was first identified in 1995 and was shown to be associated with the plasma membrane in a RhoA dependent manner (Leung et al., 1995). Various substrates for ROCK have since been identified, many of which have been implicated in actin cytoskeleton regulation.

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One of the substrates of ROCK is the regulatory light chain of myosin II (MLC). Non-muscle myosin II is a molecular motor and crosslinks actin filaments. Movement of the myosin heads along the actin filament leads to filament contraction which is essential for cellular reshaping and movement (Vicente-Manzanares et al., 2009). ROCK can phosphorylate MLC on S19 in a Ca^{2+} independent manner (Amano et al., 1996). Upon phosphorylation of the regulatory myosin light chain, myosin ATPase activity is increased leading to actomyosin contraction (Vicente-Manzanares et al., 2009). MLC phosphorylation is reversible and its dephosphorylation is mediated by myosin phosphatase. It has been reported that ROCK can also phosphorylate myosin phosphatase on its myosin phosphatase targeting subunit 1 (MYPT1) and thereby inhibit the phosphatase (Kimura et al., 1996). Thus, the kinase activity of ROCK can directly and indirectly result in higher MLC phosphorylation levels.

In addition to direct MLC phosphorylation, ROCK has been shown to phosphorylate other proteins that influence the actin cytoskeleton (reviewed in Amano et al., 2010). In mammals, one of these substrates is LIM kinase (LIMK) which has an increased kinase activity when phosphorylated at T508 in its activation loop by ROCK (Ohashi, 2000). LIMK in turn phosphorylates and inactivates the actin depolymerizing factor cofilin, which leads to the stabilization of actin filaments.

As many ROCK substrates appear to influence actomyosin in a direct as well as in an indirect manner, ROCK is considered a major regulator of the actin cytoskeleton linking RhoA activity to cytoskeletal rearrangements.

1.2 Rho associated kinase - ROCK

In mammals there are two isoforms of Rho-associated kinase, termed ROCK1 (p160 ROCK-1, ROK β) and ROCK2 (p164 ROCK-2, ROK α). Both isoforms share an overall amino acid sequence identity of 64% and 83% sequence identity for the kinase domain (Amano et al., 2010). ROCK1 levels were found to be higher in the liver, lung and testis and ROCK 2 levels were higher in the brain and skeletal muscles (Leung et al., 1996). Since the two isoforms are highly similar, I will refer to both isoforms as ROCK throughout this thesis unless a specific isoform is indicated.

ROCK is a 160 kDa protein that belongs to the family of AGC protein kinases and is structurally related to MRCK (myotonic dystrophy kinase-related CDC42-binding kinase), CRIK (citron Rho-interacting kinase) and DMPK (myotonin protein kinase) which are all implicated in cytoskeletal

regulation (reviewed in Pearce et al., 2010). All of these kinases are multidomain kinases and a schematic overview of ROCK is illustrated in Figure 1.



Figure 1. Domain organization of ROCK1.

Capped helix bundle (CHB), Shroom binding domain (SBD), Rho binding domain (RBD, split pleckstrin homology domain (PH) into which is spliced a cysteine-rich C1 domain. The caspase-3 cleavage site is indicated by a dashed line.

The kinase domain of ROCK is located at the N-terminus of the protein and structures of the ROCK1 kinase domain as well as of the ROCK2 kinase domain have been solved by X-ray crystallography (Jacobs et al., 2006) (Yamaguchi et al., 2006). These structures showed that the kinase domain forms a parallel dimer due to the N-terminal capped helix bundle (CHB) which facilitates dimerization by forming an extensive interface with the N-lobe and C-terminal extension of the kinase domain. The importance of the CHB and the C-terminal extension for the kinase domain dimerization has been confirmed by hydrodynamic studies, which showed that without either the CHB or C-terminal extension the kinase domain is monomeric (Doran et al., 2004).

The kinase domain is followed by a 700 amino acid sequence that is structurally only partly characterized. Structure prediction programs such as COILS (Lupas et al., 1991) predict this region to be exclusively coiled coil. In fact, all structures of parts of this region that have been reported so far show parallel coiled coils (Shimizu et al., 2003) (Dvorsky et al., 2004) (Tu et al., 2011) (Mohan et al., 2013). Protein interaction sites have been reported for the coiled coil region including RhoA (Blumenstein and Ahmadian, 2004) as well as Shroom 3, a member of the actin binding shroom family of proteins (Mohan et al., 2013).

Although multiple RhoA interacting domains have been proposed for the coiled coil region (Blumenstein and Ahmadian, 2004) only one structure of a RhoA binding domain (RBD) has been solved to date (Dvorsky et al., 2004). This structure revealed that two RhoA molecules bind to a parallel coiled coil fragment of ROCK1 spanning residues 947-1015. Interestingly, each RhoA molecule makes contacts with both chains of the coiled coil and the RhoA residues

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involved in the binding interface belong to the switch region, which is known to adopt different conformations depending on whether RhoA is GDP or GTP bound (Hakoshima, 2003).

At the very end of the predicted coiled coil region there is a caspase-3 cleavage site in ROCK1 (Sebbagh et al., 2001) and a granzyme B cleavage site in ROCK2 (Sebbagh et al., 2005).

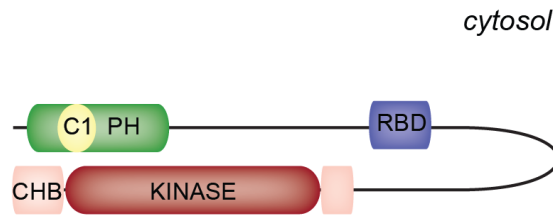
The C-terminal membrane binding domain consists of a PH domain in which a cysteine rich C1 domain is spliced into a flexible loop between the $\beta 6$ and $\beta 7$ strand ($\text{PH}_N\text{-C1-PH}_C$). Although the structures could only be solved for the isolated C1 and PH domains by NMR the chemical shifts of the entire $\text{PH}_N\text{-C1-PH}_C$ domain largely overlap with the chemical shifts of the isolated domains and therefore it can be assumed that the entire module is structurally composed of a PH domain and C1 domain (Wen et al., 2008). In addition, superposition of the PH domain with existing PH domain structures showed them to be structurally homologous. The lipid specificity for the $\text{PH}_N\text{-C1-PH}_C$ domain, however, remains somewhat mysterious. Typical C1 domains can bind the second messenger diacylglycerol (DAG) (reviewed in Colón-González and Kazanietz, 2006). However, this has not been reported for ROCK and the structure of the ROCK C1 binding pocket suggest that it is unlikely that DAG is a ligand for this domain (Wen et al., 2008). Canonical PH domains, such as that of the kinase Akt, bind specifically to 3'phosphoinositides and the ROCK PH-C1-PH domain has been shown in vitro to preferentially bind to liposomes that contain phosphatidylinositol (3,4,5)-trisphosphate ($\text{PI}(3,4,5)\text{P}_3$) (Wen et al., 2008).

Although the structures of the isolated domains have been solved for ROCK, the overall structure of the full-length protein is still unknown. Furthermore, the oligomeric state of full length ROCK is unclear. Whereas the recombinant ROCK1 and ROCK2 protein have been reported to be dimeric (Doran et al., 2004), higher order oligomers have been reported for ROCK2 isolated from rat brain (Chen et al., 2002).

1.3 Regulation of ROCK

The current view of how ROCK activity is regulated in cells is based on an autoinhibition model. This model claims that the C-terminal lipid binding domain maintains the N-terminal kinase domain and in an inactive conformation (Amano et al., 1999) (Figure 2). This autoinhibition of the kinase domain by a lipid binding domain is similar to the regulation of other AGC kinases such as protein kinase C (PKC) (Leonard et al., 2011).

AUTOINHIBITED ROCK



ACTIVE ROCK

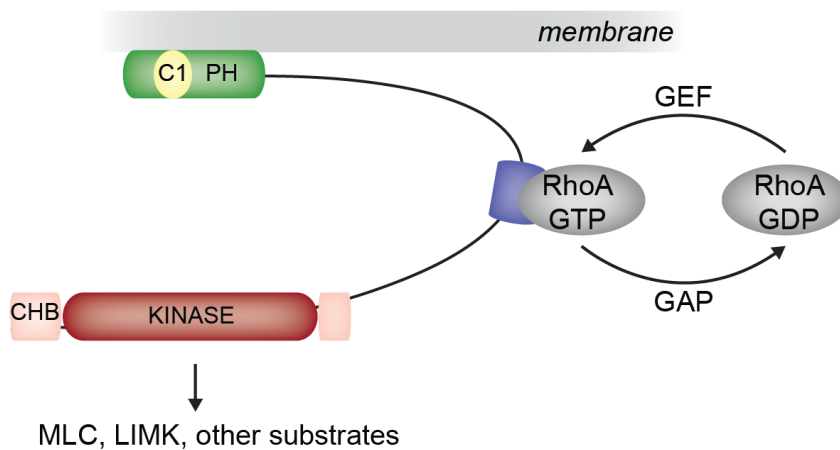


Figure 2. The autoinhibition model for ROCK

The model assumes that ROCK exists as an autoinhibited monomer with the membrane binding domain folding back onto the kinase domain. Several factors such as RhoA binding or membrane binding can release ROCK from its autoinhibited state and thereby lead to the phosphorylation of its downstream substrates.

There are several lines of evidence that support this model, most of which are based on the observation that the isolated N-terminal kinase domain has a higher kinase activity than the full length protein (Leung et al., 1996) (Amano, 1997). The evidence for direct interaction of the ROCK C-terminus with its N-terminal kinase domain stems from a study in which a C-terminal fragment of ROCK including the Rho binding domain (RBD) was shown to bind and to inhibit the activity of the isolated kinase domain in a concentration-dependent manner. Yet, this effect could only be observed when the C-terminal fragment contained the RBD and could not be observed for the lipid binding domain alone. Furthermore these observations were made using a C-terminal construct which contained two point mutations that abolish RhoA binding (Amano et al., 1999).

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In line with this autoinhibition model were subsequent reports that RhoA binding could increase ROCK kinase activity both in vitro and in vivo. This conclusion has been drawn from an increase in RhoA-independent actin stress fiber formation when RBD specific antibodies were injected into HeLa cells (Chen et al., 2002). However, human ROCK purified from blood platelets showed only a ~2 fold increase in kinase activity towards a histone H2A substrate when GTP bound RhoA was added to the kinase (Ishizaki et al., 1996). Similarly, for the recombinant protein a minor increase in full length ROCK1 or ROCK2 kinase activity was determined (less than 50% decrease in K_M) when GTP bound RhoA was added to the kinase reaction using MLC as a substrate (Doran et al., 2004).

Further support for the autoinhibition model comes from studies that showed that the cleavage of the membrane binding domain of ROCK1 by caspase-3 during apoptosis, is correlated with increased phosphorylation levels of MLC and membrane blebbing (Sebbagh et al., 2001). Similar effects were observed during granule-induced cell death, for the equivalent granzyme B cleavage of ROCK2 (Sebbagh et al., 2005) suggesting that cleavage of the membrane binding domain releases the autoinhibition of ROCK and results in a constitutively active kinase.

Finally, arachidonic acid, a polyunsaturated 20:4(ω -6) fatty acid, has been shown to increase ROCK kinase activity in vitro as well as leading to increased smooth muscle contraction in vivo. This finding suggested that arachidonic acid might bind to the C-terminal region of ROCK and thereby releases the autoinhibition of the kinase (Araki et al., 2000).

1.4 Aim of this thesis

Although the autoinhibition model is well supported by experimental data, the molecular details of how the regulatory C-terminus interacts with the N-terminal kinase domain to keep ROCK in an autoinhibited conformation are still unknown. Likewise, it is just partly understood how RhoA binding or lipid binding affects the overall conformation of ROCK and to what extent these events contribute to activate ROCK. Furthermore, it is not known whether the transition between the autoinhibited and active conformation of ROCK is only associated with big conformational changes or whether it also involves transitions in oligomeric state. This master's thesis therefore addresses the following specific aims:

- 1) *What is the oligomeric state and shape/conformation of ROCK in mammalian cells?*
- 2) *Is ROCK recruited to the plasma membrane in vivo by specific lipid second messengers?*
- 3) *What is the stoichiometry and affinity of the RhoA ROCK interaction and to what extent does RhoA binding directly increase ROCK kinase activity?*

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Materials

Chemicals were purchased from Sigma, enzymes were purchased from NEB and used as recommended, if not stated otherwise. TEV protease was made in house. Antibodies were obtained from Cell Signaling Technology. N1 and C1 vectors for expressing fluorescent fusion proteins were obtained from Clontech, pGSTparallel (pGSTII) was a kind gift of the David Waugh lab. Cos7 cells were obtained from the Baccarini lab. HeLa cells were obtained from Haplogen and the MDCK cell line was provided by the Foisner lab. *E.coli* strains used were DH5 α for cloning and BL21 (DE3) for protein expression.

2.2 Molecular biology

2.2.1 DNA amplification and construct design

Genes were amplified by PCR using KOD DNA polymerase. In order to later clone the amplified DNA into a respective vector plasmid (C1, N1, pGSTII), gene specific primers were designed, each containing an additional 5' restriction site. For the C1 vector restriction sites were XhoI Xba, for the N1 vector restriction sites were SacI and XmaI, and for cloning into the pGSTparallel vector restriction sites were BamHI and NotI.

