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Interaction of RhoD and ZIP kinase modulates actin filament assembly and focal adhesion dynamics

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ABSTRACT

RhoD is a member of the classical Rho GTPases and it has an essential role in the regulation of actin dynamics. Furthermore, RhoD also localizes to early endosomes and recycling endosomes, indicating additional roles in the regulation of endosome trafficking. A yeast two-hybrid screen identified Zipper-Interacting Protein Kinase (ZIPK) as a RhoD target. We found that RhoD interacts with ZIP kinase in a GTP dependent manner and modulates actin and focal adhesion reorganization. Interestingly, while ectopic expression of ZIPK in fibroblasts induces actin reorganization and actomyosin contraction seen as stress fiber bundling and membrane blebbing, the concomitant expression of active RhoD suppressed this phenotype. Previously, RhoD has been associated with focal adhesion regulation, and in line with this notion, we observed that ZIPK resulted in reorganization of focal adhesion and increased adhesion size. Importantly, the RhoD activity suppressed ZIPK-dependent effects on FAK activity, indicating a functional interplay between RhoD and FAK in the focal adhesion dynamics.

1. Introduction

Rho GTPases are key regulators in cellular pathways that control essential cellular functions, such as cell morphology, intracellular transport and cell migration [1]. Historically, there has been a major focus on the three classical Rho members RhoA, Rac1 and Cdc42. However, there is an increasing interest in the less studied Rho GTPases members, since there is an emerging awareness they are important signaling molecules [2–4]. We have focused on RhoD, since this Rho member harbors some unique cellular functions [5–9]. Similar to the rest of the classical Rho GTPases, RhoD binds and hydrolyses guanine nucleotides. However, in comparison to classical Rho members, RhoD has a much higher intrinsic nucleotide exchange activity [10]. This property is something RhoD shares with Rif, Wrch-1 and the tumor-associated Rac1 splice variant Rac1b [10,11]. Since the intracellular concentration of GTP exceeds the concentration of GDP by a factor of approximately 10 times, this means that RhoD is predominantly in a GTP-loaded conformation in resting cells. Till date, RhoD has not been found to be regulated by any guanine nucleotide exchange factors (GEFs) or GTPase activating proteins (GAPs), which are the conventional positive and negative regulators of Rho GTPases [2]. Intriguingly, the fact that RhoD is likely to function in a constitutively active state, suggests that its activity is under regulatory regime that is distinct from the classical GEFs and GAPs. This regime could involve regulation at the transcriptional level or by posttranslational modifications, similar to what has been found for the Rnd subfamily of Rho GTPases [12].

Several observations support the notion that the RhoD activity has a negative influence on cell migration. For instance, a study performed by Tsubakimoto et al., which employed a phagokinetic track assay, observed a decrease in cell migration in fibroblasts upon ectopically expressing the constitutively active variant RhoD/G26V [13]. Furthermore, Murphy et al., found that RhoD/G26V-expressing endothelial cells were effectively immotile, both in the presence and absence of a chemoattractant (basic fibroblast growth factor) [8]. In line with this concept, we observed that knock down of RhoD or its binding-partner WHAMM resulted in a decreased directed migration of human foreskin fibroblasts in a wound closure assay [14]. This indicated that over-activity, as well as under-activity of RhoD-dependent pathways, can affect cell migration in a negative manner. The RhoD-dependent effects on cell migration, is most likely linked to its profound effects on the organization of the actin filament system. RhoD was shown to trigger the formation of peripheral protrusions in several cell-types, including baby hamster kidney (BHK), HeLa, NIH3T3 and porcine aortic endothelial (PAE) cells [2,6]. The RhoD-related Rif also promotes the formation of long protrusions that emerge from the periphery or from the dorsal side of the cells [15]. Although, RhoD and Rif induce the same type of filopodia, there is a clear difference between the two Rho members: RhoD, but not Rif, localizes to early endosomes and has a role in endosome motility [6,8,16]. This

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clearly suggests that RhoD is a working at the interface between actin reorganization and membrane trafficking [9].

In order to gain insight into the mechanisms underlying the RhoD-dependent cellular effects, we sought to identify RhoD-binding partners that could provide clues to this regulation. The yeast two-hybrid system is a powerful tool for the study of protein: protein interactions and, using this technique, we identified Zipper-interacting protein kinase (ZIPK) as a candidate RhoD-binding protein. ZIPK, also known as death-associated protein kinase-3 (DAPK3) or DAP-like kinase (Dlk), is a member of the DAPK, serine/threonine protein kinase family, which also include DAPK, DAPK-related protein 1 (DRP-1), DAPK-related apoptosis-inducing protein kinases-1 and -2 (DRAK-1, and DRAK-2) [17–19]. Activation of these kinases is linked to death-associated cellular changes, such as membrane blebbing, cell rounding and the formation of autophagic vesicles [19]. ZIPK is considered to function as a tumor suppressor and mutations in the ZIPK gene has been found in tumors [20,21]. Interestingly, the DAPK proteins have previously not been associated with Rho GTPase signaling. In this article, we describe a novel role for RhoD in modulating ZIPK-dependent stress fibers bundling, membrane blebbing and focal adhesion dynamics.

