The Novel Cdc42 Guanine Nucleotide Exchange Factor, Zizimin1, Dimerizes via the Cdc42-binding CZH2 Domain*

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Rho family small GTPases are critical regulators of multiple cellular processes and activities. Dbl homology domain-containing proteins are the classical guanine nucleotide exchange factors (GEFs) responsible for activation of Rho proteins. Recently another group of mammalian Rho-GEFs was discovered that includes CDM (Ced-5, DOCK180, Myoblast city) proteins that activate Rac and zizimin1 that activates Cdc42 via a nonconventional GEF module that we named the CZH2 domain. We report here that zizimin1 dimerizes via the CZH2 domain and that dimers are the only form detected. Dimerization was mapped to a 200-amino acid region that overlaps but is distinct from the CZH2-binding sequences. Rotary shadowing electron microscopy revealed zizimin1 to be a symmetric, V-shaped molecule. Experiments with DOCK180 and homology analysis suggest that dimerization may be a general feature of CZH proteins. Deletion and mutation analysis indicated existence of individual Cdc42-binding sites in the zizimin1 monomers. Kinetic measurements demonstrated increased binding affinity of Cdc42 to zizimin1 at higher Cdc42 concentration, suggesting positive cooperativity. These features are likely to be critical for Cdc42 activation.

The Rho family of low molecular weight GTPases includes 22 human genes, the best known members being RhoA, Rac1, and Cdc42 (1). Rho proteins are important regulators of multiple cellular processes and activities. These include control of cell morphology, polarity, migration, adhesion to extracellular matrix proteins or other cells, proliferation, apoptosis, tumorigenesis, phagocytosis, vesicular transport, and gene transcription (2–4). Cdc42 mediates cell polarity, gene expression, cell cycle progression, and cell-cell contacts (5). The effects of Cdc42 are mediated by several effectors: actin polymerization and reorganization via activation of WASP and p65PAK proteins; microtubule reorganization via PAR-6, IQGAP1, and p65PAK; and vesicle trafficking via PAR-6 and potentially also the customer complex (6). It is not entirely clear how Rho proteins mediate such diverse and complex effects, but their selective targeting, activation, and inactivation probably play important roles.

Like other GTPases, Rho proteins are active when bound to GTP and inactive when bound to GDP. Conversion of the GDP-bound proteins to the active state is catalyzed by guanine nucleotide exchange factors (GEFs).1 For nucleotide exchange, the GEF first binds with low affinity to the GDP-bound protein and induces dissociation of GDP from this complex, which leads to formation of a higher affinity intermediate. This intermediate is then dissociated by binding of GTP (7). GEFs can therefore be distinguished from other GTPase-interacting proteins by their ability to bind preferentially to the nucleotide-depleted (ND) state compared with GTP- or GDP-bound states (8, 9). The classical GEFs for Rho GTPases share a common motif, designated the Dbl homology (DH) domain, that mediates nucleotide exchange (10). In mammals, over 60 DH domain-containing proteins have been identified, illustrating the need for selective activation of Rho proteins by different signaling pathways and under diverse conditions (11).

Another family of mammalian Rho-GEFs was recently discovered that includes CDM (Ced-5, DOCK180, Myoblast city) proteins that activate Rac, and zizimin1 that activates Cdc42 (9, 12–14). This family includes 11 mammalian genes, which, based on sequence homology, can be divided into a subfamily related to CDM proteins and a subfamily related to zizimin1. The two groups share two conserved domains that we named CZH1 and CZH2 for CDM-zizimin homology 1 and 2, respectively (9). We propose here to name collectively the superfamily CZH proteins. The CZH2 domain, also called DOCKER or DHR2, is a GEF domain that shows no sequence homology to DH domains. The function of the CZH1 domain, also named DHR1, remains unknown (13, 14).

