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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

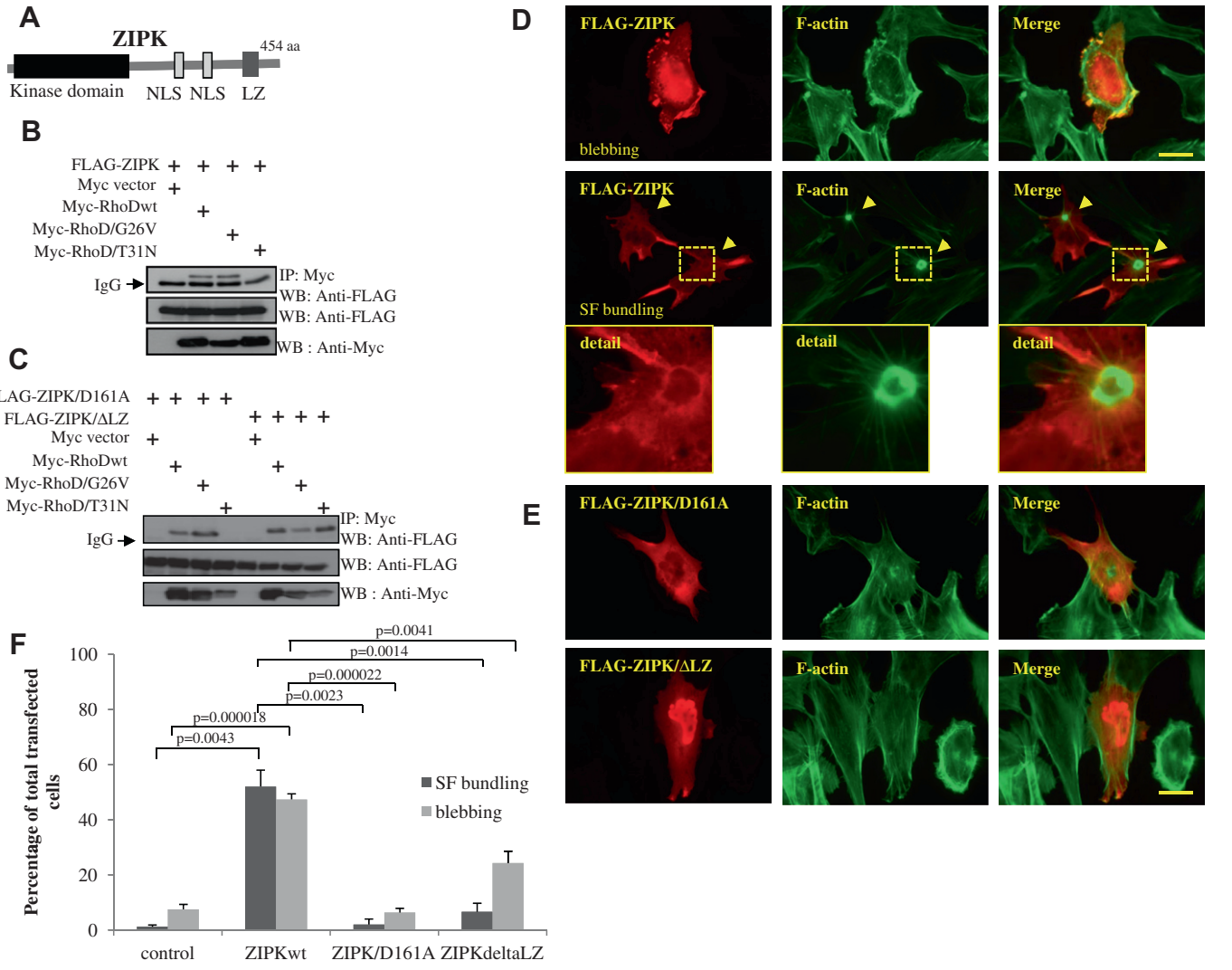

Fig. 1. ZIPK is a RhoD binding protein. (A) The domain organization of ZIPK. NLS = nuclear localization signal. (B) The interactions between constitutively active and dominant negative mutants of RhoD and ZIPK was analysed by immunoprecipitation in transiently transfected HEK293T cells. The presence of FLAG-ZIPK in the Myc precipitates was revealed by Western blotting, using anti-FLAG antibodies. (C) The interaction between the RhoD mutants and mutants of ZIPK was analysed by immunoprecipitation followed by Western Blotting to detect the ZIPK mutants in the RhoD immunoprecipitates. (D) FLAG-ZIPK transiently transfected in BJ/SV40T cells was visualized using mouse anti-FLAG antibodies followed by TRITC-conjugated anti-mouse antibodies. Filamentous actin was visualized using Alexa Fluor488-conjugated phalloidin. Scale bar, 20 μm. Magnified images show detail of the star-shaped contracted actin bundles (arrowheads). (E) FLAG-ZIPK/D161A and ZIPK/ΔLZ were transiently transfected in BJ/SV40T cells and visualized using mouse anti-FLAG antibodies followed by TRITC-conjugated anti-mouse antibodies. Filamentous actin was visualized using Alexa Fluor488-conjugated phalloidin. Scale bar, 20 μm. (F) Quantification of the cellular effects induced by ZIPK; bundling of stress fibers (SF) and membrane blebbing. At least 100 cells per experiment from three independent experiments were scored. The error bars represent standard deviation.