2. Materials and methods

2.1. Antibodies and DNA work

The following antibodies were used: mouse anti-Myc (9E10) (Convance, Princeton, NJ, USA); monoclonal mouse anti-Flag (M2) (Sigma–Aldrich, St. Louis, MO, USA); rabbit anti-Myc, mouse anti-phospho-tyrosine (PY99) and rabbit anti-FAK (Santa Cruz Biotechnology, Santa Barbara, CA, USA); rabbit anti-FAK [pY397] and anti-FAK[pY576] (BioSource-Invitrogen, Carlsbad, CA, USA); TRITC-conjugated anti-mouse, and aminomethylcoumarin acetate (AMCA)-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Alexa Fluor 488-conjugated phalloidin (Molecular Probes-Invitrogen, Carlsbad, CA, USA) was used to visualize filamentous actin. The yeast two-hybrid screen has been described before [14]. FLAG-tagged cDNA constructs encoding human ZIPK were generous gifts from Tim Haystead, Duke University, Durham, NC, USA. The construction of pRK5Myc encoding the different mutants of Murine RhoD, has been described before [14].

2.2. Cell culture, transfection and immunoprecipitation

HEK293T cells and human foreskin BJ fibroblasts stably transfected with hTERT and SV40 Large T antigen (BJ/SV40 cells) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin. (HyClone, Thermo Scientific, Waltham, MA, USA). The cells were cultured at 37 °C in an atmosphere of 5% CO_2. The cells were transfected using JetPEI reagents (PolyPlus Transfection, Illkirch, France) according to the protocol provided by the manufacturer.

For the immunoprecipitation, the transiently transfected cells were lysed on ice in Triton X-100 buffer (20 mM HEPES, pH 7.5, 0.1 M NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1% aprotinin) 24 h post transfection. The lysed cells were collected in microcentrifuge tubes and centrifuged for 15 min at 4 °C. The supernatants were incubated together with the primary antibodies for 1 h, after which the immunoprecipitates were collected on protein G-Sepharose (GE Healthcare, Uppsala, Sweden) for 1 h at 4 °C. The beads were washed three times with Triton X-100 buffer and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE); subsequently they were transferred to Nitrocellulose (Hybond C, GE Healthcare, Uppsala, Sweden). Immunoblotting analyses were performed with the antibodies as specified in the figure legends, followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare, Uppsala, Sweden). The Western blots were revealed using the Luminol immunoblotting reagent (Santa Cruz Biotechnology, Carlsbad, CA, USA).

2.3. Immunocytochemistry

The BJ/SV40T cells were seeded on coverslips and transfected using JetPEI. The cells were fixed 20–24 h post transfection in 3% paraformaldehyde in phosphate buffered saline (PBS) for 25 min at 37 °C, and washed with PBS. The cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min, washed again in PBS, and then blocked in 5% FBS in PBS for 30 min at room temperature. The primary and secondary antibodies were diluted in PBS containing 5% FBS. The cells were incubated with the primary antibodies and secondary antibodies for intervals of 1 h, followed by washing in PBS. The coverslips were mounted on microscopy slides using of Fluoromount-G (Southern Biotechnology Associates), and the cells were photographed using a Zeiss AxioVert 40 CFL microscope attached to a Zeiss AxioCAM MRm digital camera, and the AxioVision software. The cellular effects induced by ectopic expression were determined by microscopy analysis. At least 100 cells were scored for each transfection condition. The statistical analyses using Student's *t*-test throughout the study were based on experiments that had been repeated at least three times. Quantification of the mean focal adhesion size was made using the ImageJ software. Fifteen randomly selected fields of view from each condition were photographed and used for the image analysis. The experiment was repeated three times.

3. Results and discussion

3.1. ZIPK is a RhoD binding partner

Previously, we performed a yeast two-hybrid system screen with the constitutively active RhoD/G26V mutant fused to the DNA-binding domain of GAL4 as the bait, to screen a human mammary gland cDNA library fused to the GAL4 activation domain. In addition to the already described binding partner FILIP1 [14], we identified ZIPK as a potential RhoD binding-protein (Fig. 1A). ZIPK consists of a kinase domain at the N-terminus, putative nuclear localization signal (NLS) motives and a leucine-zipper (LZ) type of dimerisation domain and at the C-terminus (Fig. 1A). ZIPK been shown to regulate actin dynamics, primarily through phosphorylation of the Myosin regulatory light chain [22]. Since Rho GTPases have regulatory roles in actin dynamics, these findings make ZIPK an attractive candidate to regulate cytoskeletal reorganization downstream of RhoD. We performed an immunoprecipitation assay to study the RhoD binding-capacity. We transiently transfected FLAG-tagged ZIPK together with constitutively active (G26V) and dominant negative (T31N) mutants of RhoD in HEK293T cells. We found that ZIPK binds in a GTP-dependent manner to RhoD, since interacted with the active mutant of the GTPase and not to the dominant negative variant of RhoD (Fig. 1B). Furthermore, we tested the interaction between RhoD and a kinase-inactive mutant of ZIPK (D161A) and a mutant lacking the leucine-zipper domain (ΔLZ). We found that both mutants bound RhoD but ZIPK/ΔLZ had lost the GTP-dependency of the interaction (Fig. 1C).

3.2. RhoD activity can suppress the ZIPK-induces cell contraction and stress fiber bundling

We next wanted to study the role of ZIPK- and RhoD-induced effects on the reorganization of the actin filament system. To this