Genetic evidence in worms, flies, and mice demonstrated the necessity of CDM proteins for cell migration and engulfment of apoptotic bodies (12, 15–17). DOCK180 contributes to integrin-mediated Rac activation and cell spreading and neurite outgrowth induced by nerve growth factor (18). Another member, DOCK4, has tumor suppressor activity via regulation of intercellular junctions (19). Green plants have a distinct Rho GTPase family termed ROPs but completely lack DH proteins (20). Interestingly a zizimin homolog, named SPIKE1, was identified in Arabidopsis, and it is now known to interact with multiple ROPs (21).

We report here that zizimin1 forms V-shaped dimers via a 200-amino acid region within the CZH2 domain. Individual Cdc42-binding sites most likely exist in each subunit. Analysis of binding affinity suggests positive cooperativity. These data

1 The abbreviations used are: GEF, guanine nucleotide exchange factor; ND, nucleotide-depleted; DH, Dbl homology; WCE, whole cell extract; EM, electron microscopy; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; HA, hemagglutinin; IP, immunoprecipitation; WT, wild type; GTP-γ-S, guanosine 5'-3-O-(thio)triphosphate.
2 D. Szymanski, personal communication.
provide the first insights into the biochemical properties of CZH family exchange factors.

MATERIALS AND METHODS

Cell Culture and Transfections

COS7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum penicillin and streptomycin, all from Invitrogen. 24 h before transfection, the cells were subcultured so as to reach 50–70% confluency the next day for transfection. Unless indicated otherwise, the cells were transfected in 100-mm tissue culture dishes with 1.3 g of total DNA using Effectene (Qiagen) according to the manufacturer’s instructions. For electron microscopy and BIAcore experiments, 6 × 10⁶ cells were electroporated with 20 μg of total DNA in 0.4-ml cuvettes at 250 volts and 950 microfarads. The cells were lysed in buffer with 1% Triton X-100 and immunoprecipitated (IP) with anti-FLAG antibody. Zizimin1 levels in IPs and the corresponding WCE (representing 9% of the extract used for IP) were determined by Western blotting with anti-HA antibody (top two panels) or anti-FLAG antibody (bottom two panels).

Zizimin1 Purification

Transfected cells were collected by trypsinization, washed twice in cold phosphate-buffered saline, and lysed 10 min on ice in 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1% protease inhibitor mixture (final concentrations, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 15 μM pepstatin A, 14 μM E-64, 40 μM bestatin, 20 μM leupeptin, and 0.8 μM aprotonin; Sigma P8340). The lysates were cleared by 10 min of centrifugation at 16,000 × g. A portion of this whole cell extract (WCE) was reserved, and the rest was incubated with M2 anti-FLAG affinity beads (Sigma F2426) for 3 h, on a rocker at 4 °C. For co-immunoprecipitation experiments, the beads were washed three times in lysis buffer at 4 °C, 10 min/wash and eluted in 2× SDS-PAGE loading buffer (0.125 mM Tris, pH 6.8, 4% SDS, 20% glycerol 10% β-mercaptoethanol). In the experiment presented in Fig. 1C, cell lysate and immunoprecipitation were done in RIPA buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1% protease inhibitor mixture).

When purifying zizimin1 for electron microscopy (EM), the immunoprecipitates were washed three times in lysis buffer for 30 min each time and once in 50 mM Tris, pH 7.5, 500 mM ammonium acetate for 30 min. Zizimin1 was eluted for 30 min at 4 °C in 50 mM Tris, pH 7.5, 500 mM ammonium acetate, and 0.2 mg/ml 3× FLAG peptide (Sigma F4799). When purifying zizimin1 for surface plasmon resonance experiments, the immunoprecipitates were washed three times in lysis buffer for 30 min each time and once for 30 min in 50 mM NaCl, 1% Triton X-100. Zizimin1 was eluted 30 min at 4 °C in 50 mM Tris, pH 7.5, 500 mM NaCl, 1% Triton X-100, and 0.3 mg/ml FLAG peptide (Sigma #F3290). Some of the material was analyzed by SDS-PAGE, and the rest was supplemented with 0.5 mg/ml bovine serum albumin and dialyzed into phosphate-buffered saline.

