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Corresponding Author: Professor Jaw-Ji Yang, Ph.D.

Corresponding Author's Institution: Chung-Shan Medical University

First Author: Chih-Yang Huang

Order of Authors: Chih-Yang Huang; Li-Chiu Yang; Kuan-Yu Liu; Pao-Hsin Liao; Janet Ing-Yuh Chou; Ming-Yung Chou; Wei-Wen Lin; Jaw-Ji Yang, Ph.D.

Abstract: We found that overexpression of RhoGDI β , a Rho GDP dissociation inhibitor, induced hypertrophic growth and suppressed cell cycle progression in a cultured cardiomyoblast cell line. Knockdown of RhoGDI β expression by RNA interference blocked hypertrophic growth. We further demonstrated that RhoGDI β physically interacts with ZAK and is phosphorylated by ZAK in vitro, and this phosphorylation negatively regulates RhoGDI β functions. Moreover, the ZAK-RhoGDI β interaction may maintain ZAK in an inactive hypophosphorylated form. These two proteins could negatively regulate one another such that ZAK suppresses RhoGDI β functions through phosphorylation and RhoGDI β counteracts the effects of ZAK by physical interaction. Knockdown of ZAK expression in ZAK- and RhoGDI β -expressing cells by ZAK-specific RNA interference restored the full functions of RhoGDI β .

Response to Reviewers: COMMENTS FOR THE AUTHOR:

This paper describes an important link between ZAK and RhoGDI. However, several questions should be clarified before considering publication?

1, Overexpression of either RhoGDI or ZAK can increase cell size, but co-expression of these molecules decrease cell size (Figure 4). Can the author explain the potential mechanism for this weird phenomenon?

2, Is this related to localization or enzymatic activity?

Response to question 1 & 2:

This phenomenon is discussed in the discussion section page 19. One possible hypothesis is that binding of ZAK to RhoGDI β might change the localization and/or subcellular concentrations of both proteins as well as the biological functions of ZAK. Interference with the cellular activities of ZAK by RhoGDI β , as evidenced by the results of the pull-down assay, measurements of cell size, and growth of the transfected cells, might be partially explained by maintenance of the bound ZAK in a hypophosphorylated form. Comparison of the cell size of ZAK-expressing cells with ZAK- and RhoGDI β -expressing cells showed a relative decrease in cell size of 1.74- to 1.32-fold.

The binding of RhoGDI to ZAK also decrease the ZAK kinase activity. We also found that ZAK kinase activities were decrease in cells with the co-expression of ZAK and RhoGDI β .

3. Is there a figure limitation for JBS? The supplementary figure can be incorporated into Figure 1. It is not easy to directly read Figure 1. What is the mean for -100 or -200?

Response to question 3:

-100 and -200 are deletion of C-terminal 100 and 200 amino acids. Also added in the supplementary figure 1.

4. If the RhoGDI prefers binding to unphosphorylated ZAK, then changes of affinity between DN or constitutive ZAK to RhoGDI β should be observed in Fig. 1A.

Response to question 4:

The relative binding affinity was calculated and added in Figure 1. ZAKdn has higher affinity to RhoGDI.

5. Please show the RhoGDIbeta expression level in the cells overexpression of GDIbeta and knockdown.

Response to question 5:

The expression level of RhoGDI in the cells overexpression and knockdown is shown in Figure 2C.

6. How to measure cell size and growth? (in Material and Method section)

Response to question 6:

The measure cell size and growth were added in Material and Method section.

RhoGDI β -induced hypertrophic growth in H9c2 cells is negatively regulated by ZAK

Running title: ZAK, RhoGDI β , and Rac1 in cell growth and migration

Chih-Yang Huang^{1,2,3a}, Li-Chiu Yang^{4,5,6a}, Kuan-Yu Liu^{4a}, Pao-Hsin Liao^{4,6}, Janet Ing-Yuh Chou⁷, Ming-Yung Chou^{4,6}, Wei-Wen Lin⁸, and Jaw-Ji Yang^{4*}

**Graduate Institute of Chinese Medical Science,¹ Graduate Institute of Basic Medical Science,² China Medical University, Taichung 404, TAIWAN
Department of Health and Nutrition Biotechnology,³ Asia University, Taichung 413, TAIWAN
School of Dentistry,⁴ Institute of Medicine,⁵ Chung-Shan Medical University Hospital,⁶ Chung-Shan Medical University, Taichung 402, TAIWAN
Midwestern University Chicago College of Osteopathic Medicine⁷, Chicago, USA
Cardiovascular Center,⁸ Veterans General Hospital, Taichung 407, TAIWAN**

^aThese authors contributed equally to the paper.

***Correspondent Footnotes:**

**School of Dentistry
Chung-Shan Medical University, Taichung 402
TAIWAN**

Phone: 886-4-24718668 ext.55539

Fax: 886-4-24759065

E-mail: jjyang@csmu.edu.tw

Abstract

We found that overexpression of RhoGDI β , a Rho GDP dissociation inhibitor, induced hypertrophic growth and suppressed cell cycle progression in a cultured cardiomyoblast cell line. Knockdown of RhoGDI β expression by RNA interference blocked hypertrophic growth. We further demonstrated that RhoGDI β physically interacts with ZAK and is phosphorylated by ZAK *in vitro*, and this phosphorylation negatively regulates RhoGDI β functions. Moreover, the ZAK-RhoGDI β interaction may maintain ZAK in an inactive hypophosphorylated form. These two proteins could negatively regulate one another such that ZAK suppresses RhoGDI β functions through phosphorylation and RhoGDI β counteracts the effects of ZAK by physical interaction. Knockdown of ZAK expression in ZAK- and RhoGDI β -expressing cells by ZAK-specific RNA interference restored the full functions of RhoGDI β .

Key words: hypertrophic growth/ RhoGDI β /ZAK

Introduction

ZAK belongs to the mixed lineage kinases, a family of serine/threonine kinases that are classified as MAP3Ks and can activate the JNK and nuclear factor κ B (NF κ B) pathway (17). ZAK induces JNK activation through a dual phosphorylation kinase, JNKK2/MKK7 (22). To identify effectors of ZAK and to study the ZAK signaling cascade, we used a yeast two-hybrid system to isolate ZAK effectors from a human heart cDNA library. One of the isolated cDNAs encoded Rho GDP dissociation inhibitor beta (RhoGDI β). RhoGDI β , also known as Ly-GDI or D4-GDI, belongs to a family of Rho GDP dissociation inhibitors (12,20,23) that includes RhoGDI α , RhoGDI β , and RhoGDI γ (2,6) and is thought to regulate the activity and localization of Rho family proteins (5,7,9,11); however, only their ability to sequester RhoGTPases is well understood. RhoGDIs regulate RhoGTPase activity by inhibiting GDP dissociation, maintaining RhoGTPases in an inactive state. RhoGDI α is expressed in all cell types and appears to function as a molecular chaperone for GTPases, shuttling them between the cytosol and the plasma membrane (8). Therefore, the question remains as to whether RhoGDI β is actually a RhoGTPase regulator. A recent study indicated that stimulation of T lymphocytes and myelomonocytic cells with phorbol esters leads to RhoGDI β phosphorylation on serine/threonine residues

(10), raising the question of whether RhoGDI β is involved in a signal transduction pathway in these cells. Thus, details of the regulatory roles of RhoGDI β remain to be elucidated.

In the present study, we provide evidence that ZAK serves as a RhoGDI β kinase, and demonstrate the phosphorylation of RhoGDI β by ZAK *in vitro*, as well as the physical association between ZAK and RhoGDI β . We further show that this phosphorylation negatively regulates RhoGDI β -induced hypertrophic growth. Therefore, we speculate that the ZAK-RhoGDI β interaction maintains ZAK in a hypophosphorylated inactive form.

Materials and methods

Immunoprecipitation and detection of ZAK kinase activity

293T cells were transfected with the ZAK expression vector, and cell lysates were prepared in IP buffer (40 mM Tris-HCl, pH 7.5, 1% (w/v) Nonidet P-40 (NP40), 150 mM NaCl, 5 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl (PMSF), 20 mM NaF, proteinase inhibitors (1 mg/ml Leupeptin, 1 mg/ml Pepstatin A, 2.5 mg/ml Aprotinin) (Sigma), and 1 mM sodium vanadate). Cell extracts (200 µg) were incubated with 5 µl of anti-ZAK polyclonal antibody generated in our laboratory for 6 h at 4°C, mixed with 20 µl of protein-A Sepharose suspension, and incubated for an additional hour. Immunoprecipitates were collected by centrifugation and washed three times with IP buffer and twice with kinase buffer (20 mM Tris-HCl, pH7.4, 4 mM MgCl₂) alone. Protein kinase assays were carried out using a GST-RhoGDIβ fusion protein as the substrate. The GST-RhoGDIβ fusion protein was incubated for 15 min at 25°C with immunoprecipitated ZAK in the presence of kinase buffer (20 mM HEPES, pH 7.6, 1 mM EGTA, 1 mM (DTT), 2 mM MgCl₂, 2 mM MnCl₂, 5 mM NaF, 1 mM Na₃VO₄, 50 mM NaCl) and [γ -³²P]ATP. Following incubation, the phosphorylated GST-RhoGDIβ was boiled in SDS sample buffer, and the proteins

were subjected to SDS-PAGE on a 15% gel. The gel was dried, and phosphorylated RhoGDI β substrate was visualized by autoradiography.