The template DNA to amplify the genes stems from different sources: A synthetic gene coding for human Rock2¹⁰⁻¹³⁸⁸ gene was purchased from GenScript and was codon optimized for expression in insect cells. The template for the membrane binding domains, Rock1¹⁰²¹⁻¹³⁵⁴ Rock1¹¹¹⁹⁻¹³⁵⁴, stems from a human image clone (BC113114) purchased from OpenBiosystems. The tandem C1 (C1₂) construct was obtained by two step PCR splicing together the two C1 domains, namely Rock1¹²⁰⁵⁻¹²⁸⁸ (human) with Rock2¹²³⁷⁻¹³²⁰ (human, synthetic gene). Additionally, an 8 amino acid long peptide (VTSAANEG) links the two domains.

For the tandem eGFP-eGFP (eGFP₂) the eGFP gene was amplified from the C1 vector and cloned into the eGFP-C1 vector between the XhoI and XbaI sites. Thus the fusion protein contains the same flexible linker between the two eGFP proteins that is present in all other eGFP fusion proteins.

RhoA¹⁻¹⁸¹ was amplified from a human cDNA library generated in house. Cloning into the GST parallel vector gives a GST-RhoA¹⁻¹⁸¹ fusion construct with a TEV protease cleavage site between the N-terminal GST and the C-terminal RhoA. Cleavage of the fusion protein by TEV

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protease results in a RhoA¹⁻¹⁸¹ protein that contains 5 additional amino acids (GAMGS) at its N-terminus prior to Met1 of RhoA

The point mutation G14V in RhoA¹⁻¹⁸¹ was introduced by strand overlap extension PCR. A BFP-AKT-PH domain was constructed by T. Leonard and contains the human Akt1 residues 1-117 in a BFP-C1 vector. pBFP-C1 was generated from pEGFP-C1 by excising the eGFP gene using the flanking NheI and XhoI restriction sites and replacing it with mTag-BFP.

2.2.2 Restriction digest and ligation

Amplified DNA and vector plasmids were cleaved with the very same restriction enzymes. To increase ligation efficiency, digested vectors were loaded onto a 1% agarose gel and cut out of it. The gel-purified digested vectors were subsequently treated with antarctic phosphatase to prevent religation and increase ligation efficiency. Ligation was performed by T4 ligase.

2.2.3 Transformation, selection, sequencing

Electrocompetent *E.coli* (DH5 α) were transformed with the ligation mixes and selected on agar plates containing the respective antibiotics (ampicillin for pGSTII, kanamycin for the N1 and C1 vectors). Colonies were checked for correct ligation by colony PCR using a gene specific and vector specific primer and evaluation of the length of the PCR product on a 1% agarose gel. Colonies leading to a PCR product of right size were grown in a 4 ml culture overnight and plasmids were extracted using a mini prep kit (Sigma). All constructs were finally checked for correct insertion, reading frame and sequence of the insert by sequencing the plasmid using forward and reverses.

Chemically competent BL21 (DE3) cells were transformed with the expression vector by a 45 seconds heat shock transformation at 42°C followed by 2 minutes on ice and a recovery of 1 h at 37°C in antibiotics free LB medium.

2.3 Live cell imaging

2.3.1. Cell culture and transfection

HeLa, Cos7 and MDCK cell lines were cultured in DMEM high glucose (PAA) supplemented with 2 mM L-Glutamine and Streptomycin-Penicillin (PAA) and 10% FBS (PAA).

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Transient transfection with fluorescent fusion constructs was accomplished by the use of 1µl Lipofectamine 2000 (Life Technologies) and 0.5µg of construct DNA per chamber of a 4 chamber microscopy dish (InVitro Scientific). 8 to 20 h after transfection, cells were starved for 8 to 20 h by exchanging the medium for DMEM containing 0.1% FBS. Prior to imaging, the medium was exchanged for HBSS (Life Technologies) supplemented with 1mM pyruvate (Life Technologies).

2.3.2. Microscopy

Confocal microscopy was carried out on a Zeiss LSM 710 with a 63x oil objective. During stimulation and imaging, cells were kept at 37°C using a heated stage. Starved cells were either stimulated with 100 ng/ml (11nM) IGF-1 (LONGR³ IGF-1, Sigma) or treated with 3 to 300 µM arachidonic acid dissolved in DMSO, resulting in a final DMSO concentration of 0.5 to 1 %. Analysis of fluorescence localization and translocation was carried out using the program ImageJ.

2.4 Fluorescence size exclusion chromatography (FSEC)

2.4.1 Calibration

A Superdex 200 10/300 column was calibrated with protein standards ranging from 25-669 kDa. A partition coefficient k was calculated for every protein following the formula

$$k = \frac{V_e - V_0}{V_c - V_0}$$

where V_c is the geometric column volume, V_e is the elution volume of the protein and V_0 is the column void volume. The partition coefficient was plotted against the decimal logarithm of the molecular weight $\log_{10}(\text{Mr})$ and a linear regression was performed on the data.

2.4.2 Sample preparation, application and fluorescence readout

Cos7 cells were transiently transfected with eGFP-Rock2 fusion constructs when they reached between 60-80% confluency. Transfection was carried out using 4 µl of Lipofectamine 2000 and 2µg of DNA per well of a 6 well plate of Cos7 cells. 40 to 72 h after transfection cells were harvested and lysed. Briefly, cells were incubated with trypsin until they detached from the cell culture dish. Trypsin was inhibited by diluting the cells in 4x medium containing FBS. Cells were centrifuged at 500 g for 10 min and resuspended in isotonic buffer (20 mM Tris pH 7.4, 140 mM

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KCl, 10 mM NaCl, 10 mM β -mercaptoethanol) containing 8 μ l/ml protease inhibitor cocktail (P8849 Sigma). Cell lysis was achieved with 3 cycles of freeze thaw in liquid nitrogen. Insoluble material was removed by ultracentrifugation at 124 000 g for 15 min. The soluble fraction was loaded onto a size exclusion column (Superdex 200 10/300) equilibrated in an iso-osmotic physiological buffer (20 mM Tris pH 7.4, 140 mM KCl, 10 mM NaC, 10 mM β -mercaptoethanol). 200 μ l fractions were collected and fluorescence was read out on a TECAN Infinite F500 fluorescence plate reader. The total protein concentration was roughly estimated by absorbance at 280 nm and the sample was diluted to a 10 to 100 nM total protein concentration for FCS analysis.

2.5 Fluorescence correlation spectroscopy (FCS)

FCS was performed on a Confocor spectrofluorimeter (Carl Zeiss-Evotec, Jena, Germany) equipped with a 450 nm Argon-laser (LASOS Lastertechnik GmbH, Jena, Germany) and a long pass dichroic beam splitter (510 nm). Photons were recorded on an avalanche photodiode detector (SPCM-CD 3017) and fluorescence intensities were autocorrelated with a hardware correlator (ALV 5000, ALV, Langen, Germany). Data were analyzed with the FCS ACCESS software (Carl Zeiss-Evotec). For every sample 10 consequent measurements were recorded, each lasting a minimum of 10 seconds.

2.5.1 Confocal volume

Before each use of the instrument the confocal volume was calibrated by measuring rhodamine 6G in water. The confocal volume was calculated from the known diffusion coefficient of rhodamine 6G of $2.8 \times 10^{-10} \text{ m}^2/\text{s}$ (Magde et al 1974).

2.5.2 Diffusion coefficient

Experimental diffusion coefficients were calculated from the diffusion time and the calibrated confocal volume.

$$D_{exp} = \frac{\omega_{xy}^2}{4\tau_D}$$

where τ_D is the diffusion time derived from the autocorrelation curve, and ω_{xy} is the radius of the confocal volume waist.

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Theoretical diffusion coefficients were calculated by the Stokes-Einstein-equation

$$D_{theo} = \frac{k_B T}{6\pi\eta r}$$

where k_B is the Boltzmann's constant, T is the absolute temperature, η is the viscosity of water (0.001 Pa s) and r is the hydrodynamic radius of the protein. The hydrodynamic radius was estimated by the globular protein approximation

$$r_{theo} = \sqrt[3]{\frac{3M}{4N\pi\rho}}$$

where M is the molecular weight of the protein, N is the Avogadro constant and ρ is the volumetric mass density ($1200 \frac{kg}{m^3}$).

The diffusion coefficient of a stiff rod was calculated by

$$D_{theo} = \frac{AkT}{3\pi\eta l}$$

where A is defined by

$$A = \ln\left(\frac{l}{d}\right) + 0.312 + \frac{0.565}{\frac{l}{d}} - \frac{0.1}{(\frac{l}{d})^2}$$

with l being the length and d the diameter of the rod.

2.6 Protein purification

2.6.1 Protein expression and harvest

An overnight culture of BL21 DE3 grown in LB and ampicillin was diluted with LB medium containing ampicillin to an OD₆₀₀ of 0.05. Cells were grown at 37 °C shaking at 170 rpm. When cells reached an OD₆₀₀ of 0.4 the temperature was reduced to 26°C (RhoA¹⁻¹⁸¹) or 16°C (RhoA¹⁻¹⁸¹G14V). When cultures reached an OD₆₀₀ of 0.6 to 0.8 protein expression was induced with 0.1mM IPTG. After 5 h (RhoA¹⁻¹⁸¹) to 20 h (RhoA¹⁻¹⁸¹G14V) cells were harvested by

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centrifugation. Cell pellets were resuspended in lysis buffer (50 mM Tris pH 7.4, 150 mM KCl, 2 mM MgCl_2 , 1 mM TCEP), frozen in liquid nitrogen and stored at -80°C .

2.6.2 Cell lysis

Resuspended cell pellets (a 1L culture cell pellet was resuspended in 50 ml lysis buffer) were thawed on ice. 400 μl protease inhibitor cocktail (P8849 Sigma), lysozyme (0.2 mg/ml final concentration) and 2 μl benzonase were added and incubated for 30 min on ice. Cell lysis was completed by sonication using a Sonifier W-450D running 2 cycles, each 2 minutes at 50% power. Insoluble material was pelleted at spun down at 38 400 g.

2.6.3 Affinity batch purification

Glutathione sepharose 4B beads were equilibrated in lysis buffer (50 mM Tris pH 7.4, 150 mM KCl, 2 mM MgCl_2 , 1 mM TCEP). The soluble fraction of the lysed cells was incubated with the beads (1ml of beads per 1L culture) for 2 h at 4°C . Beads were subsequently washed three times with at least 10 times the bead volume and finally resuspended in lysis buffer (10 times volume of the beads). GST-RhoA was cleaved on the beads using TEV protease, overnight at 4°C . After separation from the beads, the supernatant was concentrated by centrifugation in an Amicon Ultra 4 (10 kDa MWCO).

2.6.4 Size exclusion chromatography

Concentrated protein samples were loaded with a 500 μl loop onto a Superdex 75 10/300 size exclusion column equilibrated in 50 mM Tris pH 7.4, 150 mM KCl, 2 mM MgCl_2 , 1 mM TCEP. 500 μl fractions were collected and analyzed by SDS-PAGE.

2.7 RP-HPLC

Ion pairing reversed phase chromatography was performed with a reverse phase Phenomenex C18 column (150 x 4.60 mm, 5 μm , 300A) connected to an EC 4/3 UNIVERSAL RP guard column (Macherey-Nagel) and operated on an ÄKTA Ettan as described before (Eberth and Ahmadian, 2009). Runs were performed in isocratic mode (20 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ pH 6.5, 10 mM Tetra-n-butylammonium bromide, 18% CH_3CN) at a flowrate of 1 ml/min. A 55 μl loop was used to inject nucleotide standards (GDP, GTP) or protein and retention was monitored by absorbance at 260 and 280 nm.

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2.8 SDS PAGE

Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed as described previously (Laemmli, 1970).

Protein samples were mixed with 2x SDS loading dye and boiled at 95 °C for 5 minutes prior to loading. Samples were run at a constant voltage of 180-200V for 1h or until the front of loading dye ran out of the gel.

2.8.1 Coomassie staining

Polyacrylamide gels were stained with SimplyBlue SafeStain (Life Technologies). Gels were destained with H₂O.

2.8.2 Immunoblot

For Western Blot analysis proteins were transferred to a nitrocellulose membrane (Whatman, Protran BA 83 0.2µm, GE Healthcare). Wet transfer was performed at room temperature using a constant voltage of 60V for 90 min and a transfer buffer (25mM Tris, 192mM Glycine, 20% methanol). After transfer membranes were blocked with 5% milk powder in TBS-T (50mM Tris pH 7.4, 150mM NaCl, 0.1% tween 20) for 1h at room temperature and subsequently incubated with primary antibody (diluted in 5% milk TBS-T) for 1 hour at room temperature or overnight at 4°C. Membranes were washed 3 times with TBS-T and then incubated with a secondary antibody-horseradish peroxidase (HRP) conjugate (diluted in 5% milk TBS-T) for 1h at room temperature. Blots were developed with the HRP substrate (ECL Select, Western Blotting Detection Reagent, Amersham GE Healthcare). Chemiluminescence was detected on a Fusion FX7 imaging system (PiqLab). Exposure times were automatically adjusted for optimal signal intensity.