Chemical Cross-linking

Cells were collected by trypsinization, washed twice in phosphate-buffered saline, and lysed in 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% protease inhibitor mixture. After 10 min of incubation on ice, the lysates were clarified by 16,000 × g centrifugation for 10 min. 5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) dissolved in water was added for 30 min at room temperature. Control samples received equal volumes of water. The reaction was stopped by addition of 5× SDS loading buffer (0.3125 mM Tris, pH 6.8, 10% SDS, 50% glycerol, and 25% β-mercaptoethanol).

Determination of Zizimin1 Molecular Weight

Sedimentation—COS7 cells, untransfected or transiently transfected with HA-tagged zizimin1, were harvested by trypsinization and washed twice in phosphate-buffered saline. The cells were lysed...
peptide, and 50% glycerol. 200-/H9262 density (0.725 for protein), were analyzed by SDS-PAGE and an-

FLAG antibody used for IP. FLAG blots are the heavy chain of the ar-

and is displayed as in els.

ments was determined by co-immunopre-

B

B

C

ments. The positions of different fragments are indicated by arrows. The very top bands in the HA or FLAG blots are the heavy chain of the FLAG antibody used for IP. D, HA-tagged zizimin11693–1878 and zizimin11775-end were expressed in COS7 cells. Cell lysates treated with (+) or without (−) 15 mM EDC were analyzed by SDS-PAGE and anti-HA Western blotting.

10 min on ice in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 1% protein inhibitors mixture. The lysates were centrifuged for 30 min at 100,000 × g, and the supernatants were collected. 500-µl aliquots of lysates or protein standards were loaded onto 11.2 ml of 5–20% sucrose gradients in lysis buffer. After 16 h of centrifugation at 270,000 × g, fractions of ~600 µl (this value varied slightly between experiments) were collected starting at the bottom. The fractions were separated by SDS-PAGE. The protein standards gels were stained with Coomassie, and the cell lysates were analyzed by Western blotting with anti-HA or anti-zizimin1. The positions of very top bands in the HA or FLAG blots are the heavy chain of the FLAG antibody used for IP. D, HA-tagged zizimin11693–1878 and zizimin11775-end were expressed in COS7 cells. Cell lysates treated with (+) or without (−) 15 mM EDC were analyzed by SDS-PAGE and anti-HA Western blotting.

Cdc42 Pull-down Assays

The cell lysates were prepared as for the immunoprecipitation experiments (see above). Glutathione S-transferase-Cdc42 pull-down was done as described (9).

Binding of Purified Zizimin1 Dimers to Cdc42

FLAG-tagged zizimin1 constructs were immunoprecipitated as described above followed by 30 min of elution at 4 °C in 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5 mg/ml bovine serum albumin, and 0.2 mg/ml 3× FLAG peptide. The eluate was diluted and supplemented to bring the final concentration to 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5 mg/ml bovine serum albumin, and 0.06 mg/ml 3× FLAG peptide. Each eluate was divided; 4% was saved as total sample, 27% was used for anti-HA IP, and 88% was pulled down with ND-Cdc42.

Surface Plasmon Resonance

Anti-mouse IgG1 was covalently immobilized on CM5 chips, then anti-FLAG monoclonal antibody (Sigma F3165) was allowed to bind to the chips followed by binding of FLAG zizimin1, purified as described above. Bacterially expressed His6-Cdc42 was purified using TALON metal affinity resin (BD Biosciences Clontech; Palo Alto, CA) and used as an analyte. Binding kinetics were measured using a BIACore 3000 at 25 °C at a flow rate of 50 µl/min. Association was followed for 2 min, and dissociation was measured for 10 min. The running buffer was 10 mM HEPES, 7.4, 150 mM NaCl, 0.05% P20, 3 mM EDTA. To regenerate the chip, Cdc42 was dissociated from zizimin1 using 35 µl of running buffer containing 10 mM GTP, 3 mM MgCl2, and no EDTA. The data were analyzed with the 1:1 model using the BIAevalution 3.1 software.