Pull-down assay

293T cells were transfected with the pEGFPC1 ZAK mutant constructs described above. Cell lysates were pre-cleared with glutathione-Sepharose beads, followed by the addition of glutathione-Sepharose beads bound to GST-RhoGDI β and incubation with rotation for at least 2 h at 4°C in pull-down (PD) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP40, 1 mM DTT, 1 mM PMSF, and protease inhibitor mixture). The beads were washed five times with PD buffer and boiled for 5 min in 30 μ l of 5 \times concentrated Laemmli buffer. The bound proteins were then resolved by electrophoresis on 10% SDS-PAGE gels, followed by immunoblotting.

SiRNA knockdown

We used the expression vector pCDNA-HU6, a derivative of pCDNA3.1/Myc-His(-) with a human U6 promoter, for expression of short hairpin RNAs (shRNAs). Two oligonucleotides, shRNA-F (36 nucleotides) and shRNA-R (41 nucleotides), were

synthesized, each of which consisted of a 19-nucleotide stem sequence. The oligonucleotides used for shRNA were as follows: ZAK460iF (GATCCGCCTCTCGGTTCCATAACCATTTC AAGAGAA), ZAK460iR (AGCTTAAAAAGCCTCTCGGTTCCATAACCATTCTCTTGAAA), ZAK1712iF (GATCCGCCAGTGGTTAGATACTCTGATTCAAGAGAT), ZAK1712iR (AGCTTAAAAAGCCAGTGGTTAGATACTCTGATCTCTTGAAT), RhoGDI β 353iF (GATCCGGGATATTGTGTCAGGCCTGATTCAAGAGAT), RhoGDI β 353iR (AGCTTAAAAAGGGATATTGTGTCAGGCCTGATCTCTTGAAT), RhoGDI β 568iF (GATCCGCTGGGAGTGGAACCTGTCGATTCAAGAGAT), and RhoGDI β 568iR (AGCTTAAAAAGCTGGGAGTGGAACCTGTCGATCTCTTGAAT).

Stable RhoGDI β -or ZAK-expressing H9c2 cell lines were transfected with these pCDNA-HU6-shRNA plasmids and a pBABE-puro plasmid as a selection marker.

Puromycin-resistant clones were further tested for expression of ZAK or RhoGDI β after addition of doxycycline to induce expression.

Cell staining and measurement and growth.

H9c2 cells were fixed and permeabilized. Actin filaments were visualized using rhodamine-labeled phalloidin. Cells were examined and photographed using a Zeiss Axioskop and a confocal microscope. The cell size was analyzed using Image-Pro Plus software.

To determine cell growth, cells were grown in plated on 6-well dish (5×10^3 /well) with the presence of doxycycline to induce the ectopic genes. Cells were count every 24 hours by hemacytometer.

Results

Isolation of a ZAK-interacting protein, RhoGDI β

A fusion protein containing the GAL4 DNA binding domain and ZAK was used as bait in a yeast two-hybrid screen for proteins that associate with ZAK from a human heart cDNA library, as ZAK is highly expressed in heart tissue. Eighty-one clones that specifically interacted with the bait were identified. Restriction enzyme and nucleotide sequence analysis revealed that two of these clones contained a 1.3-kb cDNA with a 127–amino acid open reading frame. The cDNA was amplified by RT-PCR and found to encode the 204-residue human protein RhoGDI β .

Interaction between ZAK and RhoGDI β

To test whether ZAK and the RhoGDI β identified by the yeast two-hybrid screen interact *in vitro*, we performed pull-down assays with green fluorescent protein (GFP)-tagged ZAK and glutathione *S*-transferase (GST)-tagged RhoGDI β . We observed that GST-RhoGDI β interacted with ZAK, a dominant-negative (dn) ZAK, and a constitutively active (E/E) ZAK (Fig. 1A). We also demonstrate that RhoGDI β

binding to ZAK may be mediated through a region encompassing residues 600-700 (supplementary Fig. 1) of ZAK. Furthermore, the fragment corresponding to this region of amino acids 600-700 in ZAK (ZAK 6-7) was pulled down with GST-RhoGDI β (supplementary Fig. 2), suggesting that the binding site for RhoGDI β is between residues 600-700. Taken together, these data indicate that ZAK physically interacts with RhoGDI β through the region encompassing residues 600-700, and that this interaction is independent of the kinase activity of ZAK, as the kinase-dead mutant (ZAKdn) was pulled down by GST-RhoGDI β . We also demonstrated that the relative binding affinity of RhoGDI β to ZAKdn is higher than wild-type ZAK.

We have previously shown that ZAK forms homodimers via its intrinsic leucine zipper domain in order to activate the JNK/SAPK pathway (22). Upon dimerization, ZAK phosphorylates its dimerized partner, resulting in full activation of ZAK. The data presented here (Fig. 1B) and previously indicate that overexpression of ZAK in cells results in a shift in its mobility by SDS-PAGE as a result of autophosphorylation (22). Because ZAK might exist in both unphosphorylated and hyperphosphorylated forms *in vivo*, we tested the ability of unphosphorylated and hyperphosphorylated ZAK to bind RhoGDI β . When unbound ZAK was visualized by immunoblotting,

both the unphosphorylated and hyperphosphorylated forms were detected, but only unphosphorylated ZAK was pulled down by GST-RhoGDI β , where -100 and -200 were the positive and negative binding control, respectively (Fig. 1B). Thus, our result suggests that RhoGDI β has a higher affinity for unphosphorylated ZAK.

Given the observed physical interaction of RhoGDI β with ZAK, RhoGDI β may be a kinase substrate for ZAK. We therefore investigated the potential for RhoGDI β to be phosphorylated in the presence of ZAK. An *in vitro* kinase assay demonstrated that wild-type ZAK could phosphorylate RhoGDI β , but the dominant-negative ZAK could not. Moreover, autophosphorylation of ZAK was also detected in this assay (Fig. 1C). Our results demonstrate that RhoGDI β is able to both bind to and be phosphorylated by ZAK, suggesting that the function of RhoGDI β may be regulated by ZAK.

RhoGDI β induces hypertrophic growth in H9c2 cells

To characterize the biological effects of RhoGDI β on cardiac cells, we generated stable H9c2 cardiac cells that expressed RhoGDI β under tetracycline-responsive transactivator control. In this system, the addition of the tetracycline analog doxycycline induces the expression of RhoGDI β . Expression of RhoGDI β resulted in

a marked increase in actin fiber organization (Fig. 2A), and cell size increased by approximately 2-fold compared to control cells (Fig. 2B).

Knockdown of RhoGDI β restores cell size in RhoGDI β -expressing H9c2 cells

To confirm the role of RhoGDI β in hypertrophic growth of RhoGDI β -expressing H9c2 cells, we knocked down RhoGDI β expression in these cells by vector-based stable transfection of small interfering RNAs (siRNAs) specific for RhoGDI β . Two target sequences were used, corresponding to nucleotides 353 to 373 (U6-353i) and 568 to 588 (U6-568i). The siRNA-mediated suppression of RhoGDI β expression was stable for 60 days (data not shown). RhoGDI β knockdown cells were smaller than RhoGDI β -overexpressing cells (Fig. 2C) and had less organized actin fibers (data not shown). These results demonstrate that RhoGDI β is able to modulate hypertrophic growth in H9c2 cells, and that specific knockdown of RhoGDI β decreases the enlarged cell size induced by RhoGDI β .

To assess whether the expression of RhoGDI β in H9c2 cardiac cells could influence cell cycle progression, we first set out to determine the growth rate of RhoGDI β -expressing cells compared to parental cells. A total of 5000 cells

ectopically expressing RhoGDI β were cultured in 6-well dishes. The RhoGDI β -expressing cells grew slower than the parental cells (Fig. 3A). To determine whether the slower growth of the RhoGDI β -expressing cells was due to blocking of the cell cycle, the cell cultures were trypsinized and their cell cycle distributions were analyzed by flow cytometry. RhoGDI β -expressing cells had a higher percentage of cells in G₁ (78.77%) compared to control cells (68.50%). Moreover, fewer RhoGDI β -expressing cells were in the G₂/M and S phases (6.35% and 8.71%, respectively) compared to parental cells (14.88% and 22.78%, respectively) (Fig. 3B). These results suggest that RhoGDI β or its regulated signaling pathways might be involved in regulation of G₁ checkpoint control.