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3.1 Cellular localization, shape and oligomeric state of eGFP-ROCK2

3.1.1 eGFP-ROCK2

To gain insight into the cellular localization, oligomeric state and the overall shape of ROCK, a fluorescent reporter protein was genetically linked to a synthetic human ROCK2 gene. Enhanced green fluorescent protein (eGFP) was fused to ROCK2 either N-terminally (eGFP-ROCK2) or C-terminally (ROCK2-eGFP) and the fusion protein was transiently expressed in mammalian cells and examined by confocal microscopy (Figure 3). eGFP-ROCK2 appears to be evenly distributed throughout the cytoplasm and does not appear to be associated with specific membranes or cell compartments. However, in all three cell lines, it appears to be absent from the nucleus. Transient eGFP-ROCK2 expression altered the cell morphology compared to untransfected cells, especially in HeLa and MDCK cells. The transfection efficiency and expression levels – as judged by fluorescence intensity – were generally very low compared to eGFP expression alone. eGFP-ROCK2 was subsequently expressed Cos7 cells, which gave the best fluorescence signal and displayed the least affected morphology.

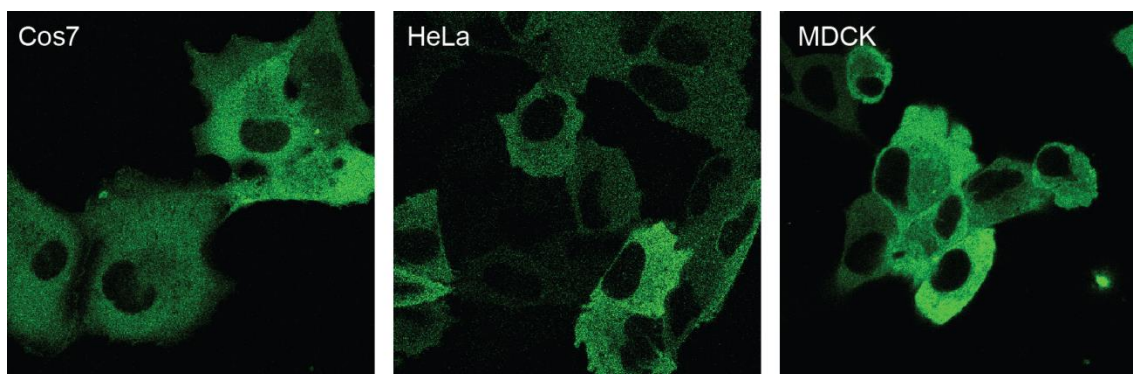


Figure 3. eGFP-ROCK2 localization inside mammalian cells

Confocal microscopy of mammalian cell lines (Cos7, MDCK, HeLa) transiently expressing eGFP-ROCK2

To obtain information about the overall conformation and oligomeric state of this eGFP-ROCK2 fusion protein a new method was implemented that is based on the application of fluorescence size exclusion chromatography (FSEC) coupled to fluorescence correlation spectroscopy (FCS). The workflow established for this is outlined in Figure 4A. The two methods provide complementary information on the size and shape of a fluorescent fusion protein. Size exclusion chromatography provides elution volumes – a measure that correlates with the size and shape of a molecule - and FCS gives the diffusion coefficients, which is also correlated with size and

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shape. Additionally, FCS can be used to determine the oligomeric state of a fluorescent fusion protein by looking at the molecular brightness of individually diffusing species.(Chen et al., 2003).

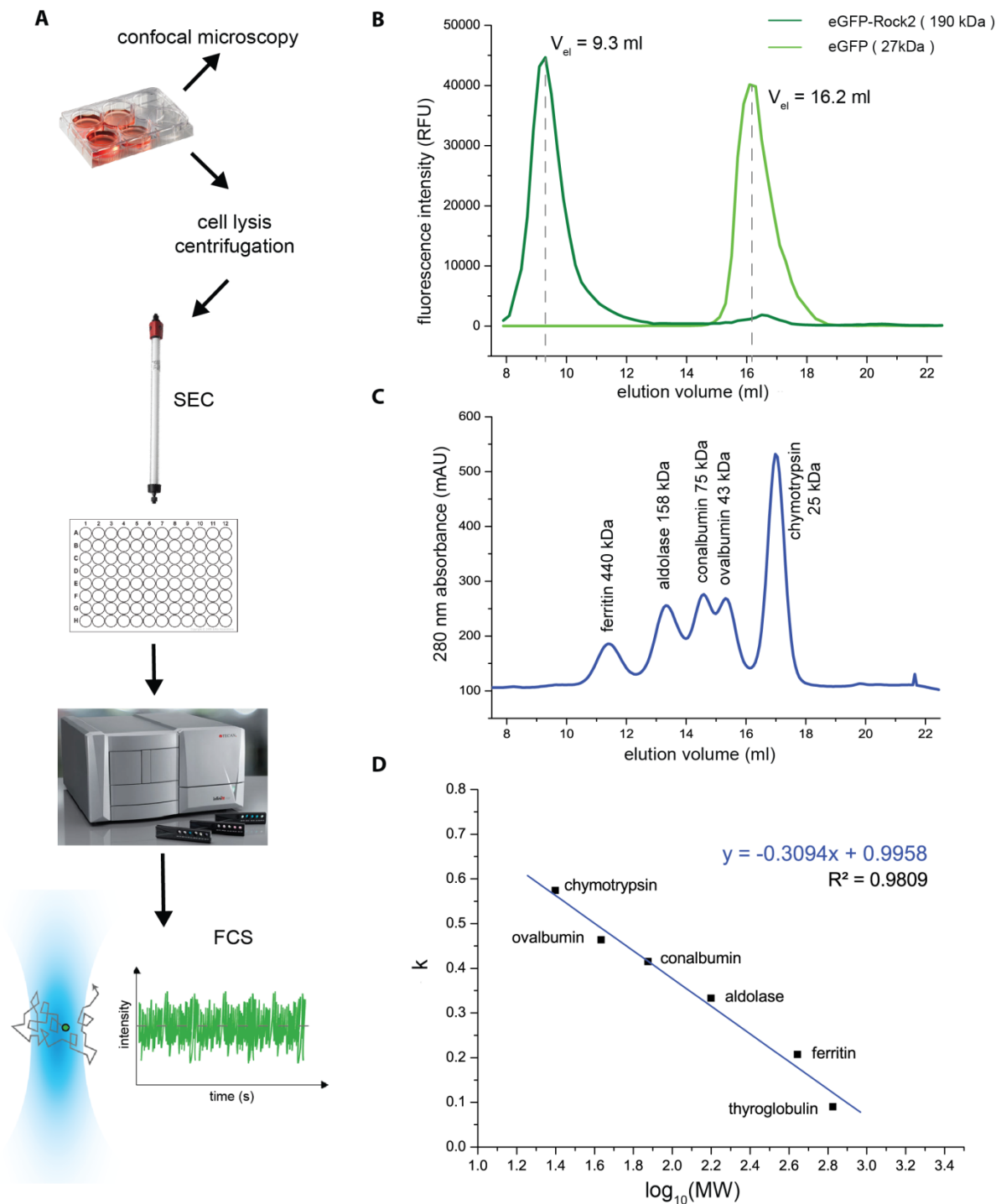


Figure 4. Workflow and FSEC

(A) Workflow established to characterize eGFP-ROCK2. Transfected cells are either imaged or lysed. Cell lysates are centrifuged and the soluble fraction is loaded onto a Superdex 200 30/100 size exclusion column. Fractions

are collected in a 96 well plate and fluorescence is read out by a TECAN Infinite 500 fluorescent plate reader. Peak fractions can be further analyzed by FCS

- (B) FSEC elution profiles for lysates of cells that were either transiently expressing eGFP or eGFP-ROCK2.
- (C) SEC elution profile of a protein standard mix on the Superdex-200 10/300
- (D) Linear regression of the logarithm of the molecular weight ($\log_{10}(\text{MW})$) and the partition coefficient (k) determined from the elution volumes

Unlike recombinant protein purification, which is based on high yield heterologous expression systems and requires multiple purification steps, this experimental setup allows one to characterize native proteins that have been expressed in mammalian cells lines at low expression levels. The only interference that these fluorescent fusion proteins encounter is a dilution in a physiological buffer.

3.1.2 Fluorescence size exclusion chromatography (FSEC)

To investigate the size and overall shape of eGFP-ROCK2 in Cos7 cells, the cell lysates were applied to a size exclusion column. The elution profiles for cell lysates transfected with either eGFP-ROCK2 or eGFP show two symmetric peaks at very different elution volumes (Figure 4B). This suggests that, in mammalian cell lysates, both proteins adopt a single overall state. To evaluate the size of each fluorescent species the size exclusion column was calibrated with globular protein standards of known molecular weight. Thus a protein standard mix (Figure 4C) and a thyroglobulin solution were applied to the column and a linear regression (materials and methods 2.4.1) of the partition coefficient k against $\log_{10}(\text{Mr})$ was calculated (Figure 4D).

	MW (kDa)	$\log_{10}(\text{MW})$	V_{el} (ml)	k
thyroglobulin	669	2.83	9.6	0.09
ferritin	440	2.64	11.4	0.21
aldolase	158	2.2	13.3	0.33
conalbumin	75	1.88	14.6	0.42
ovalbumin	43	1.63	15.3	0.46
chymotrypsin	25	1.40	17.0	0.57
eGFP	34	1.54	16.2	0.52
eGFP-ROCK2	995	3.00	9.3	0.07

Table 1. Calibration of Superdex 200 10/300

The table summarizes the molecular weight of known protein standards (**black**), the experimentally determined elution volumes and partition coefficient of known protein standards, eGFP and eGFP-ROCK2 (**green**) and the molecular weight of eGFP and eGFP-ROCK2 as deduced from the linear regression (**purple**).

For eGFP the calibration gives a molecular weight of 34.2 kDa (Table 1), which agrees well with the expected molecular weight of an eGFP monomer (27 kDa). The elution volume of eGFP-ROCK2 is outside the calibration range. Extrapolating the regression line predicts a globular protein of approximately 1 MDa (Table 1, Figure 4B and 4D) which is more than 5 times the molecular weight of an eGFP-ROCK2 monomer (190 kDa). This finding rules out the possibility

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that eGFP-ROCK2 is a globular monomer or dimer in Cos7 cells but rather suggests it to be an oligomeric and/or extended protein.

3.1.3 Fluorescence correlation spectroscopy (FCS)

The peak fractions of the FSEC were analyzed by FCS and two representative autocorrelation curves are shown in Figure 5A and 5B. All curves were fit with a one component fit that well described the experimental data (Figure 5A and 5B, residuals). This further strengthens the observation that eGFP-ROCK2 in Cos7 cells exists as a single species with a well-defined conformation and oligomeric state. The experiment was carried out for both the N-terminal (eGFP-ROCK2) as well as C-terminal (ROCK2-eGFP) fusion proteins and eGFP alone was always measured in parallel. Between the experiments the diffusion coefficients of eGFP varied from 8.5 ± 0.2 to $9.0 \pm 0.5 \cdot 10^{-11} \text{ m}^2/\text{s}$. (Table 2, Figure 5C), which is in very close agreement with the reported diffusion coefficient of $8.7 \cdot 10^{-11} \text{ m}^2/\text{s}$ (B.R. Terry, E.K. Matthwes, 1995) and $9.32 \pm 0.22 \cdot 10^{-11} \text{ m}^2/\text{s}$ (Hink et al., 2000). The diffusion coefficients of eGFP-ROCK2 and ROCK2-eGFP were found to be $2.7 \pm 0.1 \cdot 10^{-11} \text{ m}^2/\text{s}$ and $2.5 \pm 0.1 \cdot 10^{-11} \text{ m}^2/\text{s}$, respectively. This demonstrates that the position of the eGFP tag (N-terminal or C-terminal) does not considerably influence the overall size and shape of ROCK2.

For perfectly globular proteins a theoretical diffusion coefficient can be estimated from the molecular weight (see 2.5.2). It should be pointed out that for globular proteins the diffusion coefficient is not linearly dependent on the molecular weight of the protein. Table 2 and Figure 5C give an overview of the experimental and theoretical diffusion coefficients for eGFP and eGFP-ROCK2. From these calculations it is clear that eGFP-ROCK2 and ROCK2-eGFP have lower diffusion coefficients than would be predicted for a globular pentameric protein.