DNA Constructs

HA-tagged zizimin1 constructs were cloned in the pEF4-Myc-His-C vector (Invitrogen) as described (9).

FLAG-tagged Zizimin1 Fragments—A linker coding for FLAG
followed by glycine (DYKDDDDKG) and a KpnI site was constructed using the oligonucleotides 5′-GCCACCATGGATTACAAGGATGACGATGACAAGGGTGGTACC-3′ and 5′-GATCGGTACCACCCTTGTCATC-GTCATCCTTGTAATCCATGGTGGCGTAC-3′. It was cloned into pEF-4-Myc-His-C using the KpnI and BamHI sites to generate pEF-FLAG-KPN. Zizimin1 fragments were amplified by PCR and cloned in pEF-FLAG-Kpn using the KpnI and NotI sites.

FLAG Full-length Zizimin1—A sequence coding for FLAG followed by two glycines (GCCACCATGGATTACAAGGATGACGATGACAAGGGTGGTACC) was added to zizimin1 before its first codon using PCR, and the FLAG-zizimin1 insert was cloned into pEF4-Myc-His-C using the KpnI and NotI sites.

Zizimin11865–1866—Zizimin1 amino acids 1865–1866 were mutated from YI to AA using the site-directed mutagenesis kit QuikChange XL (Stratagene, La Jolla, CA) and the primers 5′-GATCTGGATTCTAAGTATGCCGCGGCCCAGGTGACTCACGTCATC-3′ and 5′-GATGACGTGAGTCACCTGGGCCGCGCATACTTAGAATCCAGATC-3′. This mutation creates a SacII site. A fragment containing the mutation was subcloned into the pEF-FLAG-zizimin1 and pEF-HA-zizimin1 vectors and sequenced.

His6-Cdc42—the cDNA coding for human wild type Cdc42 was amplified by PCR using the primers 5′-TCTGGATCCGGGAGGAGGACAGACAATTAAGTGTGTTGG-3′ and 5′-AAAGAATTCTCATACACACACACTTGG-3′. The insert was cloned into pRSET-B (Invitrogen) using the BamHI and EcoRI sites. All of the constructs were confirmed by sequencing.

FLAG- and HA-tagged DOCK180 were from Dr. Kodi Ravichandran (The Beirne Carter Center for Immunology Research and the Department of Microbiology and Pharmacology, University of Virginia, Charlottesville, VA).

Antibodies
Anti-zizimin1 antibody was raised in rabbits immunized with a peptide bearing the first 12 amino acids using standard procedures. The resultant sera were affinity purified on columns conjugated to the same peptide. Anti-HA monoclonal antibody was from Covance (Princeton, NJ; MMS-101P).

RESULTS
Zizimin1 Oligomerizes—Sequence analysis of zizimin1 indicated potential coiled coil structures in the last 100 amino acids (9). These structures are often implicated in oligomerization, which prompted us to test whether zizimin1 exists as higher order complexes. We therefore transfected COS7 cells with HA-tagged zizimin1 alone or together with FLAG-tagged protein. When cell lysates were immunoprecipitated with antibody to FLAG, HA-zizimin1 was efficiently precipitated from cells co-expressing FLAG-zizimin1 but not from control cells that expressed only HA-zizimin1. These results demonstrate self-association of zizimin1 (Fig. 1B).

As an independent assay for oligomerization, lysates of cells overexpressing HA-zizimin1 were treated with the zero-length cross-linking reagent EDC that links amino and carboxylic acid...
groups when they are within close proximity (22). The samples were then analyzed by SDS-PAGE and Western blotting with anti-HA. Cross-linking induced the appearance of a single major higher molecular mass band (Fig. 1D). No higher oligomers were detected. Fibronectin and zizimin1 have similar monomer molecular weights (fibronectin, 263; zizimin1, 236), and plasma fibronectin is a disulfide-bonded dimer; thus, fibronectin runs under reduced or nonreduced conditions was used as a marker for monomers and dimers, respectively (arrows on Fig. 1D). The EDC-induced zizimin1 band had the same mobility as the fibronectin dimer, suggesting that zizimin1 forms oligomers, most likely dimers.