Effects of RhoGDI β on expression of cell cycle regulatory proteins

The expression levels of G₁ cyclins (cyclin D and E) are rate-limiting for cell cycle progression (15). However, by western blot analysis, neither cyclin D nor E levels were changed in the low proliferative capacity RhoGDI β -expressing cells (Fig. 3C). Because cell cycle progression commences in G₁ with assembly and activation of cyclin D-CDK4/6, followed by the subsequent activation of cyclin E-CDK2 in the G₁/S transition, we also studied the effect of RhoGDI β on G₁ cell cycle regulatory

proteins. RhoGDI β expression also had no effect on the activity of the cyclin D-CDK4/6 complex in H9c2 cells; however, the kinase activity of cyclin E-CDK2 complexes decreased in the RhoGDI β -expressing cells (Fig. 3C). Because the activities of these CDK complexes can be regulated by the cyclin-dependent kinase inhibitors (CKIs) p21^{Waf1/Cip1} and p27^{Kip1}, we measured their expression levels. We determined that the protein levels of both p21^{Waf1/Cip1} and p27^{Kip1} were increased in the RhoGDI β -expressing cells (Fig. 3C). However, the high p21^{Waf1/Cip1} expression levels did not decrease the activity of the cyclin D-CDK4/6 complex in these cells. Studies by other investigators have indicated that elevated CKI expression levels are similarly associated with vascular smooth muscle (18) and mesangial cell hypertrophy (1). Our observations suggest that the level of p21^{Waf1/Cip1}, which is regulated by RhoGDI β or its signaling pathways, might play a role in the response to hypertrophic stimuli. This role, however, appears to be dissociated from the regulation of cyclin D-CDK4/6 activity by p21. Furthermore, levels of p27^{Kip1} were elevated in RhoGDI β -expressing cells, and expression was inversely correlated to the kinase activity of cyclin E-CDK2 (Fig. 3C). These results suggest that expression of p27^{Kip1} might be associated with cell proliferation.

RhoGDI β -induced hypertrophic growth is regulated by ZAK

Given our finding that RhoGDI β proteins can associate with and be phosphorylated by ZAK, we hypothesized that the function of RhoGDI β is regulated by ZAK. We used a bi-directional tetracycline-responsive transactivator vector to regulate the expression of RhoGDI β or ZAK (wt or dn), or both RhoGDI β and ZAK (wt or dn), in H9c2 cells, and then cell sizes were measured. Consistent with previous studies (13,14), ZAK-expressing or RhoGDI β -expressing cells showed an increase in cell size of approximately 1.7- or 2.5-fold, respectively (Fig. 4A), and also underwent actin fiber reorganization (data not shown). The co-expression of both ZAK and RhoGDI β in H9c2 cells resulted in a decrease in cell size from 2.5-fold to 1.2-fold relative to the parental cells. ZAKdn-RhoGDI β cells did not have a substantially decreased cell size relative to RhoGDI β -expressing cells (Fig. 4A). These results suggest that the kinase activity of ZAK might play a negative regulatory role in the hypertrophic growth functions of RhoGDI β in cardiac cells.

Overexpression of both RhoGDI β and ZAK reverses RhoGDI β -regulated cell arrest

As the expression of RhoGDI β induced G1 arrest in H9c2 cells, but expression of ZAK had no effect on cell cycle progression and ZAK was able to regulate

RhoGDI β functions, we next studied the effects of ZAK on RhoGDI β 's regulation of cell growth rate by examining the growth rate in ZAK- and RhoGDI β -expressing cells compared with RhoGDI β -expressing cells. Cells expressing both ZAK and RhoGDI β grew substantially faster than RhoGDI β -expressing cells (Fig. 4B). Moreover, ZAKdn- and RhoGDI β -expressing cells demonstrated the same growth rate as RhoGDI β -expressing cells.

To determine the role of ZAK in RhoGDI β -induced hypertrophic growth in H9c2 cells, we compared the cell sizes of ZAK siRNA-, ZAK- and RhoGDI β -expressing cells (ZAKGRi2), scrambled ZAK siRNA-, ZAK- and RhoGDI β -expressing cells (ZAKGRCi), and ZAK- and RhoGDI β -expressing cells. The expression of ZAK siRNA, but not scrambled ZAK siRNA, in cells overexpressing ZAK and RhoGDI β increased cell size to the same degree as observed for RhoGDI β -expressing cells (Fig. 4C).

Discussion

We identified RhoGDI β as a ZAK-interacting protein in a yeast two-hybrid screen. This led us to investigate whether and how they might regulate one another's functions. RhoGDI α was first identified in bovine brain cytoplasm on the basis of its ability to inhibit GDP dissociation from RhoGTPases. RhoGDI β , the second GDI identified, shares 67% amino acid identity with RhoGDI α and is almost exclusively expressed in hematopoietic lineages (5). In contrast to RhoGDI α , RhoGDI β might bind to and inhibit GDP dissociation from purified RhoGTPases *in vitro*, and appears to be at least 10-fold less potent than RhoGDI α (21). Moreover, it has been reported that RhoGDI β can be phosphorylated on tyrosine (16). Therefore, questions arise with regard to whether RhoGDI β is an actual regulator of cellular RhoGTPases, its role in the signaling pathway, and its regulation. Upon introduction of RhoGDI β into rat cardiac H9c2 cells, the cells exhibited hypertrophic growth and a retarded cell cycle. We also demonstrated that RhoGDI β is phosphorylated by ZAK *in vitro*. It is striking that the co-expression of ZAK and RhoGDI β in H9c2 cardiac cells resulted in inhibition of the biological functions of RhoGDI β , indicating that not only does RhoGDI β potentially physically interact with ZAK, but it may also be negatively regulated by ZAK via phosphorylation. Several studies have also demonstrated that

RhoGDI β is cleaved at its N terminus during apoptosis in a caspase-dependent manner, and that the cleaved RhoGDI β is retained in the nuclear compartment (19).

This suggests that RhoGDI β could function in the nucleus.

Numerous studies have implicated RhoGDI β phosphorylation/dephosphorylation in the regulation of RhoGTPases/RhoGDI β complex dissociation and mediation of RhoGTPase activation (3,4). We found that RhoGDI β was able to associate with a mixed lineage kinase, ZAK, resulting in phosphorylation of RhoGDI β . We are currently mapping the phosphorylation sites of RhoGDI β . To further study the role of ZAK in regulating the activity of RhoGDI β , we used a bi-directional tet-on inducible system to express both ZAK and RhoGDI β in H9c2 cardiac cells. Our results demonstrate that the hypertrophic growth phenotype was inhibited by coexpression of ZAK and RhoGDI β ; however, we did not observe an inhibitory effect for the dominant-negative ZAK. Clearly, the kinase activities of ZAK are necessary for the negative regulation of RhoGDI β functions, including G1 cell cycle arrest and hypertrophic growth.

The physical interaction of ZAK and RhoGDI β results in subsequent inactivation of RhoGDI β by phosphorylation, and might require docking interactions. The

RhoGDI β docking domain is localized to the last 100 C-terminal residues of ZAK.

One possible hypothesis is that binding of ZAK to RhoGDI β might change the localization and/or subcellular concentrations of both proteins as well as the biological functions of ZAK. Interference with the cellular activities of ZAK by RhoGDI β , as evidenced by the results of the pull-down assay, measurements of cell size, and growth of the transfected cells, might be partially explained by maintenance of the bound ZAK in a hypophosphorylated form. Comparison of the cell size of ZAK-expressing cells with ZAK- and RhoGDI β -expressing cells showed a relative decrease in cell size of 1.74- to 1.32-fold. We also found that ZAK kinase activities were decrease in cells with the co-expression of ZAK and RhoGDI β (data not show). In addition, the fact that the growth rate of ZAK- and RhoGDI β -expressing cells was similar to ZAKdn-expressing cells and faster than RhoGDI β -expressing cells indicates that RhoGDI β inhibits the biological functions of ZAK.