Table 2. Diffusion coefficients

Experimental and theoretical diffusion coefficients are given in $10^{-11} \text{ m}^2/\text{s}$.

experimental		theoretical	
D_{eGFP}	8.5 ± 0.2 to	D_{eGFP}	10.3
	9.0 ± 0.5	$D_{\text{eGFP-ROCK2}}$	5.4
$D_{\text{eGFP-ROCK2}}$	2.7 ± 0.1	$D_{\text{eGFP-ROCK2 (2x)}}$	4.3
$D_{\text{ROCK2-eGFP}}$	2.5 ± 0.1	$D_{\text{eGFP-ROCK2 (3x)}}$	3.7
		$D_{\text{eGFP-ROCK2 (4x)}}$	3.4
		$D_{\text{eGFP-ROCK2 (5x)}}$	3.2

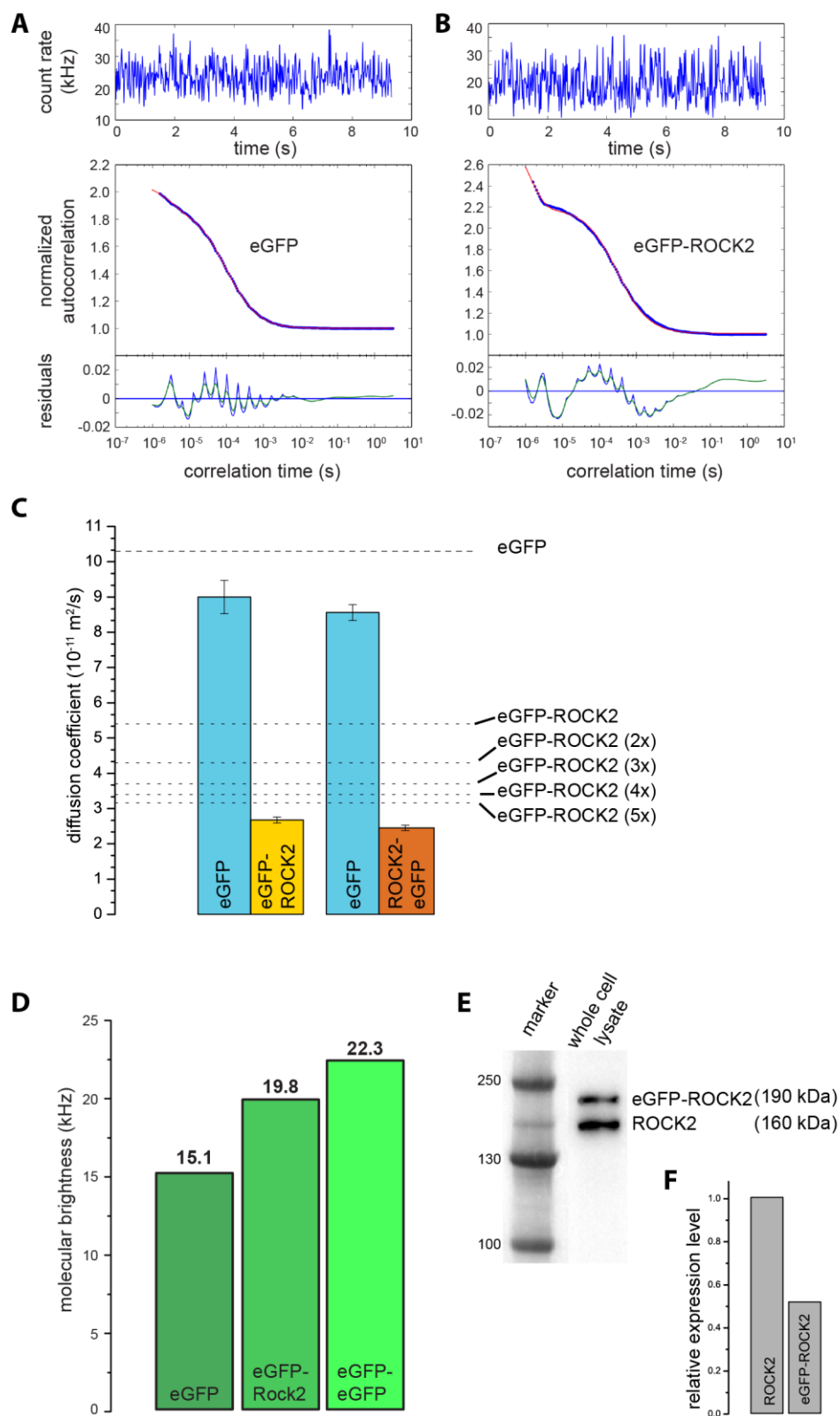


Figure 5. FCS applied to eGFP-ROCK2

(A, B) Representative autocorrelation curves for (A) eGFP and (B) eGFP-ROCK2

(C) Diffusion coefficients determined in two independent experiments. In every experiment the diffusion coefficient of eGFP and eGFP-ROCK2 or ROCK2-eGFP were determined in parallel. Each species was

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- measured at least 10 times and deviations between each of the consequent measurements are represented by the error bars. The theoretical diffusion coefficients of eGFP and oligomers of eGFP-ROCK2 are drawn as dashed lines and indicated on the right.
- (D) Molecular brightness analysis of eGFP, eGFP-ROCK2 and eGFP₂.
 - (E) Western blot performed on whole cell lysates transfected in the same manner as for FSEC-FCS analysis.
 - (F) Expression levels of endogenous ROCK2 and eGFP-ROCK2. Immunoblot signal (E) was quantified and normalized to ROCK2 signal.

The oligomeric state of eGFP-ROCK2 was further investigated by analyzing the molecular brightness of the eGFP fluorophore. The molecular brightness is dependent on the number of fluorophores per particle diffusing in the confocal volume. It has been previously shown that each fluorophore associated to the diffusing particle equally contributes to the molecular brightness of the diffusing species (Chen et al., 1999). Thus, if the brightness of eGFP and oligomeric eGFP is known, the oligomeric state of eGFP-ROCK2 can be determined. For this purpose the system was calibrated by measuring the brightness of eGFP as well as the brightness of the eGFP-eGFP fusion protein (eGFP₂) (Figure 5D). Unfortunately, the values obtained are not in the linear range and the eGFP-eGFP brightness is not double but 1.5 times the eGFP brightness and optimization of the optical setup (reduction in laser intensity) is required to overcome this problem. Nevertheless, the molecular brightness of eGFP-ROCK2 clearly lies between the brightness for monomeric and dimeric eGFP.

To address whether the intermediate brightness value could be caused by heterodimerization between ectopically expressed eGFP-ROCK2 and endogenous ROCK2, the relative expression levels of both proteins were investigated. Western blot analysis of whole cell lysates revealed that eGFP-ROCK2 expression is around 50% of the endogenous ROCK2 expression (Figure 5E and 5F).

3.2 Membrane recruitment of ROCK

Membrane recruitment of the ROCK lipid binding domains was investigated by live cell imaging. In this assay, ROCK lipid binding domains were transiently expressed as fluorescent fusion proteins in mammalian cell lines. Cells were treated with PI3K agonists or arachidonic acid and membrane translocation was monitored in vivo by enrichment of the fluorescence signal at the plasma membrane. Since membrane enrichment is hard to quantify, translocation was measured as cytosolic depletion of fluorescence signal.

3.2.1 PI3K

It was previously reported that, in vitro, the PH_N-C1-PH_C domain of ROCK1 binds to liposomes containing folch lipids (brain lipid extract). Binding of the entire lipid binding domain was shown to be more efficient than for the isolated C1 and PH domains. Additionally the PH_N-C1-PH_C domain preferentially bound artificial liposomes (75:25% PC:PS) containing 5% PI(3,4,5)P₃ over liposomes of other phosphoinositide compositions (Wen et al., 2008).

To test whether PI(3,4,5)P₃ dependent membrane translocation occurs in vivo, HeLa cells were transfected with an ROCK1-eGFP construct that contained the C terminal PH_N-C1-PH_C domain as well as an additional 100 N-terminal residues (ROCK1¹⁰²⁴⁻¹³⁵⁴-eGFP). These residues are predicted to form a coiled coil and would therefore be expected to dimerize the membrane binding domains, which might better resemble the ROCK membrane binding in the cell than the isolated PH_N-C1-PH_C. The cells were cotransfected with an AKT PH domain construct (BFP-AKT1¹⁻¹¹⁷). Production of PI(3,4,5)P₃ in the plasma membrane was induced by adding 100 ng/ml IGF-1.

Although membrane translocation could be monitored for the AKT PH domain, the ROCK¹⁰²⁴⁻¹³⁵⁴-eGFP did not appear to translocate (Figure 6A and 6B) suggesting that ROCK is not responsive to PI(3,4,5)P₃ alone in vivo and thus may not be downstream of PI3K signaling, as has been reported previously (Park et al., 2008).

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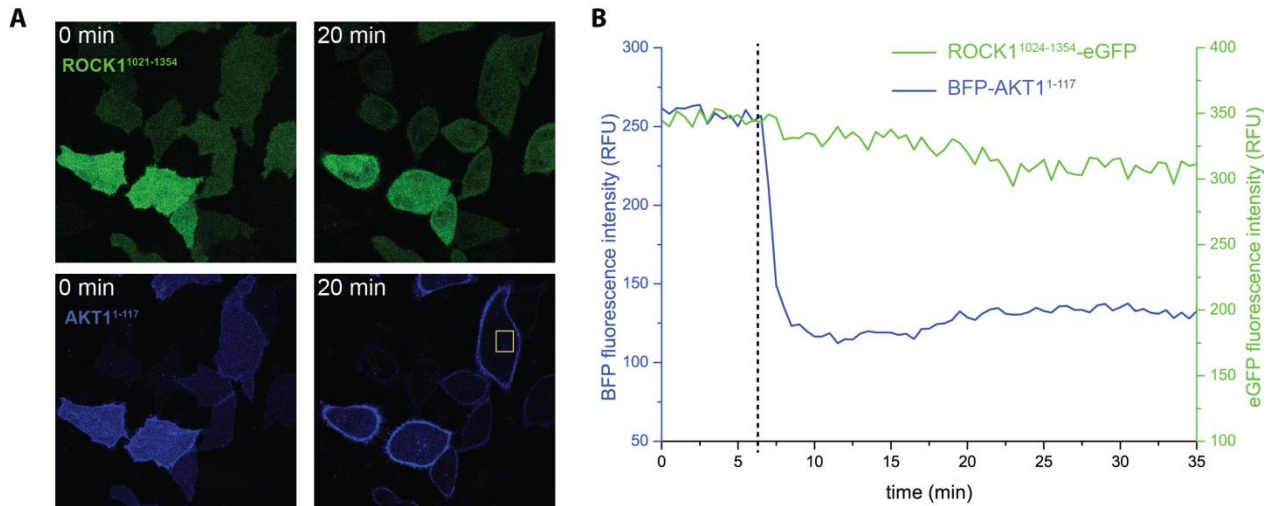


Figure 6. ROCK1¹⁰²⁴⁻¹³⁵⁴ does not translocate to the plasma membrane compared to AKT¹⁻¹¹⁷
 (A) Confocal microscopy of HeLa cells co-transfected with eGFP-ROCK1¹⁰²⁴⁻¹³⁵⁴ and BFP-AKT1¹⁻¹¹⁷. Two time points are shown and correspond to the times in (B). At 6 min, cells were treated with 100 ng/ml IGF-1.
 (B) Cytosolic depletion of fluorescence signal. The mean fluorescence intensity was measured within a region (yellow box) indicated in (A). 100ng/ml IGF treatment is indicated (dashed line)

3.2.2 Arachidonic acid

It was previously reported that arachidonic acid increases ROCK kinase activity in vitro (Araki et al., 2000). However, it is unclear, how ROCK might interact with arachidonic acid and how this interaction would increase kinase activity. A specific ligand for the ROCK C1 domain is not known although it has been reported to be an atypical C1 domain, which does not respond to the DAG mimic phorbol ester (Wen et al., 2008).

To test whether arachidonic acid could be a ligand for the ROCK C1 domain, a tandem C1 construct was designed (C1₂). The rationale for using a C1 tandem rather than isolated C1 domains is based on our finding that ROCK is exclusively dimeric inside cells (see 3.1.). A C1 tandem construct addresses the possibility that avidity might play an important role in ligand binding and therefore better resembles the plasma membrane translocation of full-length ROCK. This approach was successfully applied before to demonstrate the specific membrane recruitment of the atypical C1 domain of the centralspindlin subunit MgcRacGAP to plasma membranes (Lekomtsev et al., 2012). To avoid recombination problems during cloning the ROCK1 C1 domain was fused to the ROCK2 C1 domain with a flexible linker. Although the domains stem from different isoforms, their amino acid sequence is highly conserved.