**Mapping the Oligomerization Domain**—To identify sequences in zizimin1 required for oligomerization, the interactions between FLAG-tagged full-length zizimin1 and HA-tagged deletion mutants were tested by co-immunoprecipitation. Deletion of the C-terminal 191 amino acids (∆1879-end) modestly reduced the association, whereas deletion of 373 amino acids (∆1696-end) nearly abolished the interaction (Fig. 1B). When lysates containing the HA-tagged C-terminal deletion constructs were treated with EDC, the more slowly migrating species was decreased for zizimin1_11693-end and further diminished with zizimin1_1696-end (Fig. 1D).

To test whether residues 1696–1878 are sufficient for oligomerization, we co-expressed FLAG and HA forms of a similar fragment (1693–1878) and performed the co-immunoprecipitation and cross-linking assays. HA-zizimin1_1693–1878 precipitated with FLAG-zizimin1_1693–1878, but not with a FLAG-tagged zizimin1 pleckstrin homology domain that was used as a negative control (Fig. 2B). EDC treatment induced a slower migrating band compatible with dimeric zizimin1_1693–1878 (Fig. 2D). These results show that zizimin1 amino acids 1696–1878 encompass the core sequence required for oligomerization, whereas amino acids 1879–2069 enhance the association. The potential coiled-coil lies at the last hundred amino acids and thus is outside the main oligomerization motif. Efforts to further map the interactions involved in dimer formation were unsuccessful because neither the 1693–1777 nor the 1775–2069 fragments showed significant dimer formation by either chemical cross-linking or co-immunoprecipitation (Fig. 2, B–D).

**DOCK180 Oligomerization**—As mentioned in the introduction, this family of GEFs can be divided into DOCK180-related and zizimin-related proteins. The amino acids from position 1696 to the end are part of the CZH2 domain, which is conserved among all family members. This homology raises the question whether the DOCK180-related proteins also oligomerize. We therefore expressed FLAG-tagged together with HA-tagged DOCK180 and assayed co-immunoprecipitation. HA-DOCK180 was detected in anti-FLAG immunoprecipitates in the presence of FLAG-DOCK180 but not in its absence (Fig. 1C). Thus, DOCK180 also self-associates, suggesting that this property is conserved throughout the CZH family.

**Zizimin1 Is a Dimer**—To determine whether zizimin1 assembles into dimers or higher oligomers, hydrodynamic properties were assayed. Lysates of nontransfected or zizimin1-overexpressing COS7 cells were analyzed by both gel filtration and sedimentation on sucrose gradients. The resultant fractions were analyzed by Western blotting to detect zizimin1. Globular protein standards were separated in parallel. These measurements showed that endogenous zizimin1 had a Stokes radius of 8 Å and a sedimentation coefficient of 12.6 S (Fig. 3). When overexpressed at moderate levels, the main peak of HA-zizimin1 had a Stokes radius of 13.6 S but showed a tail toward a higher molecular weight. Neither endogenous nor HA-zizimin1 had any protein detectable at the position expected for monomers. Calculations of the molecular weight based on these values (see “Materials and Methods”) yielded a molecular weight for zizimin1 of 480 kDa. Based on amino acid composition, the monomer mass of zizimin is 236 kDa; thus hydrodynamic measurements indicate that zizimin1 exists mainly as a dimer. However, when expressed at high levels, HA-zizimin1 ran as much higher aggregates (not shown), suggesting that higher order oligomers are possible under some conditions.