Acknowledgments

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References

1. Abdel-Wahab N., Weston B.S., Roberts T. and Mason R.M. Connective tissue growth factor and regulation of the mesangial cell cycle: role in cellular hypertrophy. *J Am Soc Nephrol* 13(10):2437-2445, 2002.
2. DerMardirossian C. and Bokoch G.M. GDIs: central regulatory molecules in Rho GTPase activation. *Trends Cell Biol* 15(7):356-363, 2005.
3. DerMardirossian C., Rocklin G., Seo J.-Y. and Bokoch G.M. Phosphorylation of RhoGDI by Src Regulates Rho GTPase Binding and Cytosol-Membrane Cycling. *Mol. Biol. Cell* 17(11):4760-4768, 2006.
4. DerMardirossian C., Schnelzer A. and Bokoch G.M. Phosphorylation of RhoGDI by Pak1 mediates dissociation of Rac GTPase. *Mol Cell* 15(1):117-127, 2004.
5. Dovas A. and Couchman J.R. RhoGDI: multiple functions in the regulation of Rho family GTPase activities. *Biochem J* 390(Pt 1):1-9, 2005.
6. Dransart E., Olofsson B. and Cherfils J. RhoGDIs revisited: novel roles in Rho regulation. *Traffic* 6(11):957-966, 2005.
7. Faure' J. and Dagher M.-C. Interactions between Rho GTPases and Rho GDP dissociation inhibitor (Rho-GDI). *83(5):409-414*, 2001.
8. Fukumoto Y., Kaibuchi K., Hori Y., Fujioka H., Araki S., Ueda T., Kikuchi A. and Takai Y. Molecular cloning and characterization of a novel type of regulatory protein (GDI) for the rho proteins, ras p21-like small GTP-binding proteins. *Oncogene* 5(9):1321-1328, 1990.
9. Golovanov A.P., Chuang T.H., DerMardirossian C., Barsukov I., Hawkins D., Badii R., Bokoch G.M., Lian L.Y. and Roberts G.C. Structure-activity relationships in flexible protein domains: regulation of rho GTPases by RhoGDI and D4 GDI. *J Mol Biol* 305(1):121-135, 2001.
10. Gorvel J.P., Chang T.C., Boretto J., Azuma T. and Chavrier P. Differential properties of D4/LyGDI versus RhoGDI: phosphorylation and rho GTPase selectivity. *FEBS Lett* 422(2):269-273, 1998.
11. Groysman M., Hornstein I., Alcover A. and Katzav S. Vav1 and Ly-GDI Two Regulators of Rho GTPases, Function Cooperatively as Signal Transducers in T Cell Antigen Receptor-induced Pathways. *J. Biol Chem* 277(51):50121-50130, 2002.
12. Harding M.A. and Theodorescu D. RhoGDI2: a new metastasis suppressor gene: discovery and clinical translation. *Urol Oncol* 25(5):401-406, 2007.
13. Huang C.Y., Chueh P.J., Tseng C.T., Liu K.Y., Tsai H.Y., Kuo W.W., Chou M.Y. and Yang J.J. ZAK re-programs atrial natriuretic factor expression and

- induces hypertrophic growth in H9c2 cardiomyoblast cells. *Biochem Biophys Res Commun* 324(3):973-980, 2004.
14. Huang C.Y., Kuo W.W., Chueh P.J., Tseng C.T., Chou M.Y. and Yang J.J. Transforming growth factor-beta induces the expression of ANF and hypertrophic growth in cultured cardiomyoblast cells through ZAK. *Biochem Biophys Res Commun* 324(1):424-431, 2004.
 15. Hunter T. and Pines J. Cyclins and Cancer II: Cyclin D and CDK inhibitors come of age. *Cell* 79:573-582, 1994.
 16. Kasper B., Tidow N., Grothues D. and Welte K. Differential expression and regulation of GTPases (RhoA and Rac2) and GDIs (LyGDI and RhoGDI) in neutrophils from patients with severe congenital neutropenia. *Blood* 95(9):2947-2953, 2000.
 17. Liu T.-C., Huang C.-J., Chu Y.-C., Wei C.-C., Chou C.-C., Chou M.-Y., Chou C.-K. and Yang J.-J. Cloning and Expression of ZAK, a Mixed Lineage Kinase-Like Protein containing a Leucine-Zipper and a Sterile-Alpha Motif. *Biochem Biophys Res Commun* 274:811-816, 2000.
 18. Okamoto K., Kato S., Arima N., Fujii T., Morimatsu M. and Imaizumi T. Cyclin-dependent kinase inhibitor, p21Waf1, regulates vascular smooth muscle cell hypertrophy. *Hypertens Res* 27(4):283-291, 2004.
 19. Ota T., Maeda M., Sakita-Suto S., Zhou X., Murakami M., Takegami T. and Tatsuka M. RhoGDIbeta lacking the N-terminal regulatory domain suppresses metastasis by promoting anoikis in v-src-transformed cells. *Clin Exp Metastasis* 23(7-8):323-334, 2006.
 20. Ota T., Maeda M., Suto S. and Tatsuka M. LyGDI functions in cancer metastasis by anchoring Rho proteins to the cell membrane. *Mol Carcinog* 39(4):206-220, 2004.
 21. Scherle P., Behrens T. and Staudt L.M. Ly-GDI, a GDP-dissociation inhibitor of the RhoA GTP-binding protein, is expressed preferentially in lymphocytes. *Proc Natl Acad Sci U S A* 90(16):7568-7572, 1993.
 22. Yang J.-J. Mixed lineage kinase ZAK utilizing MKK7 and not MKK4 to activate the c-Jun N-terminal kinase and playing a role in the cell arrest. *Biochem Biophys Res Commun* 297:105-110, 2002.
 23. Zhang B. Rho GDP dissociation inhibitors as potential targets for anticancer treatment. *Drug Resistance Updates* 9(3):134-141, 2006.

Figure legends

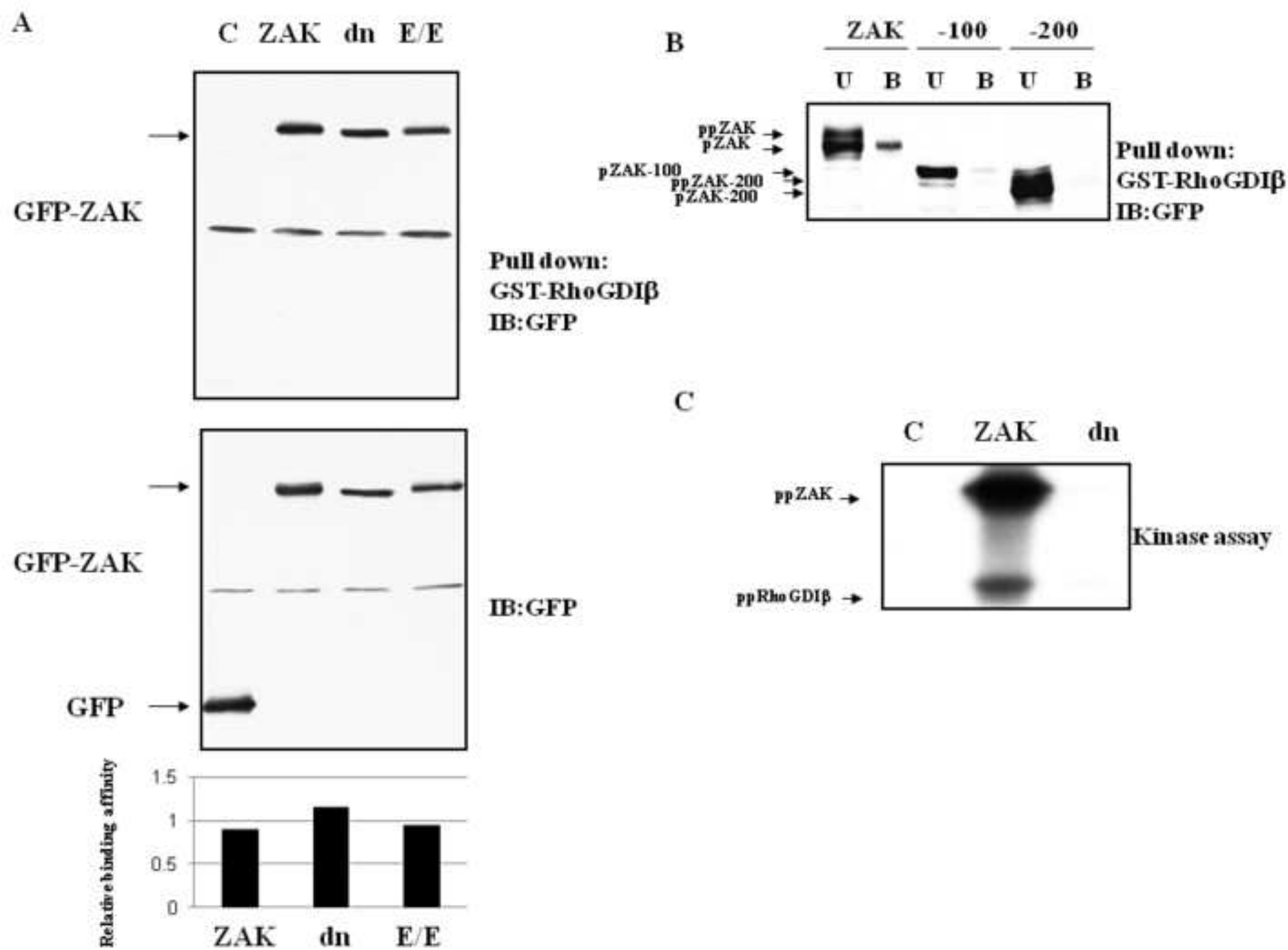
Fig. 1. Differential binding of ZAK to RhoGDI β and phosphorylation by ZAK. 293T cells were transiently transfected with the indicated GFP-tagged wild-type or mutant ZAK. After 48 h, cell lysates were harvested and 300 μ g of total lysate was used in a pull-down assay with glutathione-Sepharose bead-bound GST-RhoGDI β . (A) Wild-type ZAK (ZAK), dominant-negative (dn) ZAK, or constitutively active (E/E) ZAK. Bottom panel shows the binding affinity of RhoGDI β to different forms of ZAK. The relative binding affinity was calculated by the amount of bound form divided by the total loading of RhoGDI β . (B) Binding of RhoGDI β to ZAK may maintain ZAK in a hypophosphorylated, inactive state. Unbound (U) ZAK in cell lysates consisted of both hyperphosphorylated (ppZAK) and hypophosphorylated (pZAK) forms. The hypophosphorylated form of ZAK was pulled down by GST-RhoGDI β . (C) ZAK induces autophosphorylation and phosphorylation of GST-RhoGDI β . Cell lysates from 293T cells transfected with pEGFPC1 empty vector (C), wild-type ZAK (ZAK), or dominant-negative ZAK (dn) were immunoprecipitated with anti-GFP. The immunopurified ZAK or ZAKdn was incubated with GST-RhoGDI β in the presence of [γ - 32 P]ATP. Data shown are representative of three independent experiments.