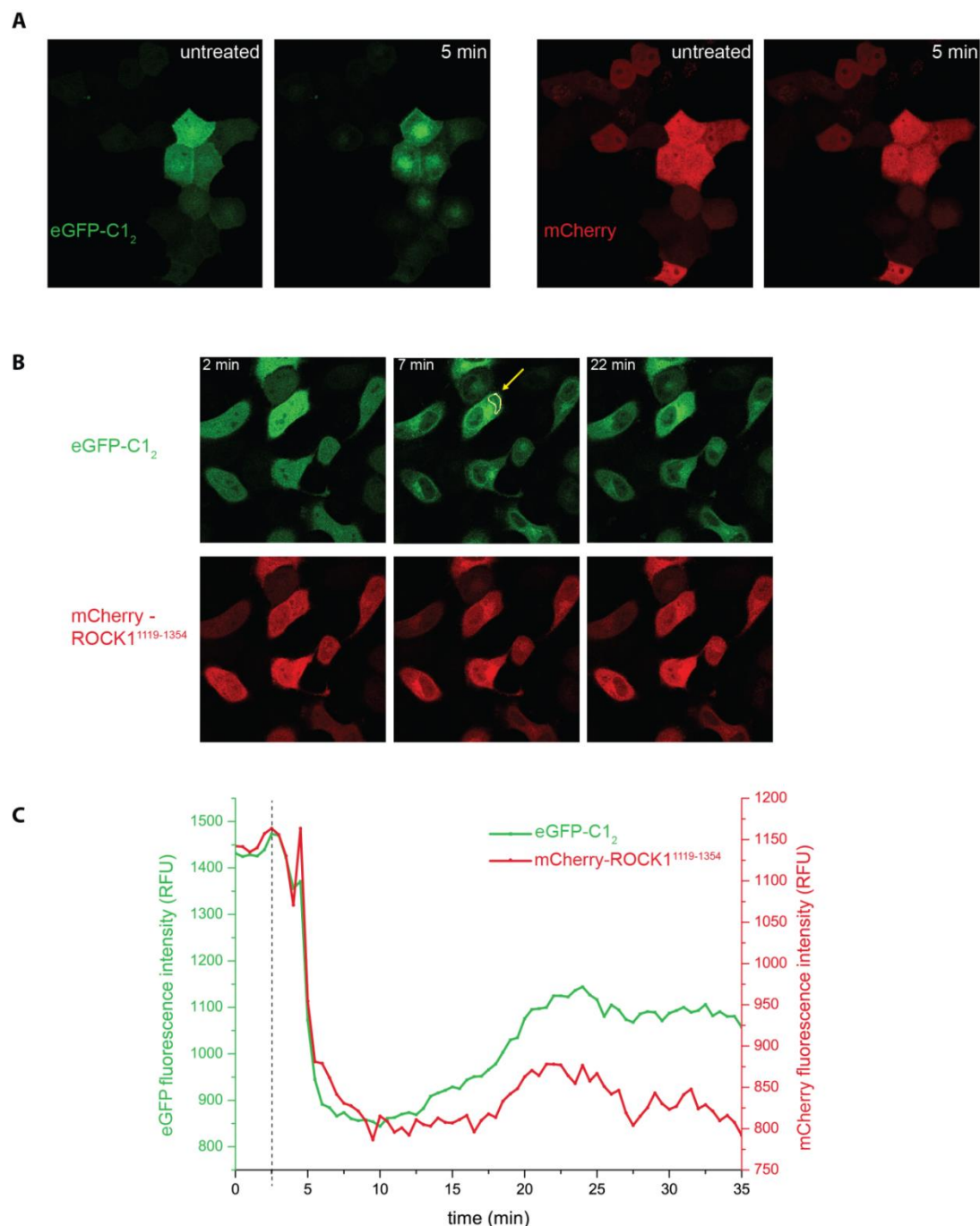


Figure 7. Arachidonic acid translocates a C1 tandem construct to internal membranes

- (A) MDCK cells were co-transfected with eGFP-C1₂ and mCherry alone. Before arachidonic acid treatment eGFP-C1₂ and mCherry were evenly distributed throughout the cell (untreated). After addition of 150 μ M arachidonic acid however, eGFP-C1₂ is enriched at the Golgi apparatus and partly on the nuclear and plasma membranes whereas mCherry is still evenly distributed throughout the cell. Cells are shown before (untreated) and 5 min after arachidonic acid treatment.
- (B) HeLa cells were co-transfected with eGFP-C1_{2x} and a ROCK1 PH_N-C1-PH_C domain construct (mCherry-ROCK1¹¹¹⁹⁻¹³⁵⁴) and treated with 150 μ M arachidonic acid. Both the C1 tandem and the PH-C1-PH domain enriched on internal structures.
- (C) Translocation was measured as cytosolic depletion. The depletion was monitored in a region of the cytoplasm is indicated by an yellow arrow in (B).

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The C1 tandem construct was expressed in MDCK cells and confocal microscopy showed that it localized throughout the cytoplasm and nucleus. Surprisingly, upon arachidonic acid treatment, the eGFP-C1₂ construct enriched mostly at internal membranes, and to a minor extent at the plasma and nuclear membranes (Figure 7A). However, a co-transfected fluorescent protein (mCherry) did not translocate at all. This indicates that the translocation induced by arachidonic acid is specific for the tandem C1 domain protein. When cells were co-transfected with eGFP-C1₂ and a ROCK1 construct containing the entire PH_N-C1-PH_C domain (mCherry-ROCK1¹¹¹⁹⁻¹³⁵⁴) membrane recruitment was observed for this construct (Figure 7B). Furthermore, membrane translocation of mCherry-ROCK1¹¹¹⁹⁻¹³⁵⁴ showed very similar kinetics to eGFP-C1₂. This is surprising given that the tandem construct contains two C1 domains whereas the PH_N-C1-PH_C contains only one C1 domain.

Surprisingly, arachidonic acid treatment did not result in the translocation of eGFP-ROCK2 to any membrane either in Cos7 or in MDCK cells (Figure 8A and 8B). A further control experiment showed that arachidonic acid treatment translocated PKC βII to the plasma membrane (Figure 8C), whereas the C1₂ translocated primarily to internal membranes (Figure 7A). The C1 domains of PKC βII bind specifically to DAG (Leonard et al., 2011) suggesting that arachidonic acid indirectly elicits PKC βII translocation.

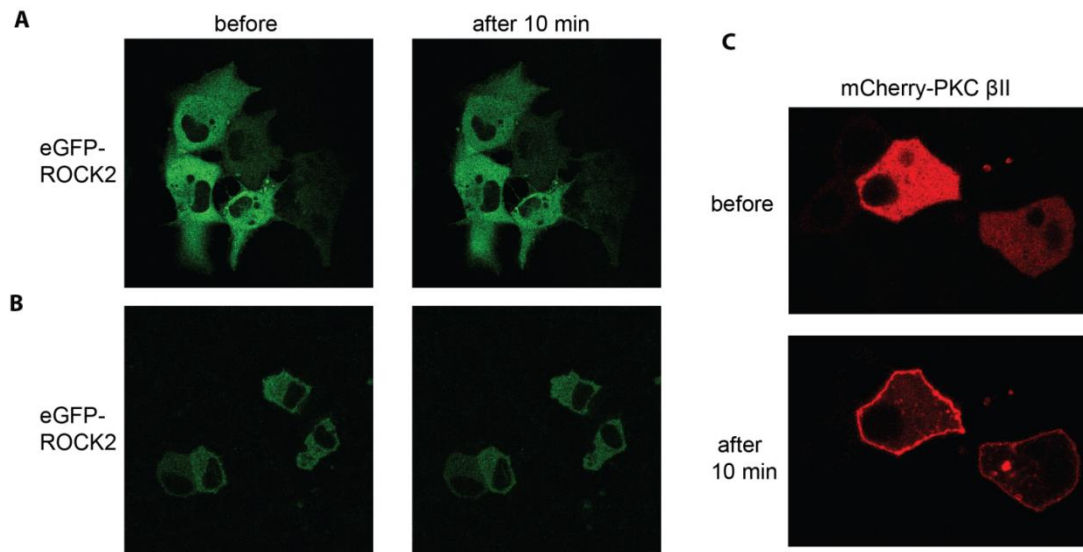


Figure 8. Effect of arachidonic acid treatment on ROCK2 and PKC βII

- (A) Cos7 cells transfected with eGFP-ROCK2 were treated with 150 μM arachidonic acid. Shown are images of right before and after 10 min of arachidonic acid treatment.
- (B) MDCK cells transfected with eGFP-ROCK2 were treated with 150 μM arachidonic acid. Shown are images of right before and after 10 min of arachidonic acid treatment.
- (C) MDCK cells expressing mCherry-PKC βII were treated with 150 μM arachidonic acid. An image before and 10 min after the treatment is shown.

3.3 RhoA purification

RhoA has been reported to increase ROCK kinase activity towards its substrates. From the crystal structure of RhoA complexed with the RBD of ROCK1, it is unclear how RhoA binding might influence kinase activity. However, the stoichiometry and affinity of RhoA to full-length ROCK has not been determined. To identify the affinity and stoichiometry of GTP bound RhoA for full length ROCK2 and to investigate whether binding directly increases the enzymatic activity of the kinase, RhoA was recombinantly produced in *E.coli* and purified to homogeneity.

3.3.1 RhoA purification

Human RhoA is a 193 residue protein. For ease of purification it was C-terminally truncated at amino acid 181, to avoid problems with its C-terminal CAAX box which can be lipidated and hence anchor RhoA to membranes (Zhang and Casey, 1996) (Shao et al., 2003). The purification was carried out as described previously for the crystal structure of the Gpp(NH)p RhoA¹⁻¹⁸¹ ROCK1⁹⁴⁷⁻¹⁰¹⁵ complex (Dvorsky et al., 2004). In addition to wild type RhoA¹⁻¹⁸¹, a mutant RhoA protein, RhoA¹⁻¹⁸¹G14V, was expressed and purified. This mutation is known to render RhoA constitutively active and has been shown to reduce the intrinsic GTPase activity of RhoA (Longenecker et al., 2003).

RhoA¹⁻¹⁸¹ and RhoA¹⁻¹⁸¹G14V were expressed as N-terminal GST fusion proteins in *E.coli*. After TEV cleavage of the GST tag the protein was purified by size exclusion chromatography (Figure 9A and 9B) and fractions were analyzed for purity by SDS PAGE (Figure 9C and 9D). Purification conditions and the SEC elution profile of the wild type and mutant RhoA¹⁻¹⁸¹ were very similar. In both cases RhoA elutes as a single, symmetric peak eluting at a volume expected for a 20 kDa protein.

Analysis of the peak fractions by SDS PAGE and Coomassie staining shows that RhoA¹⁻¹⁸¹ is almost exclusively a single band that runs at a molecular weight of ~ 17 kDa which is a bit lower than expected for a 20 kDa protein (Figure 9C). In contrast, the RhoA¹⁻¹⁸¹G14V peak fractions appeared as three discrete bands (Figure 9D), and it is so far unclear what the explanation is for the appearance of these three bands.

The ratio of the 260 nm absorbance to the 280 nm absorbance (A₂₆₀/A₂₈₀) as shown for RhoA¹⁻¹⁸¹ ranged from 0.9 to 1.1. For a pure protein, a A₂₆₀/A₂₈₀ value of ~ 0.7 would be expected. However, GTP has an A₂₆₀/A₂₈₀ ratio of 1.5 due to its purine base guanine. The

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A260/A280 absorbance ratio for purified RhoA¹⁻¹⁸¹ therefore indicates that it is nucleotide bound, consistent with previous observations for purified small GTPases (Eberth and Ahmadian, 2009).