end, we transfected FLAG-tagged ZIPK in BJ/SV40T cells and stained the cells for filamentous actin. We found that ZIPK induced a dramatic reorganization of the actin filament system. This was seen as a condensation of stress fibers into thick bundles, often localized in a doughnut-shaped assembly with stress fiber bundles emanating from the ring in a star-like fashion (Fig. 1D quantification in F), similar to what has been reported in HeLa cells [22]. Ectopic expression of ZIPK also induced membrane blebbing, however, we rarely noticed membrane blebbing and stress fiber bundling in the same cells, instead the phenomena appeared mutually exclusive (Fig. 1D). In addition, the membrane blebbing was not associated with loss of cell adhesion. This unique actin-bundling activity was clearly dependent on the ZIPK kinase activity, since a kinase deficient mutant (ZIPK/D161) was unable to induce actin stress fiber bundling (Fig. 1E and F). In addition, the ZIPK mutant lacking the leucine zipper domain (ΔLZ) did not induce stress fiber bundling, however the membrane blebbing activity was less affected (Fig. 1F). In addition, we noticed that the wild-type and kinase-inactive variants of ZIPK were localized to the cytoplasm, in contrast to the ΔLZ mutant, which was predominantly localized in the cell nucleus (Fig. 1D and E). This indicates that the LZ domain is important for the regulation of the subcellular localization of ZIPK.

Interestingly, wild-type or the constitutively active mutant RhoD cotransfected with ZIPK effectively suppressed the ZIPK-induced stress fiber bundling (Fig. 2A and C). The constitutively active RhoD/G26V also suppressed membrane blebbing, effectively reverting the phenotype to normal fibroblast cells, however, the wild-type RhoD did not suppress the ZIPK-induced blebbing. In contrast, the dominant negative mutant RhoD/T31N was unable to suppress the ZIPK-dependent stress fiber bundling as well as membrane blebbing (Fig. 2B and C), demonstrating that the effect is dependent on the GTP-bound status of RhoD. Similarly, RhoD harboring a mutation in the membrane targeting CAAX motif of