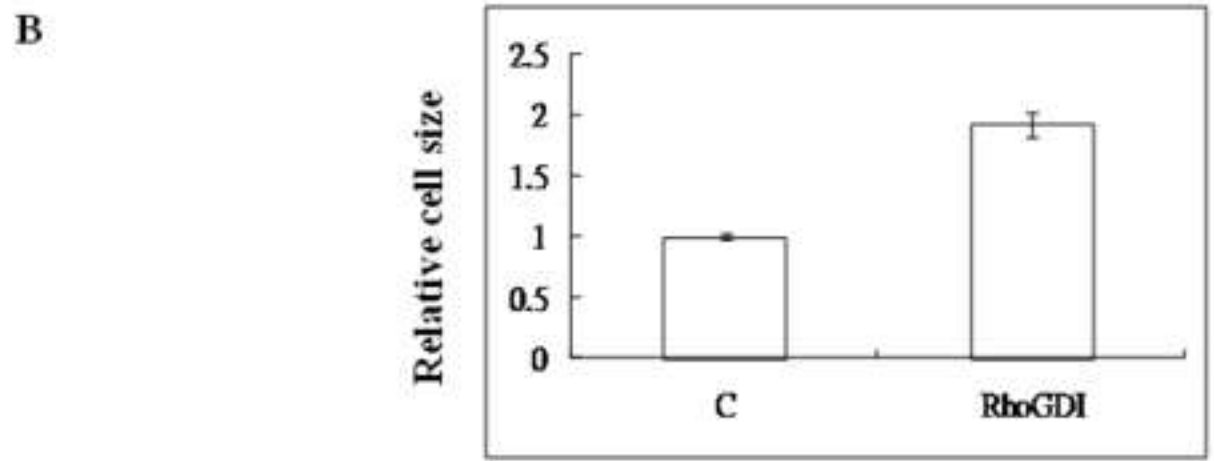
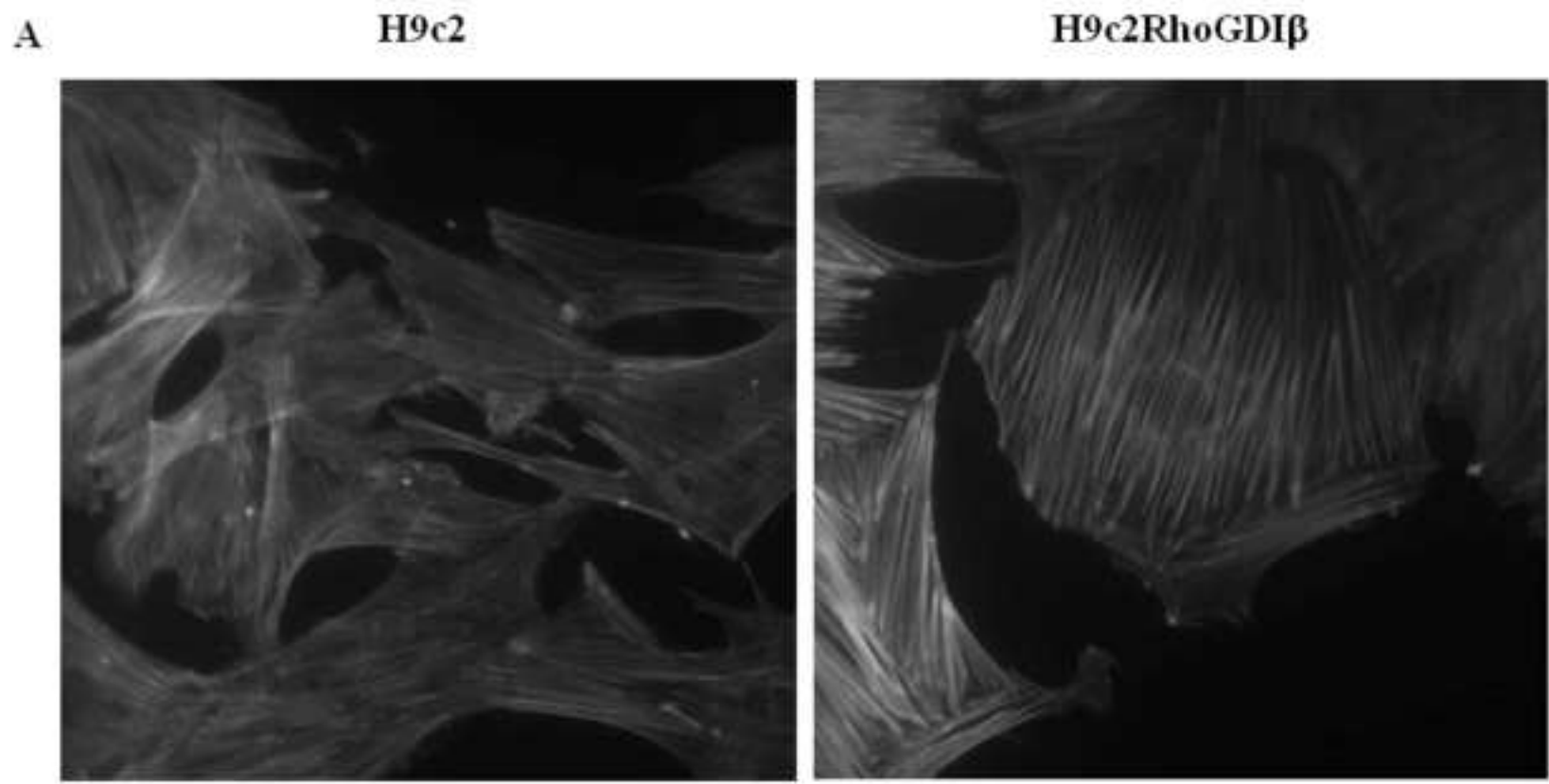
Fig. 2. RhoGDI β expression in H9c2 cells induces hypertrophic growth. (A) H9c2 cells stably transfected with empty vector (pTet) or pTet-RhoGDI β were grown in 10% fetal bovine serum with doxycycline for 3 days. The transfected H9c2 cells were fixed and immunostained with rhodamine-conjugated phalloidin. (B) Relative cell size was measured after 3 days of growth of the transfected H9c2 cells in the presence of doxycycline. (C) Quantification of the cell size of H9c2 RhoGDI β -expressing cells that also expressed RhoGDI β siRNA (U6-353i or U6-568i) or scrambled siRNA (U6-Ci).

Fig. 3. RhoGDI β expression in H9c2 cells retards cell cycle progression. (A) Growth rate of H9c2 cells stably transfected with pTet or pTet-RhoGDI β grown in 10% fetal bovine serum containing doxycycline. (B) Analysis of RhoGDI β -expressing cells by flow cytometry. RhoGDI β -expressing H9c2 cells were grown in 10% fetal bovine serum with doxycycline for 3 days, and cells were stained with propidium iodide and subjected to flow cytometry. The percentages of cells in the different cell cycle phases were calculated using ModFit software. Experiments were performed three times and gave similar results. (C) Expression and activities of various regulatory cell cycle proteins in RhoGDI β -expressing cells. Total cell extract was analyzed by western