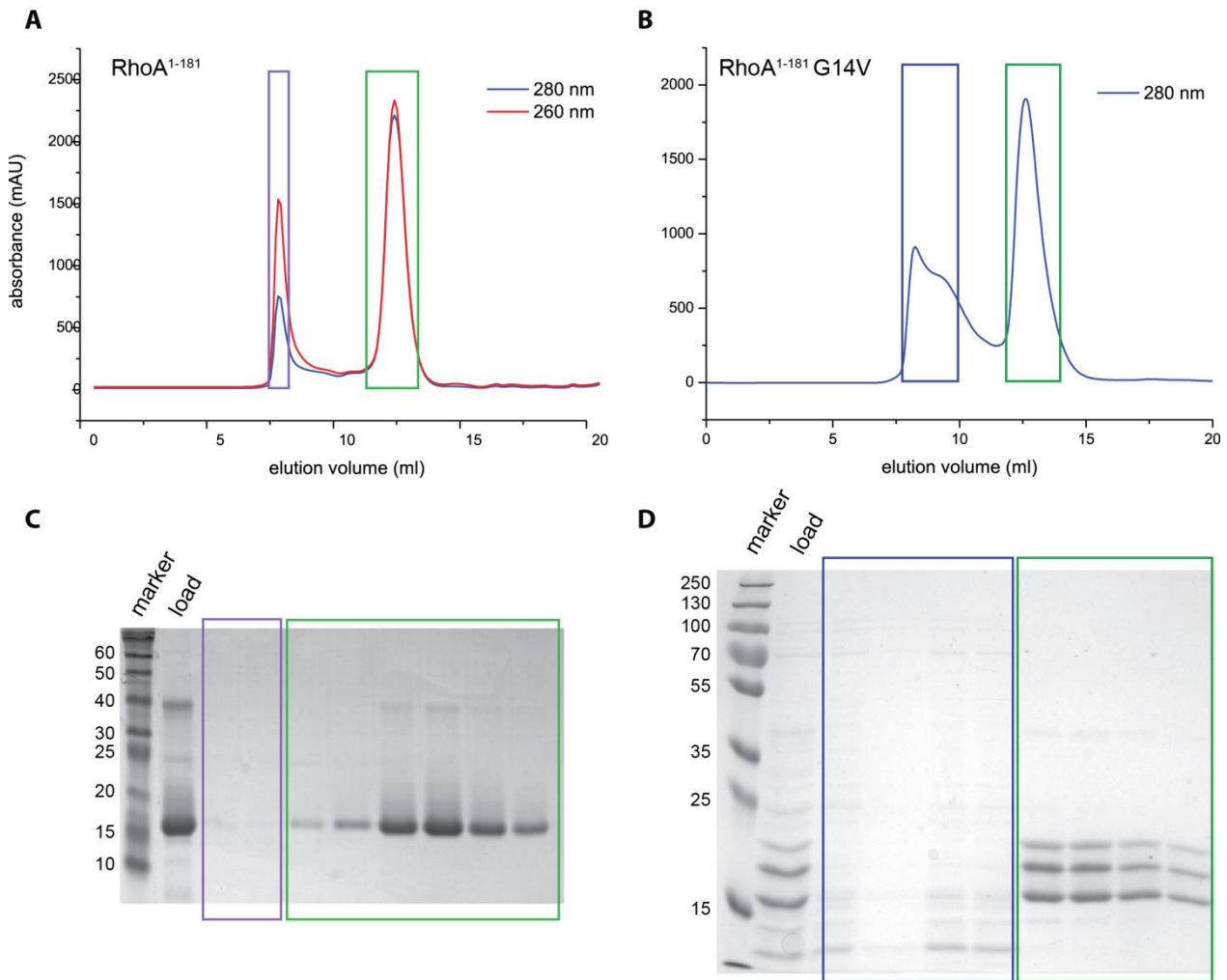


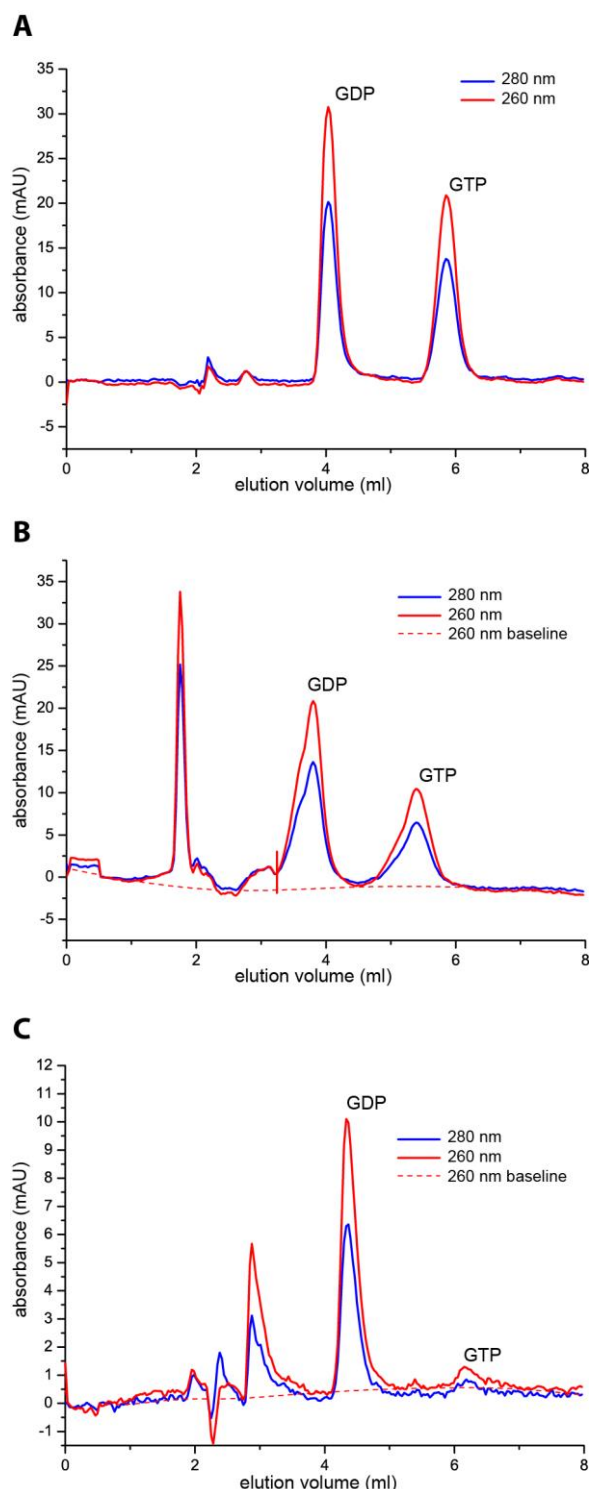
Figure 9. RhoA¹⁻¹⁸¹ and RhoA¹⁻¹⁸¹G14V purification

- (A) Size exclusion chromatography (Superdex 75 10/300) of RhoA¹⁻¹⁸¹ after cleavage of the affinity tag. Fractions (boxes) were analyzed by SDS PAGE (C)
 (B) Size exclusion chromatography (Superdex 75 10/300) of RhoA¹⁻¹⁸¹G14V after cleavage of the affinity tag. Fractions (boxes) were analyzed by SDS PAGE (D).
 (C) Coomassie staining of a 15% polyacrylamide gel showing the load, void and RhoA¹⁻¹⁸¹ peak fractions.
 (D) Coomassie staining of a 12% polyacrylamide gel showing the load, void and RhoA¹⁻¹⁸¹G14V peak fractions.

3.3.1 Nucleotide state analysis

To use the recombinant RhoA in further experiments such as binding experiments it is crucial to determine what nucleotide this protein is bound to. In particular, it has been reported that the dissociation constant of ROCK1⁹⁴⁷⁻¹⁰¹⁵ and RhoA¹⁻¹⁸¹ change by one order of magnitude depending on RhoA¹⁻¹⁸¹ being GDP or GTP bound (Blumenstein and Ahmadian, 2004). To

determine the nucleotide status of RhoA for subsequent kinase assays an ion-pairing RP HPLC assay was established to separate and quantitate the nucleotides GDP and GTP (Figure 10A).



A C18 reverse phase column was calibrated with GDP and GTP standards (Figure 10A). Purified RhoA¹⁻¹⁸¹G14V was applied to the RP-HPLC right after purification (Figure 10B) and after one week at 4°C (Figure 10C). Immediately following purification, RhoA¹⁻¹⁸¹G14V was found to be 60% GDP (76 μ M) and 40% GTP bound (51 μ M). After one week, however, GDP was still detectable whereas GTP was barely detected. This is in line with previous studies that report that the G14V mutation reduces but does not fully abolish GTPase activity (Longenecker et al., 2003). Since RhoA¹⁻¹⁸¹G14V was not purified to such high purity as wild type RhoA¹⁻¹⁸¹, and still contains intrinsic GTPase activity, further experiments ought to be carried out with wild type RhoA¹⁻¹⁸¹ and a non-hydrolyzable GTP analog (see 4.4.2).

Figure 10. Ion pairing RP HPLC

(A) Calibration of the RP column by GDP and GTP standards (each 50 μ M)
 (B) Purified RhoA¹⁻¹⁸¹G14V
 (C) Purified RhoA¹⁻¹⁸¹G14V after it was stored for one week at 8 degrees

Note that not all runs were performed the same day, and elution volumes differ between runs. However, each elution volume was verified by a GDP/GTP standard mix.

4 DISCUSSION

4.1 Characterization of ROCK2 by FSEC-FCS

Classical recombinant protein purification is based on heterologous expression systems (ranging from *E.coli* to mammalian cell lines) that aim to yield high amounts of protein in liter scale cultures. For characterization, the protein has to be purified from cell lysates by a two to three step purification procedure including e.g. affinity, ion exchange and size exclusion chromatography. During these steps the protein is subjected to different buffers and conditions (ionic strengths, pH, imidazole concentrations etc.) that might affect its native structure. The method applied in this thesis fundamentally differs from this workflow, since it analyzes small amounts of native fluorescent fusion proteins from cell lysates by FSEC and subsequent FCS.

The successive application of FSEC and FCS on cell lysates has – to my knowledge - not been reported before, although both methods are well established and widely used. In this thesis, these methods were coupled to gain information about the size and shape of a fluorescent fusion protein which was evaluated by the elution volume obtained by FSEC and the diffusion coefficient obtained by FCS. Furthermore, molecular brightness analysis was carried out by FCS to investigate the oligomeric state of a fluorescent fusion protein. Coupling both techniques provides some advantages in comparison to protein purification and characterization as well as some minor pitfalls that shall be discussed here.

One of the major advantages of this setup is intrinsic to fluorescence detection itself which is characterized by high sensitivity and low limit of detection due to extremely little background signal. Thus, very small amounts of unpurified fluorescent fusion protein suffice for analysis. For instance, for eGFP-ROCK2 one well of a six well plate of ~ 20% transfected Cos7 cells was enough to be detected well by FSEC and FCS (Figure 4, Figure 5). In contrast to a multistep protein purification the FSEC-FCS approach can be very quickly performed. Cell lysates only require ultracentrifugation (15 min), size exclusion chromatography (1 h) and recording of fluorescence fluctuation spectra (several minutes).

The fact that FSEC requires only small amounts of protein, few resources and little time, makes it particularly interesting for constructs and buffer screenings. Crystallographers, for instance, use FSEC to screen for constructs and lysis buffers to obtain well expressed, stable and monodisperse proteins. These properties are a prerequisite for crystallization and can be evaluated by comparing FSEC elution profiles (Kawate and Gouaux, 2006).

FCS is a single molecule detection method that detects single diffusing particles and can be easily applied to any solution containing a fluorescent dye. Due to the confocal volume of around 1 femtoliter, concentrations in the range of 1-100 nM are required for FCS, which makes it possible to analyze proteins at relatively low concentrations. In fact, for abundant proteins or highly expressed proteins the FSEC peak fractions might even require further dilution prior to FCS analysis as was the case in this study for eGFP. In principle, FCS is not restricted to dilute cell lysates but has been often applied to living cells (Haustein and Schwille, 2003). This clearly has the advantage that proteins can be investigated in their respective cellular environment. However, in the crowded environment of the cell recording of FCS spectra requires more experience than in dilute solutions. Furthermore, diffusion coefficients inside the cell are often altered by anomalous or confined diffusion, which is especially common for membrane associated proteins (Haustein and Schwille, 2003). Although anomalous diffusion in many cases resembles the actual situation in the cell and carries important information (e.g. low affinity protein protein interactions) it hampers the possibility to use the diffusion coefficient as a proxy for the size and shape of a fluorescent fusion protein. In this respect the application of FCS on diluted cell lysates as presented here is a tradeoff that seeks to characterize the native protein but at the same time to be easy to handle and to accurately determine the diffusion coefficient.

Apart from the diffusion coefficient, FCS can be used to investigate the oligomeric state of a fluorescent fusion protein. Chen et al have convincingly demonstrated that molecular brightness can be used to monitor protein oligomerization inside cells (Chen et al., 2003). The reasoning for adding this easily accessible parameter into the established FSEC-FCS workflow is that molecular brightness is independent of the shape of the protein. Elution volumes obtained by FSEC and diffusion coefficients obtained by FCS only depend on the overall size and shape of the protein and are thus incapable to discriminate between the contribution of protein extension and oligomerization. This is especially problematic if a protein deviates substantially from a globular protein. The molecular brightness, however, is not at all related to the dimension of the protein but only depends on the number of fluorophores associated with the protein (Chen et al., 1999).

The experimental setup presented here has some limitations that have to be considered, too. Most importantly, it must not be forgotten that genetically linking a fluorescent protein like eGFP to a protein might influence the native conformation of the protein of interest. Although the influence of the tag can never be fully excluded, tagging the protein independently at both termini can strengthen the confidence in the obtained data.

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To maintain the proteins in their native state, the cells were lysed and size exclusion chromatography was performed in a buffer that recapitulates the ionic strength, pH and reducing environment of the cytoplasm. This means, however, that only proteins that are soluble under these conditions are amenable to the FSEC-FCS analysis. Proteins that are tightly associated with membranes might be pelleted by ultracentrifugation and are therefore lost for analysis. In principle it is possible to solubilize such proteins by using other buffers (e.g. containing detergents) and FSEC has been successfully performed on integral membrane proteins (Kawate and Gouaux, 2006), though, it is difficult to know for sure whether these proteins are still in their native state.

Finally, it should be taken into account that cellular proteins are substantially diluted upon cell lysis and during size exclusion chromatography. Therefore, all low affinity interactions a protein might have inside the cell are unlikely to be present in the FSEC peak fraction. Very high affinity interactions, however, remain. So far, there is no evidence that eGFP-ROCK2 eluted in complex with other proteins, but this could be possible for high affinity interactions and constitutive protein complexes and should be considered for any analysis. On the other hand, if a protein elutes in complex with e.g. other proteins the size and shape and the stoichiometry of this complex would be very important information too. In principle, size exclusion chromatography is widely applied in the purification of tight protein complexes, but it remains to be determined how well protein complexes can be characterized with the coupled FSEC-FCS approach applied here.

Taken together, the coupled FSEC and FCS analysis allows one to characterize the size and shape of native fluorescent fusion proteins by two complementary measures: elution volumes and diffusion coefficients. Additionally, one can determine oligomeric state in a size and shape independent manner. This simple, rapid and economical approach is particularly interesting for characterizing non globular proteins under conditions similar to their native cellular environment.

4.2 ROCK is a constitutive dimer in mammalian cells

The elution profile and the diffusion coefficient suggest that eGFP-ROCK2 exists in one overall conformation that is highly extended and/or oligomeric. Yet, the molecular brightness lies between a monomer and dimer brightness, which might be reasonably interpreted as an equilibrium of monomers and dimers. However, it can be assumed that monomer and dimer would have different sizes and therefore different diffusion constants. The FSEC elution profile

as well as the fact that the autocorrelation function can be well fitted by a single component fit strongly contradict the possibility of a monomer dimer equilibrium and indicate a single oligomeric species.