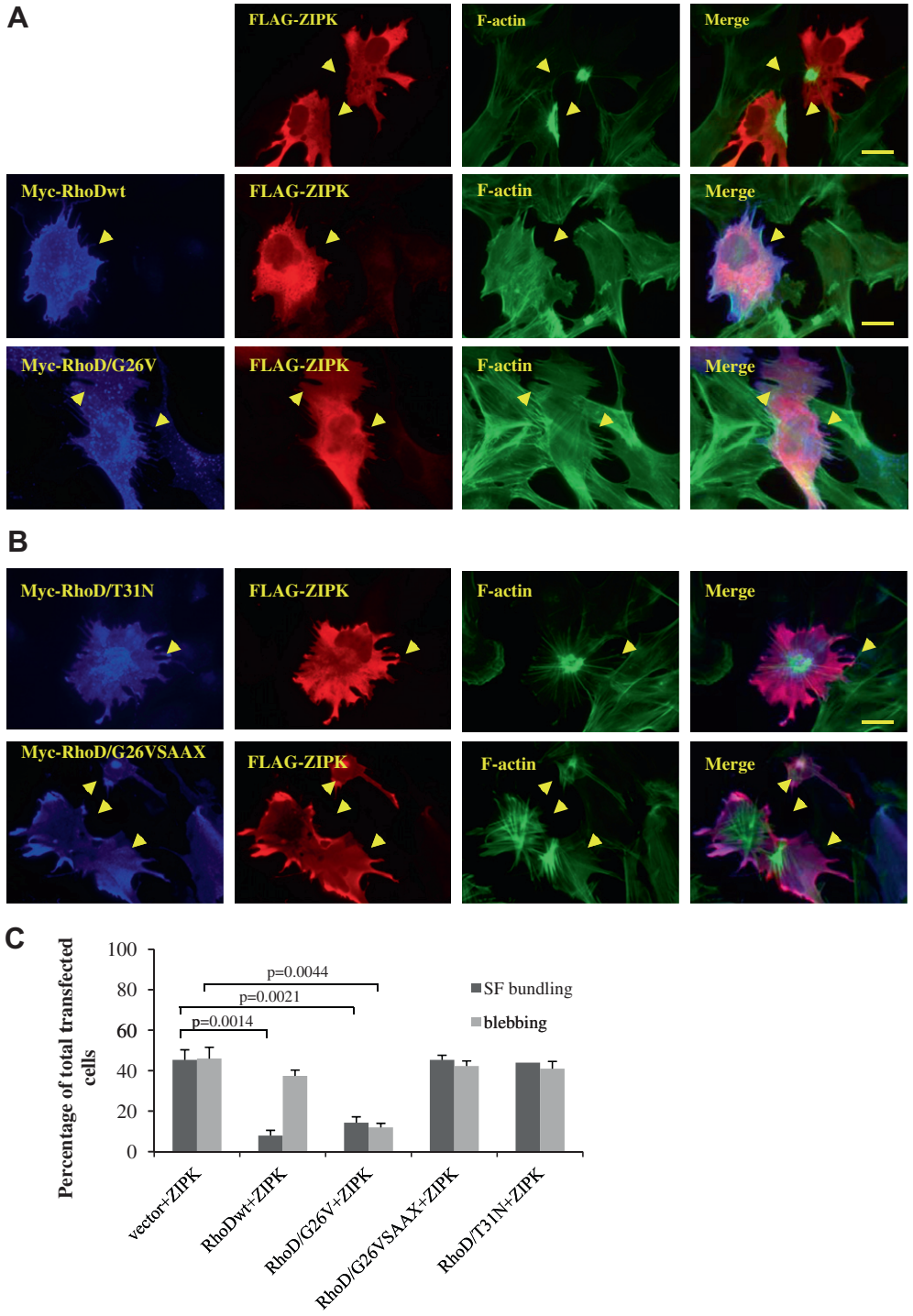

Fig. 2. RhoD suppresses the ZIPK-induced stress fiber dissolution. (A) FLAG-ZIPK was transiently transfected alone or together with RhoDwt or RhoD/G26V in BJ/SV40T cells. FLAG-ZIPK was visualized using mouse anti-FLAG antibodies followed by TRITC-conjugated anti-mouse antibodies. Myc-tagged RhoD was visualized using rabbit anti-Myc antibodies followed by AMCA-conjugated anti-rabbit antibodies. Filamentous actin was visualized using Alexa Fluor488-conjugated phalloidin. Arrowheads denote transfected cells. Scale bar, 20 μm. (B) FLAG-ZIPK was transiently transfected together with RhoD/T31N or RhoD/G26VSAAX in BJ/SV40T cells. FLAG-ZIPK was visualized using mouse anti-FLAG antibodies followed by TRITC-conjugated anti-mouse antibodies. Myc-tagged RhoD was visualized using rabbit anti-Myc antibodies followed by AMCA-conjugated anti-rabbit antibodies. Filamentous actin was visualized using Alexa Fluor488-conjugated phalloidin. Scale bar, 20 μm. Arrowheads denote transfected cells. (C) Quantification of the cellular effects induced by ZIPK; bundling of stress fibers (SF) and membrane blebbing. At least 100 cells per experiment from three independent experiments were scored. The error bars represent standard deviation.

RhoD (RhoD/SAAX) was unable to suppress the ZIPK phenotype, implicating membrane targeting of RhoD as an additional critical parameter (Fig. 2B and C). We could not find that ZIPK significantly affected the RhoD-dependent filopdia formation or RhoD localization to endosomes (Fig. 2A). ZIPK was not found in the RhoD-positive early endosomes, suggesting that ZIPK interacts with RhoD in other subcellular compartments, most likely at the plasma membrane.