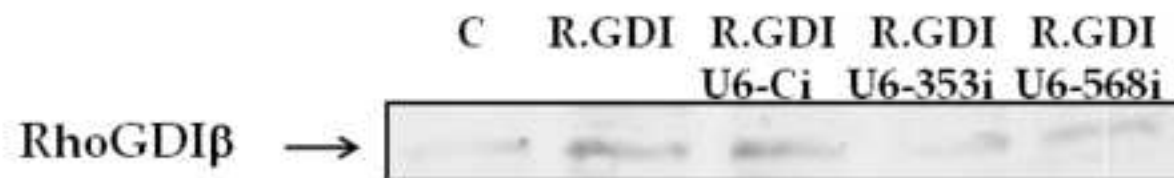
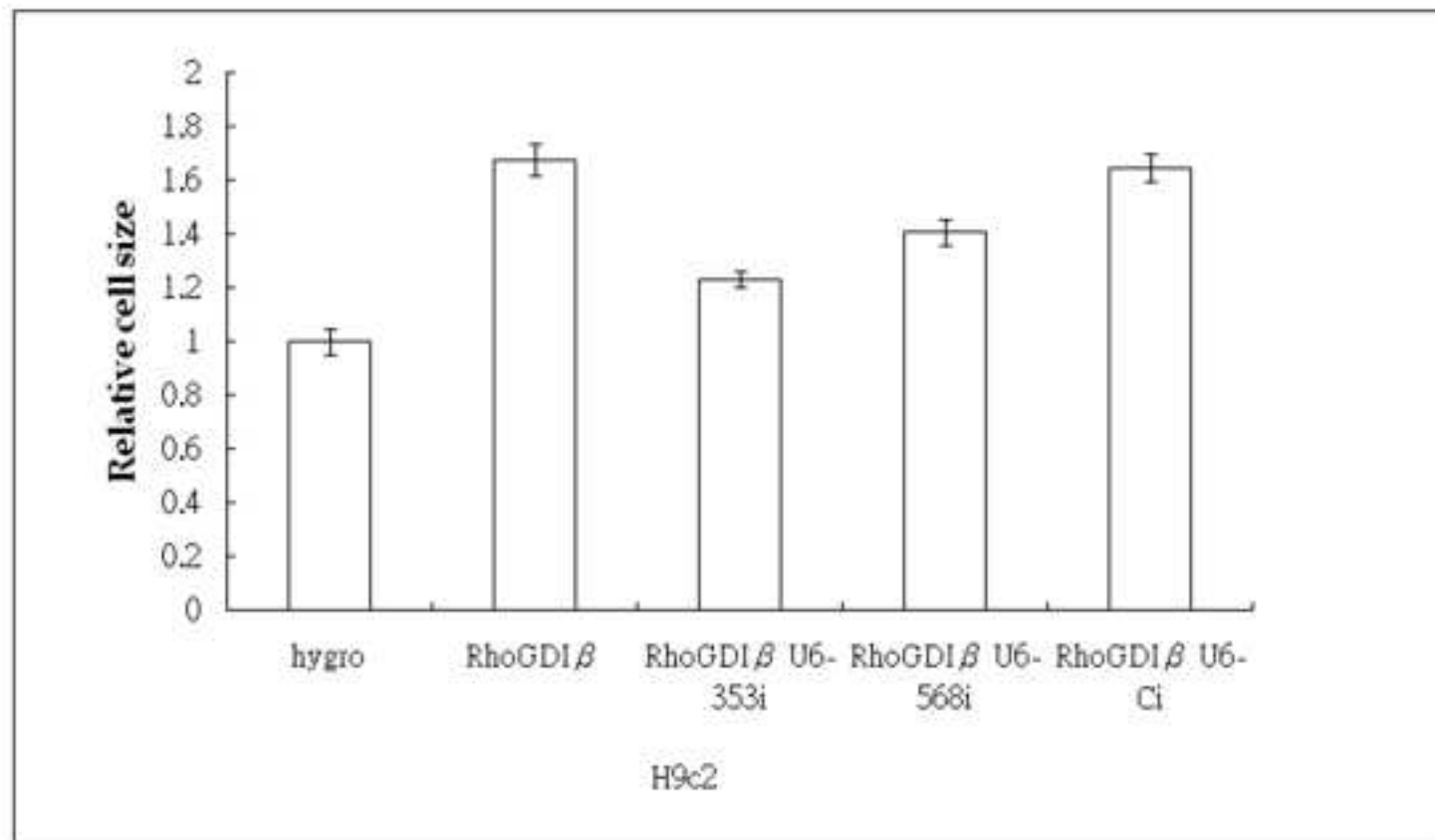
blotting and probing with the specific antibodies indicated. Cyclin D or E was immunoprecipitated from RhoGDI β -expressing cells and analyzed for associated histone H1 kinase activity. JNK activity was analyzed by immunoprecipitating JNK and assessing its activity using GST-c-jun as a substrate.

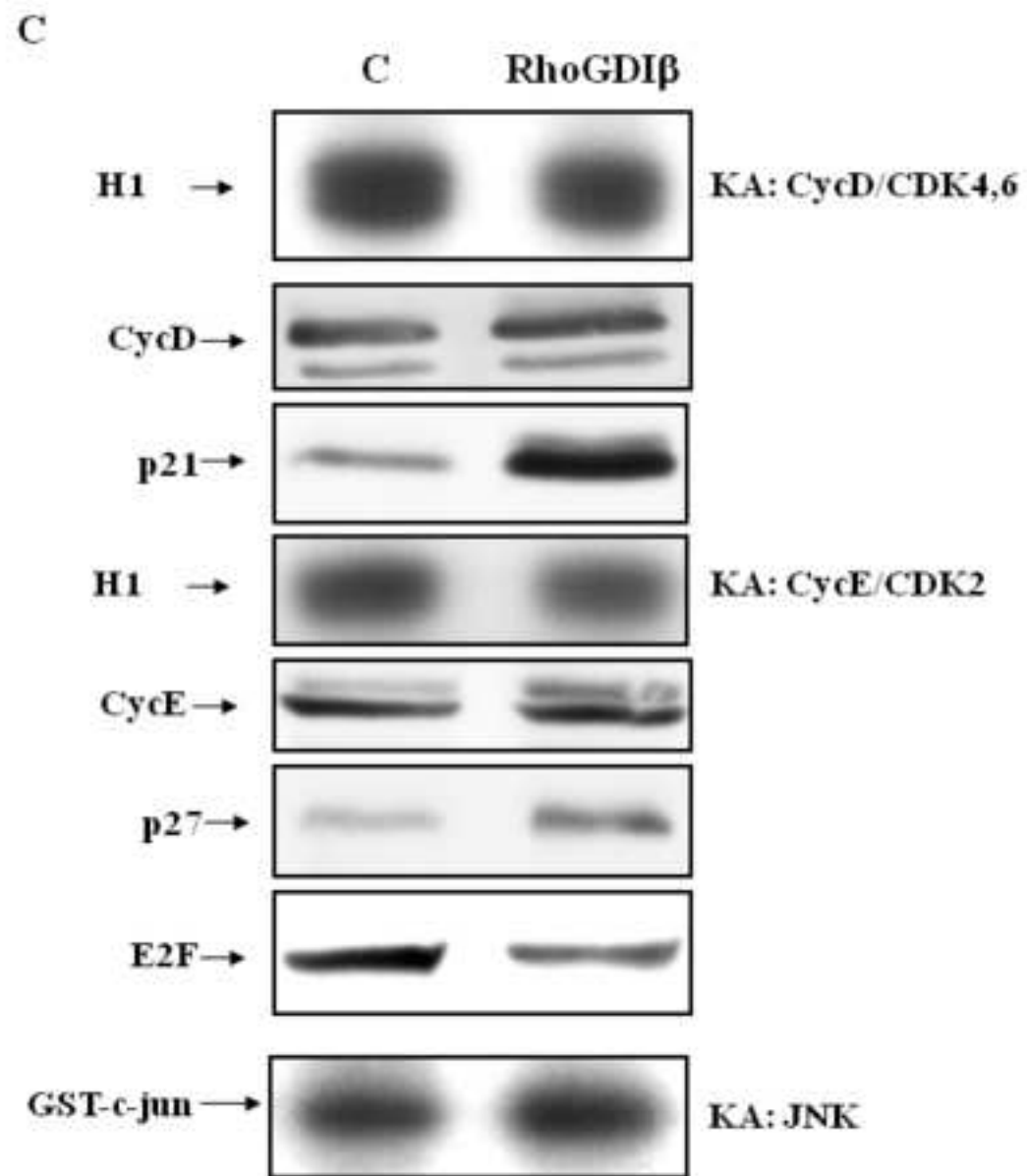
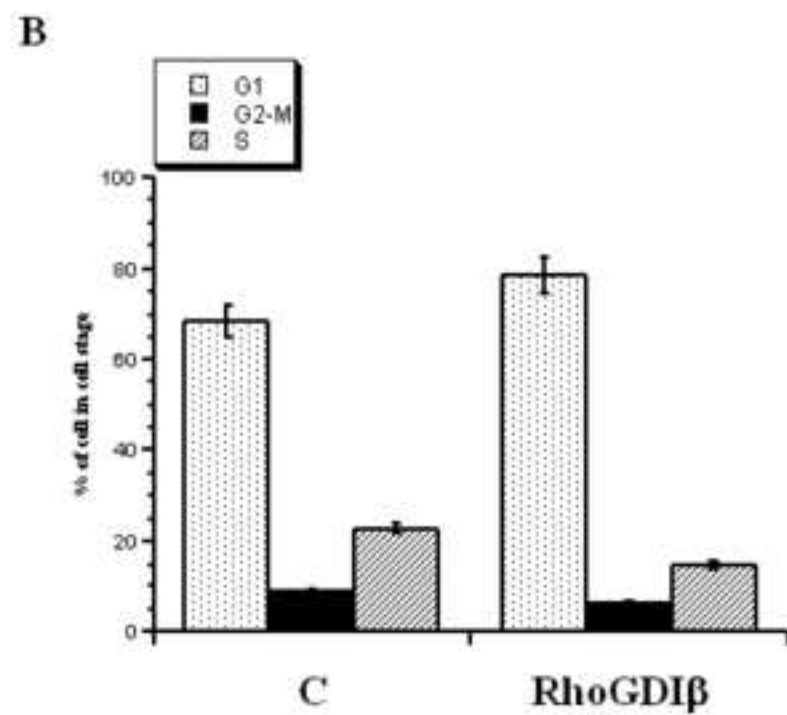
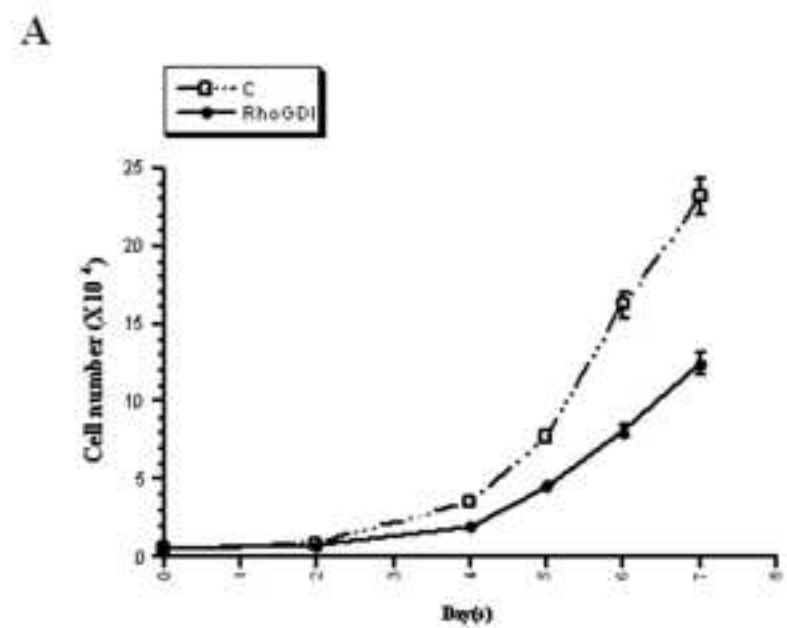
Fig. 4. ZAK reverses the effects of RhoGDI β induction of hypertrophic growth, cell migration, and suppression of cell cycle progression in H9c2 cells. (A) H9c2 cells or H9c2 cells that ectopically expressed ZAK, dominant-negative ZAK (ZAKdn), RhoGDI β , ZAK and RhoGDI β , or ZAKdn and RhoGDI β under the control of a bi-directional tetracycline response element were grown in 10% fetal bovine serum with doxycycline for 3 days, and cell size was determined. (B) The growth rate of the same cells shown in (A) grown in 10% fetal bovine serum with doxycycline. (C) SiRNA knockdown of ZAK restores the hypertrophic growth induced by RhoGDI β in ZAK- and RhoGDI β -expressing cells (ZAKGRi2).