**Rotary Shadow Electron Microscopy**—To view the zizimin1 complex, the protein was overexpressed in COS7 cells at levels where it remains primarily dimeric according to hydrodynamics, purified by FLAG immunoprecipitation, rotary-shadowed, and examined by EM. Analysis by SDS-PAGE and Coomassie staining demonstrated that the protein ran as a single major band (not shown). EM images revealed symmetric, V-shaped structures with varying angles between the two arms (Fig. 4). Although the resolution of the technique does not allow a detailed analysis of protein structure, it appears that the arms may be composed of several globular domains. EM images are therefore compatible with zizimin1 being a dimer held together at the C terminus. EM images of control samples from mock-transfected cells lacked the V-shaped structures (not shown).

**Individual Cdc42-binding Sites in Zizimin1 Monomers**—The zizimin1 CZH2 domain mediates both the interaction with Cdc42 and dimerization. The relationship between dimerization and Cdc42 binding was therefore explored. Because zizimin1 appears to exist exclusively as a dimer, we tested whether monomers that contain individual Cdc42-binding sites can function independently. To obtain a zizimin1 point mutant deficient in Cdc42 binding, we mutated tyrosine 1865 and isoelucine 1866, which are conserved in all CZH family members, to alanine. A parallel mutation in DOCK180 was found to inhibit the interaction with Rac (14). The Zizimin1AA mutation almost completely suppressed Cdc42 binding (Fig. 5A). Zizimin1AA showed a modest (~3-fold) decrease in its ability to dimerize with WT protein compared with WT/WT dimers (Fig. 5B). To purify Zizimin1WT/Zizimin1AA heterodimers, HA-tagged Zizimin1WT was co-expressed with FLAG-tagged Zizimin1AA in COS7 cells. FLAG-tagged zizimin1 was isolated by FLAG immunoprecipitation and elution; this purified material included both FLAG-Zizimin1AA/HA-Zizimin1WT heterodimers and FLAG-Zizimin1AA/FLAG-Zizimin1AA homodimers. One portion of this material was used for a pull-down assay with nucleotide-depleted Cdc42,
and the other portion was immunoprecipitated with anti-HA. Bound proteins were analyzed by Western blotting with anti-HA. Under these conditions, zizimin1 WT/AA heterodimer bound Cdc42, whereas zizimin1 AA/AA did not (Fig. 5C).

When HA-Zizimin1/FLAG-Zizimin1WT dimers were prepared and analyzed in parallel, binding of the WT/AA heterodimer was about 3.5-fold less than WT/WT protein. These results demonstrate that although the affinity is reduced, zizimin1 can still bind Cdc42 when the binding site in one monomer is mutated. Thus, each monomer has its own Cdc42-binding site.

The relationship between zizimin1 dimerization and Cdc42 binding was also addressed by testing the zizimin11775–2069 construct that dimerizes very poorly as determined by cross-linking with EDC and co-immunoprecipitation (Fig. 2, C and D). Under the same conditions, a longer fragment, zizimin11693–2069, dimerized very well (compare WCE to IP signals for the two fragments; Fig. 2C). Interestingly, Cdc42 binding by these fragments showed the opposite behavior. Both fragments bound Cdc42 specifically, but zizimin11775–2069 bound nucleotide-depleted Cdc42 4.5-fold better than zizimin11693–2069 (Fig. 5D). Thus, monomeric zizimin1 is fully competent to bind Cdc42, strongly supporting the idea that each zizimin1 subunit within the dimer has a Cdc42-binding site.

Analysis of Cdc42 Binding by Surface Plasmon Resonance—To determine the affinity and kinetics of zizimin1 binding to Cdc42, the interaction was analyzed using a BIAcore system. Zizimin1 was immobilized on the chip surface, and binding of nucleotide-depleted Cdc42 in the aqueous phase was assessed by the increase in surface plasmon resonance. No binding was detected when GDP-loaded Cdc42 was used or if zizimin1 was absent, demonstrating specificity. 