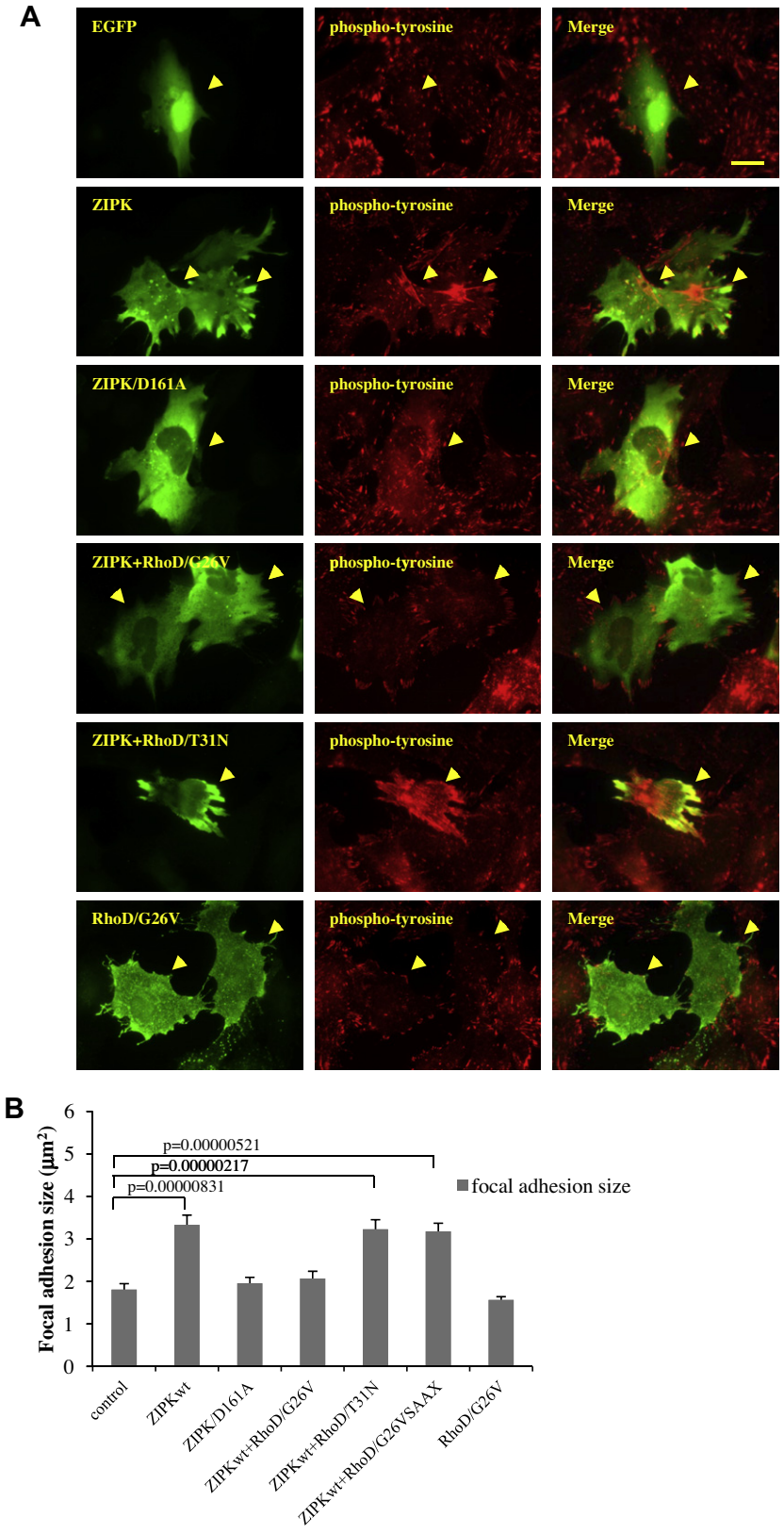

Fig. 3. ZIPK-induced focal adhesion dissolution requires an intact kinase activity. (A) EGFP (control), Myc-RhoD/G26V, FLAG-ZIPK or FLAG-ZIPK/D161A was transiently transfected in BJ/SV40T cells, or FLAG-ZIPK was transiently transfected together with Myc-RhoD/G26V or Myc-RhoD/G26DSAAX in BJ/SV40T cells. Transfected cells were visualized using rabbit anti-FLAG antibodies followed by Alexa Fluor488-conjugated anti-rabbit antibodies. Focal adhesion proteins were visualized with phospho-tyrosine-specific mouse antibodies followed by TRITC-conjugated anti-mouse antibodies. Scale bar, 20 μm. FLAG-ZIPK was visualized by rabbit anti-FLAG antibodies followed by Alexa Fluor488-conjugated anti-mouse antibodies. Focal adhesion proteins were visualized with a phospho-tyrosine-specific mouse antibody followed by TRITC-conjugated anti-mouse antibodies. Scale bar, 20 μm. (B) Quantification of the mean focal adhesion size, 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. The error bars represent standard error of the mean.