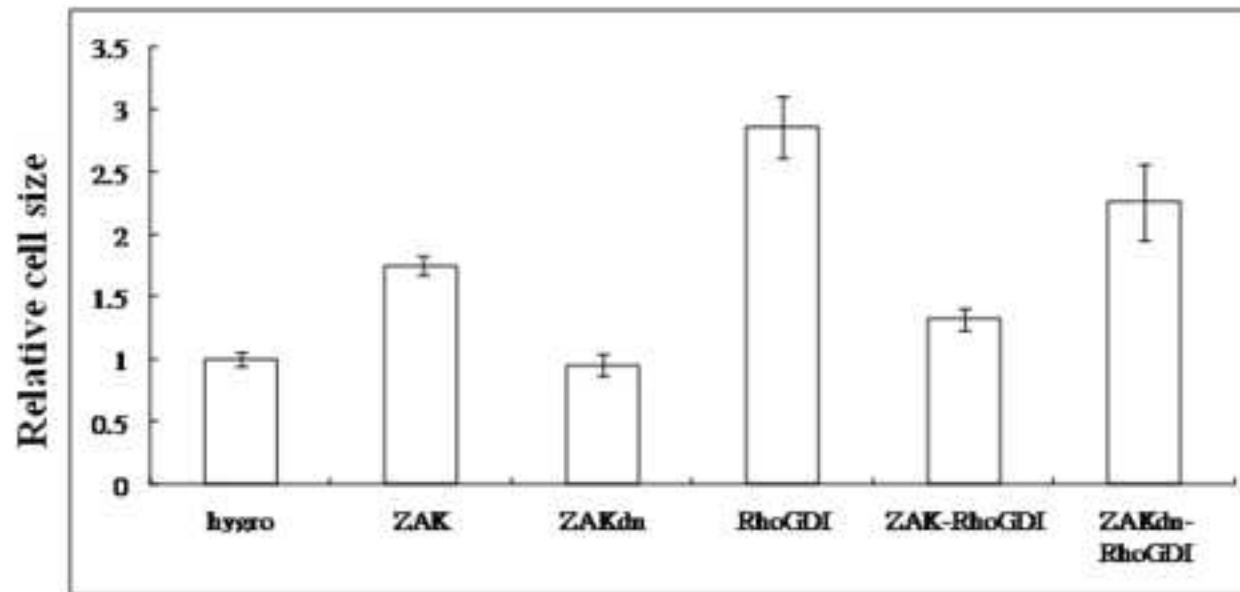


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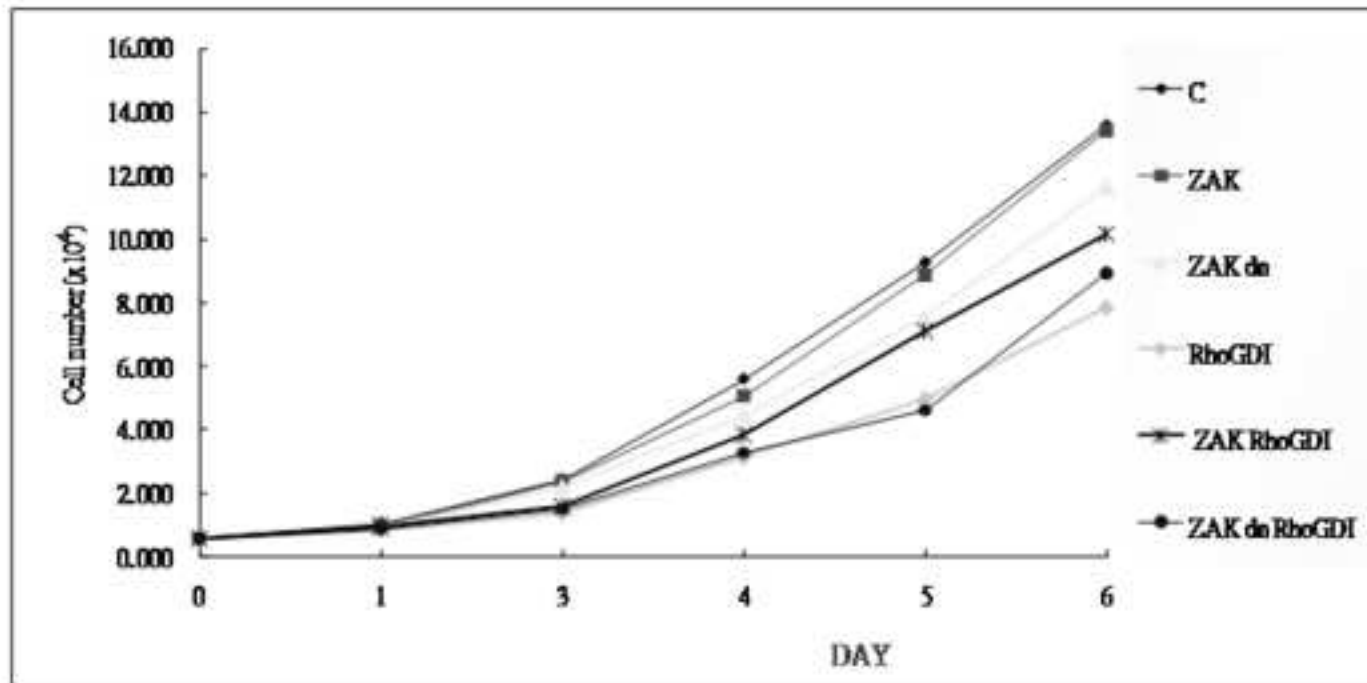