Another explanation for the intermediate brightness value observed might well be that eGFP-ROCK2 transiently expressed in Cos7 cells forms heterodimers with endogenous ROCK2 protein present in these cells. This alternative explanation is in line with the similar expression level of eGFP-ROCK2 compared to endogenous ROCK2 in Cos7 cells (Figure 5E and 5F). Given the fact that eGFP-ROCK2 expression is around 50% of the endogenous ROCK2 expression it is likely that eGFP-ROCK2 and endogenous ROCK2 form heterodimers inside cells, which would lead to an intermediate brightness value. One possibility to test this would be to knock down the endogenous ROCK2 protein while ectopically expressing eGFP-ROCK2 and such experiments are currently ongoing.

It can be concluded that eGFP-ROCK2 is most likely a constitutive dimer. Since eGFP does not dimerize under these conditions, dimerization must be an inherent property of ROCK2. However, this dimer must be highly extended due to the low diffusion coefficient and the anomalous FSEC elution volume.

These observations are in line with previous reports showing that ROCK1 is a homodimer in Cos7 cells as judged by crosslinking studies on myc-tagged ROCK1 (Garg et al., 2008) and the fact that recombinant ROCK1 and ROCK2 are exclusively dimeric by equilibrium sedimentation studies (Doran et al., 2004). Likewise, unpublished data from our lab shows that recombinantly expressed and purified human ROCK2 from insect cells is exclusively dimeric – as evaluated by static light scattering. Rotary shadowing electron microscopy (EM) revealed that dimeric ROCK2 forms a highly extended structure (Trübestein, unpublished data, Figure 11A), consisting of globular domains separated by a long rod. One end of this rod consists of a globular structure and the other end of two slightly displaced individual globular structures. The rod that connects both globular ends of the protein has a length of 107 nm. Taking into account the known structures of ROCK, that showed a parallel dimeric kinase domain, parallel coiled coil regions, and monomeric PH_N-C1-PH_C domains, an overall structural model of native ROCK can be derived (Figure 11B).

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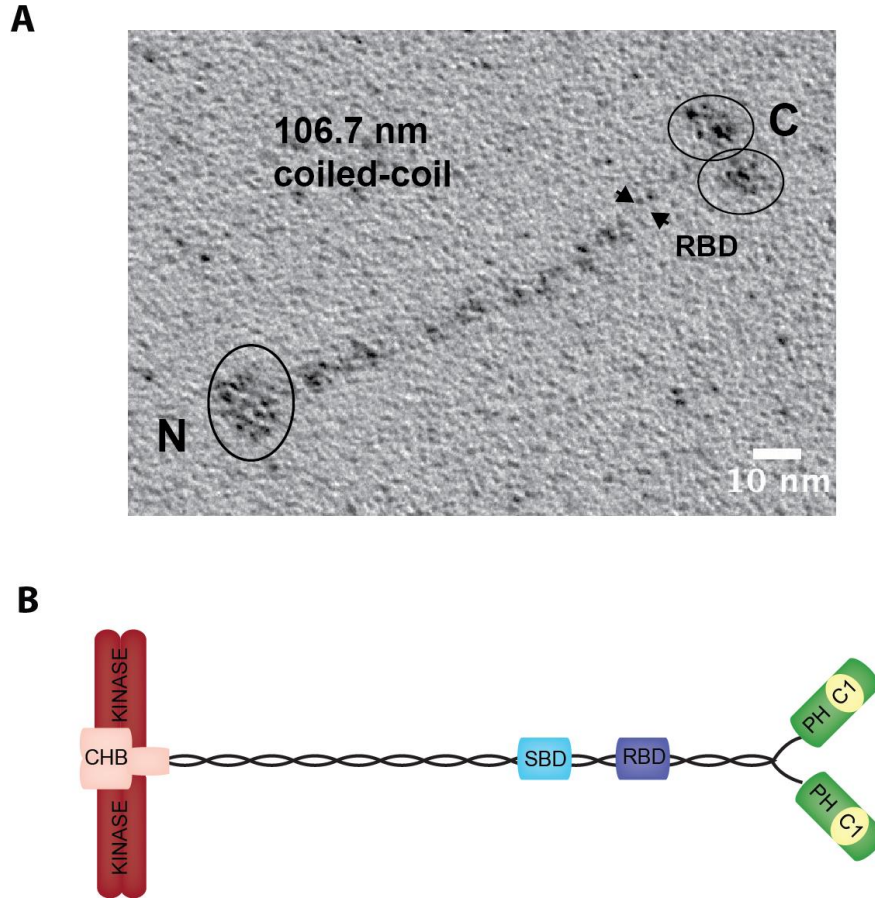


Figure 11. Structural model of ROCK

- (A) Rotary shadowing electron microscopy of recombinant human ROCK2. The N- and C-termini were assigned due to the fact, that one end has one globular domain, and the other end has two globular domains. The coiled coil between the two ends is 106.7 nm and the RBD on the coiled coil is indicated by two black arrows.
- (B) Structural model derived from known structures, and the EM images. The N-terminal kinase domains form parallel dimers via the capped helix bundles and their C-terminal extensions, the SBD and RBD are both parallel coiled coils and the N-terminal PH_N -C1- PH_C domains are both individual domains.

The diffusion coefficient of this extended structure can be approximated by the diffusion coefficient of a stiff rod (see 2.5.2). Using the maximum length of 122 nm observed by EM and the typical diameter of a coiled coil of 1 nm gives a stiff rod diffusion coefficient of $1.8 \cdot 10^{-11} \text{ m}^2/\text{s}$. This theoretical diffusion coefficient is lower than the experimental value determined for eGFP-ROCK2 and ROCK2-eGFP (2.7 ± 0.1 and $2.5 \pm 0.1 \cdot 10^{-11} \text{ m}^2/\text{s}$). This might be due to the fact, that eGFP-ROCK2 has some flexibility within the coiled coil region whereas the approximation assumes it to be completely stiff. Indeed, on the EM images one can find several molecules that appear to be bent at different positions of the coiled coil. Nevertheless, the similar diffusion coefficients suggest that the native eGFP-ROCK2 molecule actually exists in a largely extended conformation but with some conformational flexibility within the coiled coil.

4.3 Factors that control ROCK signaling

The structural model presented here contradicts the autoinhibition model (see 1.3). In particular, the EM images (Figure 11A) indicate that there is no interaction between the C-terminal PH_N-C1-PH_C membrane binding domains and the N-terminal kinase domains. Whilst it cannot be entirely ruled out that such an interaction exists inside cells, the FSEC-FCS data presented in this thesis suggest that this is unlikely the case (3.1). Thus, on the basis of this structural model, membrane binding and/or RhoA binding would not be expected to activate the kinase. This raises the question whether the catalytic activity of ROCK has to be necessarily regulated at all. ROCK could exist as a constitutively active kinase, whose intrinsic kinase activity is not altered by lipids, RhoA or any other factor. Lipid binding and RhoA binding, however, might still be essential for other aspects of ROCK signaling, including correct subcellular localization.

4.3.1 Membrane recruitment

It has been reported that the ROCK2 PH_N-C1-PH_C domain binds to folch (brain extract) lipids (Wen et al., 2008) which has also been observed for full length ROCK2 (Trübestein, unpublished data). However, it is unclear whether ROCK binds to membrane embedded lipid second messengers, which would enable ROCK to respond to certain signaling pathways.

As shown in Figure 6 the ROCK1 PH_N-C1-PH_C domain did not efficiently translocate in response to the PI3K agonist IGF-1. This is in line with previous reports that showed that the mouse ROCK1 PH domain has a lower affinity for PI(3,4,5)P₃ than other PH domains and does not translocate to the plasma membrane in response to PDGF unlike other PH domains (Park et al., 2008).

Arachidonic acid in turn, translocated a C1₂ construct and a PH_N-C1-PH_C to the Golgi apparatus and to the nuclear membrane (Figure 7). Surprisingly, both constructs showed very similar translocation kinetics, although one construct is comprised of two and the other construct comprised of only one C1 domain. However, arachidonic acid did not translocate the full length ROCK protein, but translocated PKC βII to the plasma membrane. PKC βII is a conventional PKC and has two C1 domains that both respond to the second messenger DAG. PKC βII has an additional C2 domain that engages with phospholipids in a Ca²⁺ dependent manner. Regardless of what exactly causes PKC βII to translocate to the plasma membrane in response to arachidonic acid, it seems to be mechanistically different from the C1₂ translocation that was

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mostly directed towards internal membranes. These observations make it unlikely that arachidonic acid is a specific C1 domain ligand for ROCK.

Structurally, it is hard to conceive how arachidonic acid, a polyunsaturated 20:4(ω -6) fatty acid, can be a very specific ligand, since it consists of a poly unsaturated acyl chain and a carboxyl head group. Nevertheless, it is long known that arachidonic acid which is present in cells mostly in phospholipids and when released from phospholipids by phospholipases can indeed act as a second messenger. Subsequently, arachidonic acid can be metabolized into many different products belonging to the leukotrienes and prostaglandins and these also play important roles in several signaling processes (reviewed in Di Marzo, 1995). In vivo it is therefore very hard to distinguish whether i) the ligand for the C1 domain is arachidonic acid or one of its numerous derivatives and ii) whether translocation is elicited by this ligand or by downstream signaling events.

Finally, it should be mentioned that the concentration of arachidonic acid used in these experiments was very high, ranging from 3-300 μ M. These high concentrations were used because of previous reports in which arachidonic acid concentrations of 60 μ M and 300 μ M were required to substantially increase ROCK kinase activity and to induce smooth muscle contraction, respectively (Araki et al., 2000). Although it cannot be reliably estimated to what extent arachidonic acid partitions into different cellular membranes, one could speculate that it is present in membranes in quite substantial amounts. Due to its four cis-double bonds the acyl chain is kinked, and it is known that these unsaturated acyl chains are present in some phospholipids that constitute the cellular membranes. In particular, the degree of unsaturated acyl chains is essential for the properties of the respective membrane. By adding large amounts of an unsaturated fatty acid to a cell membrane it might be possible to actually alter the membrane properties and therefore change the affinity for lipid binding domains in a very unspecific manner.

Again, it is not known whether ROCK can sense second messengers by its lipid binding domains and it could well be that ROCK does not bind second messengers at all. In this case the PH_N-C1-PH_C might only serve the function of tethering ROCK to membranes in a rather unspecific manner, although the fact that eGFP-ROCK2 appears to be evenly distributed throughout the cytoplasm (Figure 3) somewhat contradicts this view. Whatever the role of membrane binding is, it is unlikely to be linked to kinase activation. Experiments in our lab have shown that in vitro

liposome binding of purified ROCK2 does not increase the intrinsic kinase activity (Trübestein, unpublished data).

4.3.2 RhoA binding

Another important factor for ROCK regulation is the small GTPase RhoA. As described in 3.3. RhoA¹⁻¹⁸¹ was recombinantly expressed and purified and a method to analyze its nucleotide state was established. This is a prerequisite for subsequent in vitro experiments that seek to investigate the binding affinity and stoichiometry of the RhoA-ROCK complex, the conformational change that such binding might induce, and the influence of RhoA binding on the catalytic activity of the kinase.

Since RhoA¹⁻¹⁸¹G14V could not be purified to satisfactory purity as judged by SDS PAGE (Figure 9), future experiments will be carried out with wild type RhoA¹⁻¹⁸¹. However, since it is essential that RhoA is in a GTP bound state any perturbations due to the intrinsic GTPase activity of RhoA have to be avoided. Therefore, experiments ought to be carried out with RhoA¹⁻¹⁸¹ bound to a non-hydrolysable GTP analog such as Gpp(NH)p. Well established protocols are available for nucleotide exchange (Eberth and Ahmadian, 2009) and the exchange efficiency can be monitored by the implemented ion-exchange RP HPLC method (Figure 10). It will be particularly interesting to determine the stoichiometry with which Gpp(NH)p-RhoA¹⁻¹⁸¹ binds full-length ROCK and whether Gpp(NH)p-RhoA¹⁻¹⁸¹ binding influences the overall structure of ROCK.

If binding of RhoA is restricted to the RBD, though, and does not result in big conformational rearrangements, as our model predicts, it is hard to rationalize how binding to this region would actually increase the activity of an 80 nm distant kinase domain. Whatever the structural effect of RhoA binding is, once the RhoA affinity is determined the influence of RhoA binding to the kinase activity can be experimentally tested. It should be stated again, that previous reports showed that RhoA binding increases ROCK kinase activity only ~2 fold in vivo and vitro. The structural model presented here predicts that binding of RhoA to ROCK will not influence its intrinsic catalytic properties, but the effect of RhoA binding on ROCK activity remains to be experimentally tested under our conditions.