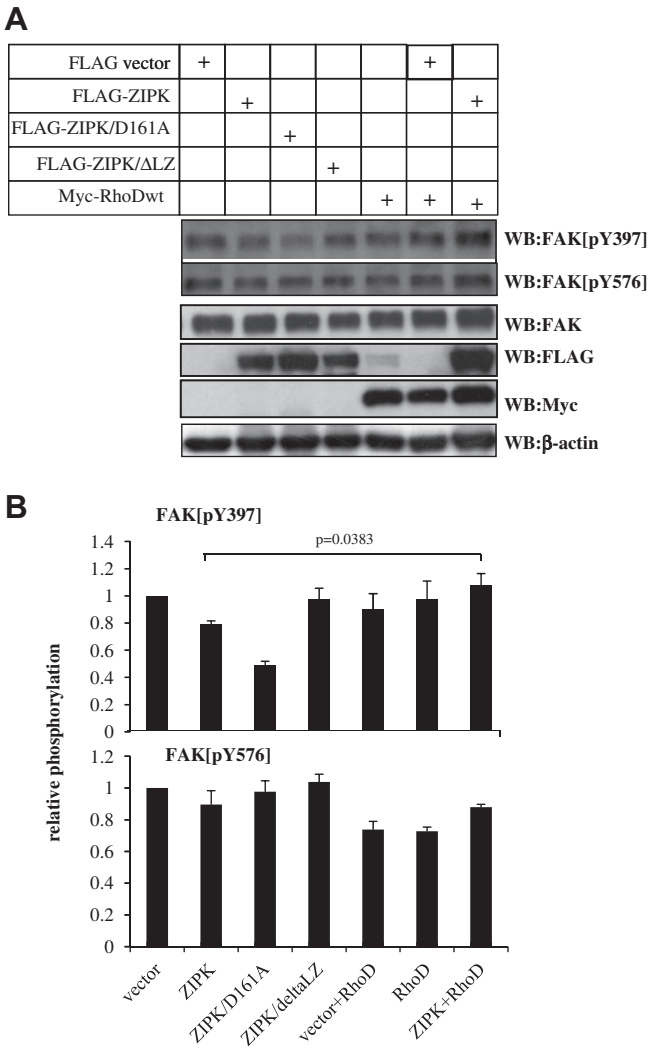

Fig. 4. ZIPK- and RhoD-dependent effects on FAK tyrosine phosphorylation. (A) The DNAs depicted in the figure were transiently transfected into BJ/SV40T cells. FAK phosphorylation at amino-acid residue Y397 or Y576 was determined by Western blotting using rabbit phosphospecific antibodies. (B) The alteration in phosphorylation relative to the vector control was determined by densitometry analysis using the ImageJ software. The data represent the mean of four experiments. The error bars represent standard error of the mean.

3.3. RhoD can modulate the ZIPK-induced focal adhesion dissolution

Since ZIPK had such a profound effect on stress fiber organization, we decided to analyze the effect on focal adhesion organization. To this end, we analyzed transiently transfected BJ/SV40T cells for focal adhesion assembly. ZIPK induced in a reorganization of focal adhesion components resulting in a dramatic increase in the focal adhesions size (from 1.8 μm^2 to 3.3 μm^2). This effect was dependent on an intact kinase activity, since the kinase inactive mutant ZIPK/D161A did not increase the focal adhesion size (Fig. 3A and B). Again, the simultaneous expression of constitutively active RhoD/G26V suppressed the ZIPK-dependent increase in focal adhesion size. This effect was dependent on the GTP-bound status and membrane targeting of RhoD, since neither the RhoD/T31N nor the RhoD/G26VSAAX mutants were unable to suppress ZIPK-induced focal adhesion size (Fig. 3A and B).

Focal adhesions dynamics is clearly associated with the activity of focal adhesion kinase (FAK). FAK is activated by integrin through disruption of an auto-inhibited conformation and FAK activation is positively correlated with the phosphorylated status of tyrosine residue 397 (Y397) [23,24]. ZIPK-expression in BJ/SV40T resulted in a marked decrease phospho-Y397; notably, the kinase deficient ZIPK/D161A also resulted in decreased phospho-Y397 (Fig. 4A and B). In contrast, the ZIPKΔLZ mutant did not result in decreased of phospho-Y397. Expression of RhoD alone, did not result in any significant alteration in Y397 phosphorylation, importantly, it suppressed the ZIPK-dependent decrease in phospho-Y397 (Fig. 4A and B). In contrast to phosphorylation on Y397, phosphorylation on tyrosine residue 576 was not significantly affected by ZIPK or RhoD expression (Fig. 4A and B).

4. Conclusions

We provide data demonstrating that RhoD interacts with ZIPK in a GTP-dependent manner and modulates stress fiber and focal adhesion reorganization. Rho GTPases have not previously been identified as binding partners for the DAPK proteins. However, there are indications that ZIPK can be regulated by Rho-dependent pathways via phosphorylation and activation by the RhoA-target Rho kinase 1 (ROCK1) [25]. Thus, our data suggest the existence of an intricate cross-talk between ZIPK activating signaling cues via RhoA/ROCK1 and inactivating signalling cues via RhoD. It will be of great future interest to study if additional DAPK members bind small GTPases, and to learn more about the signalling networks intercalating the activities of Rho GTPases and DAPK family kinases.

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