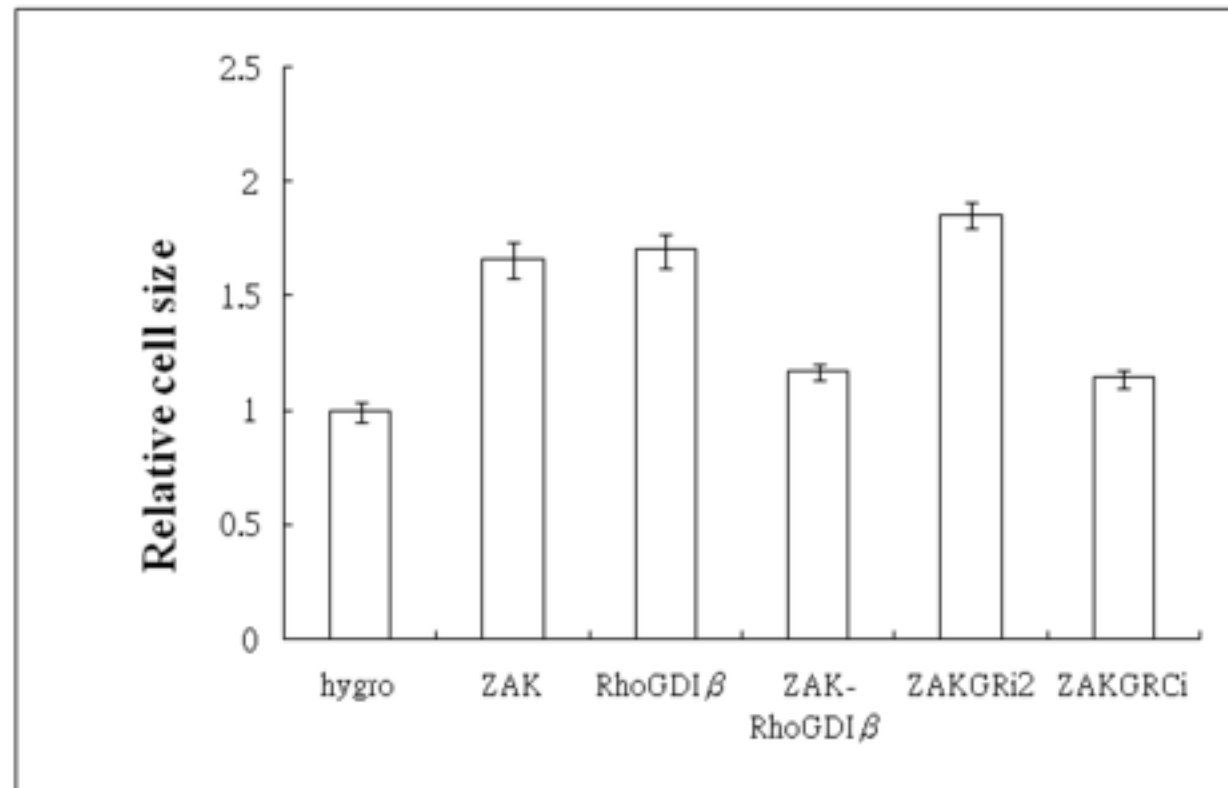
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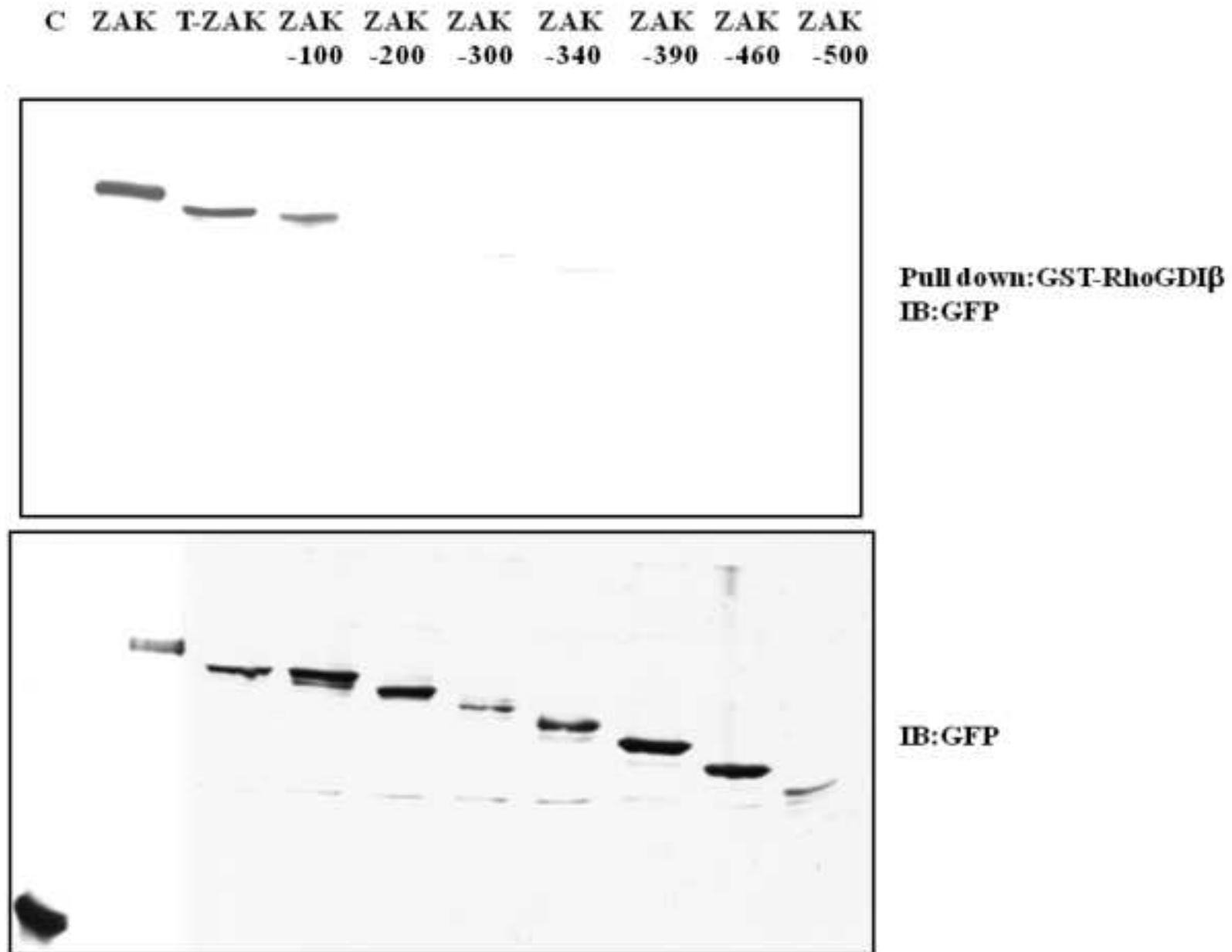


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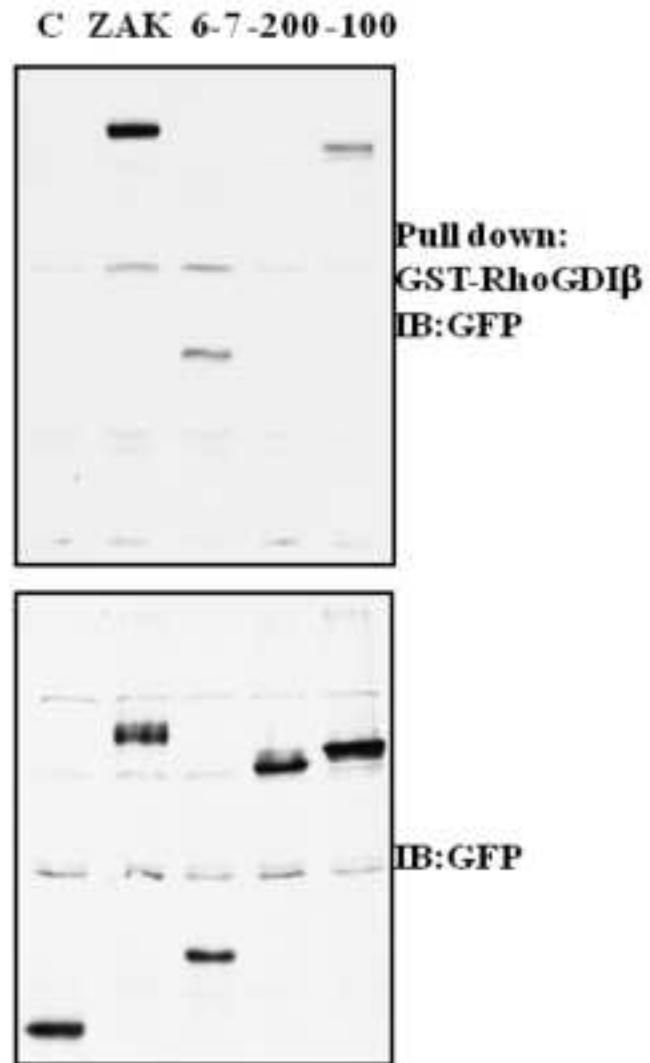


C





Supplementary Fig. 1: Binding of GST-RhoGDI β in a GST-pull down assay with ZAK-6-7. ZAK-100 (-100) is the positive control, and ZAK-200 (-200) is the negative control.



Supplementary Fig. 2: Series of different carboxyl terminal pEGFPC1-ZAK deletion mutants.