Another aspect of RhoA that has not been considered so far, is that the full length RhoA protein carries a C-terminal CAAX box motif. This motif can be geranyl-geranylated and RhoA is thereby

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tethered to cell membranes (Zhang and Casey, 1996) (Shao et al., 2003). Therefore, membrane association of RhoA is likely to be an important factor that does not activate ROCK but anchors it more tightly to the membrane. This anchoring would be regulated by the nucleotide state of RhoA. Functionally, this sort of membrane association is a very relevant feature since it does circumvent the necessity of a very specific ligand for the ROCK membrane binding domains. Therefore, the specificity for ROCK membrane anchorage could be simply determined by the different pools of GEFs GAPs and GDIs at different cellular membranes rather than lipid ligands.

However, membrane tethering by RhoA challenges the proposed structural model of ROCK. Assuming that RhoA binding is restricted to the RBD and does not induce conformational changes in ROCK, the position of the RhoA membrane anchor relative to the ROCK dimer can be estimated from the known crystal structure (Dvorsky et al., 2004). Although the complex of ROCK1⁹⁴⁷⁻¹⁰¹⁵ with Gpp(NH)p bound RhoA¹⁻¹⁸¹ is lacking 13 C-terminal residues including the CAAX motif which is required for lipidation of RhoA, it can be clearly seen that the RhoA C-termini project along the coiled coil toward the C-terminal membrane binding domains of ROCK (Figure 12A and 12C). In the absence of a conformational change this makes it highly unlikely that the dimeric rod shaped ROCK that is lipid anchored by RhoA could bind to membranes in a perpendicular manner, since the 100 residues of coiled coil between the RBD and the PH_N-C1-PH_C domain would not be accommodated between RhoA and the membrane. On the other hand, the two C-termini of the two RhoA molecules point into different directions relative to the plane of the coiled coil cross section (Figure 12B). Therefore it is hard to conceive how RhoA ROCK could be oriented parallel to the plane of the membrane without introducing large asymmetries into the membrane docked RhoA-ROCK-complex.

The structural model presented therefore poses clear questions about the nature of membrane binding. To address this it will be important to determine the affinity and stoichiometry of GTP bound RhoA for the full-length ROCK and to what extent does this interaction influence the overall structure of ROCK.

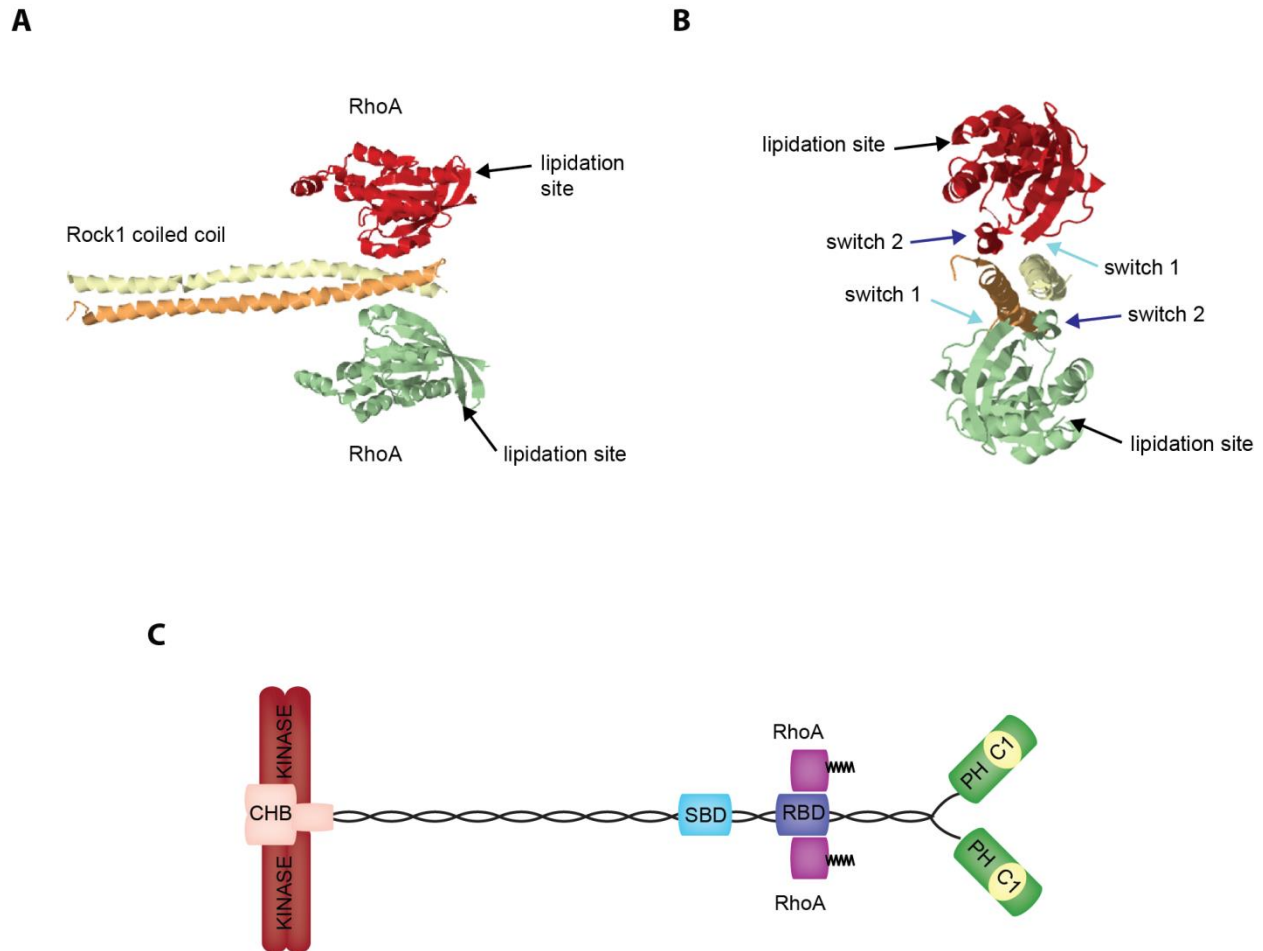


Figure 12. Membran anchoring of RhoA contradicts the structural model of ROCK

- (A) Crystal structure of ROCK1⁹⁴⁷⁻¹⁰¹⁵ with Gpp(NH)p bound RhoA¹⁻¹⁸¹ (Dvorsky et al., 2004). The C-terminus of each RhoA molecule that precedes the missing lipidation site is indicated by a black arrow.
- (B) Same as in (A) but rotated by 270 degrees in the plane of the paper. It can be well seen that both RhoA molecules interact with each chain of the coiled coil by their two switch regions (blue arrows). The C-termini lacking the lipidation sites is on top of this structure but point to opposing directions for each RhoA molecule.
- (C) Structural model including two RhoA molecules that bind to ROCK in the same way as in the crystal structure. The geranyl-geranyl lipid anchor is indicated as a zig zag line.

4.3.3 Other factors

Apart from the factors discussed so far, there may be others that regulate ROCK kinase activity *in vivo*. For instance, other proteins could activate or inhibit ROCK. Evidence for the inhibition by other proteins has been proposed in a study that showed that the regulatory region of the protein kinase Raf-1 inhibits ROCK2 activity *in vivo* (Niault et al., 2009).

A common feature of many AGC kinases such as AKT and PKC is that their catalytic activity is substantially increased by phosphorylation of two sites within the activation loop and the

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hydrophobic motif of the kinase domain (reviewed in Pearce et al., 2010). In ROCK, however, the hydrophobic motif phosphorylation site (T405 in ROCK2) has not been shown to be phosphorylated but instead is critical for dimerization of the kinase domain (Couzens et al., 2009). Phosphorylation at the activation loop (T249 for ROCK2) has not been reported either. Crystal structures of the ROCK kinase domain revealed that the unphosphorylated ROCK activation loop adopts a very similar conformation to that found in active AGC kinases. Activation loop phosphorylation is therefore appears not to be required for kinase activity (Yamaguchi et al., 2006) (Jacobs et al., 2006). This finding is exceptionally supported by the fact that related kinases such as MRCK have an unphosphorylatable valine residue at this position, but still adopt an active activation loop conformation that is similar to the ROCK activation loop (Heikkila et al., 2011).

There is evidence that scaffolding proteins regulate ROCK by controlling its subcellular localization. Proteins of the Shroom family, in particular Shroom 3 can bind to ROCK in its coiled coil region (Mohan et al., 2013). Shroom 3 is a large protein of 2000 residues, and is comprised of a PDZ domain, an actin binding SD1 domain, and a ROCK interacting SD2 region (Mohan et al., 2012). Shroom can be found in apical junctional complexes (AJC) and it has been proposed that Shroom 3 functions as a scaffold that recruits ROCK to AJCs. This view is supported by knockdown studies on chicken embryos and MDCK cells that suggests that the Shroom 3 ROCK interaction regulates MLC phosphorylation and is fundamental for neural tube closure and neuroepithelial planar remodeling, respectively (Nishimura and Takeichi, 2008).

Given the structural model presented here and the fact that there are so many different factors such as membranes, small GTPases and scaffolding proteins, which interact with ROCK, it is tempting to speculate that ROCK function is not regulated at all by kinase domain activation, but rather by subcellular localization. At first glance it might appear unlikely that a constitutively active kinase would not have severe effects on the cell. However, if ROCK was only specific enough for its substrates, no major effect on other signaling processes would be expected. On the other hand, if ROCK is only present at certain sites inside the cell, a tight regulation of kinase activity might not be necessary. Interestingly, some of the cytoskeletal substrates of ROCK such as MLC are only present at very specific locations inside the cell. For instance, the cortical actomyosin is located in very close proximity to the plasma membrane and is known to be a place where small GTPases are highly active. Finally, scaffolding proteins such as Shroom 3 might play an important role in co-localizing ROCK and its substrates at very specific sites in the

cell and thereby increasing ROCK substrate phosphorylation without any change in the intrinsic catalytic activity of the ROCK kinase domain.

This thesis has provided data that leads to a structural model of ROCK inside cells and this model contradicts the current autoinhibition model of ROCK. This thesis therefore proposes that ROCK activity is not controlled by regulating the kinase activity itself but by bringing into contact ROCK and its downstream substrates to specific subcellular locations in a temporarily controlled manner. However, whether this model is able to describe simultaneous RhoA and membrane binding remains to be tested in future experiments.

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Zusammenfassung

Das Aktin-Zytoskelett ist entscheidend für viele zelluläre Prozesse und wird kontrolliert durch kleine GTPasen der Rho Familie. Ein bekanntes Mitglied ist RhoA, welches so grundlegende Prozesse wie Zellmigration und Zellkontraktion reguliert. RhoA kontrolliert das Aktin-Zytoskelett durch verschiedene Effektoren, wobei die Rho assoziierte Kinase (ROCK) besonders bedeutend ist, weil sie sowohl direkt als auch indirekt auf Aktomyosin wirken kann. Während die Wichtigkeit der ROCK Substrate bekannt ist und ihre Folgen auf das Aktin-Zytoskelett gut beschrieben sind, weiß man nur wenig über die Regulation der katalytischen Aktivität von ROCK. Zusätzlich zur Funktion als RhoA-Effektor, kann ROCK auch an Membranen und an Gerüstproteine binden. Die gegenwärtige Auffassung ist, dass die ROCK Kinasedomäne von den regulatorischen, lipid- und RhoA-bindenden Domänen inhibiert wird. Allerdings sind die genauen molekularen Einzelheiten dieses Autoinhibitionsmechanismus' unbekannt.

Diese Masterarbeit untersucht drei verschiedene Faktoren (oligomerer Zustand, Membranbindung und RhoA-Interaktion), die entscheidend für die ROCK-Signaltransduktion sind und versucht - mit Hilfe von komplementären biophysikalischen und zellbiologischen Ansätzen - aufzugliedern - in welchem Umfang diese zur Regulation von ROCK beitragen.

Durch die Anwendung einer neuen Methode, die Fluoreszenz-Größenaußschlusschromatographie (FSEC) mit Fluoreszenz-Korrelationsspektroskopie (FCS) verknüpft, konnte die Größe/Konformation und der oligomere Zustand von nativem ROCK2 untersucht werden. Die Daten legen den Schluss nahe, dass ROCK2 ein sehr ausgedehntes und ausschließlich dimeres Protein ist. Signalmoleküle des PI3K-Signalwegs oder Arachidonsäure führen nicht zur Membrantranslokation von ROCK2, obwohl frühere Berichte die Vermutung zuließen, dass sie spezifische Lipidliganden von ROCK sein könnten. Rekombinantes RhoA wurde gereinigt und eine Methode etabliert, die es ermöglicht das RhoA gebundene Nukleotid zu bestimmen. Dies ist eine grundlegende Voraussetzung für weitere Experimente um die RhoA-ROCK Bindung zu charakterisieren.

An Hand dieser Erkenntnisse und anderen experimentellen Ergebnissen der Forschungsgruppe, kann ein Strukturmodell für ROCK abgeleitet werden. Dieses Modell trifft die Vorhersage, dass die intrinsische ROCK Kinaseaktivität nicht durch Autoinhibition reguliert wird. Diese Ergebnisse bedingen eine Revidierung des Wirkmechanismus durch den ROCK essentielle zelluläre Prozesse kontrolliert. Wir schlagen vor, dass die Aktivität von ROCK durch die zelluläre Lokalisierung von ROCK und seiner Substrate reguliert ist.

Curriculum Vitae

Research Experience

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