On rates and off rates were measured and used to calculate the equilibrium binding constant. At concentrations of Cdc42 equal or higher than 100 nM, the average apparent equilibrium constant was $14.6 \pm 2.2$ nM and showed no significant changes over this range of Cdc42 concentrations. At 50 nM Cdc42, however, the affinity was $\sim 4$-fold weaker (Fig. 6A). This difference resulted

FIG. 5. Individual Cdc42-binding sites in zizimin1 monomers. A. Zizimin1WT or Zizimin11693–2069 (AA), both FLAG-tagged, were expressed in COS7 cells and lysates prepared. Pull-down (PD) assays were performed with ND-Cdc42. Zizimin1 in pull-downs and the corresponding WCE was detected by Western blotting with anti-FLAG. The WCE represent 10% of the amount used for PD. B. HA-Zizimin1WT was co-expressed with FLAG-Zizimin1WT or FLAG-Zizimin1AA in COS7 cells and immunoprecipitated with anti-FLAG. HA-zizimin1 in the immunoprecipitates was assayed by Western blotting with anti-HA. HA-zizimin1 bands were quantified by densitometry. The ratio in the IP compared with WCE was calculated, and the values are displayed below the HA blots. C. COS7 cells were transfected with HA-zizimin1 together with FLAG-zizimin1 or FLAG-Zizimin1AA. The lysates were immunoprecipitated with anti-FLAG and the bound proteins were eluted with FLAG peptide. The eluates, defined as total, were subsequently used for anti-HA immunoprecipitation or pull-down assays with ND-Cdc42. The HA-zizimin1 level in these fractions was determined by Western blotting and quantified by densitometry. The ratio of HA-zizimin1 in the pull-downs compared with the totals was calculated. The total samples represent 16% of the amount used for IP and 6% of the amount used for PD. D, lysates of COS7 cells expressing HA-tagged zizimin11693-end or zizimin11775-end were used in pull-down assays with ND or GTPyS-loaded Cdc42. The samples were assayed by anti-HA Western blotting and quantified by densitometry. The ratio of HA-zizimin1 in the WCE compared with ND-Cdc42 pull-down was calculated. The WCE represents 10% of the amount used for the pull-downs.

FIG. 6. Analysis of Cdc42 binding to zizimin1. Full-length zizimin1, purified from COS7 cells, was immobilized on BIAcore chips, and the binding to increasing concentrations of ND-Cdc42 was measured. The values are the means from at least six experiments ± standard error of the mean. Statistical significance of the differences obtained between the 50 nm and the higher concentrations was analyzed using paired Student's t test. A, equilibrium dissociation constant. B, on rate. C, off rate. Here $p$ was below 0.05 only for the comparison between the 50 and 100 nm concentrations.
from changes in both the on and off rates (Fig. 6, B and C). The increase in affinity at higher concentrations suggests positive cooperativity in Cdc42 binding to zizimin1. We also carried out surface plasmon resonance measurements using the zizimin1<sub>1512-end</sub> fragment that contains only the CZH2 domain. Cdc42 binding showed similar affinity and behavior at low Cdc42 concentrations (not shown), indicating that cooperativity is a property of the isolated CZH2 domain. Unfortunately, at Cdc42 concentrations much below 50 nM, the signal to noise ratio was too low to allow meaningful measurements (not shown); thus Hill coefficients to characterize the cooperativity could not be accurately determined.

**DISCUSSION**

These results demonstrate that zizimin1 dimerizes via an area in its unconventional GEF domain. Zizimin1 monomers were not detected even in cell extracts that are highly diluted compared with cytoplast, indicating that dimers form with high efficiency. To test whether dimerization affects the activity of zizimin1 in cells, we attempted to disrupt it by expressing fragments containing the dimerization domain (zizimin1<sub>1695–1878</sub> or zizimin1<sub>1663-end</sub>). Expression of these fragments, however, did not disrupt dimerization of the full-length protein. We speculate that this may result from the facts that zizimin dimers appear to be very stable and the fragments self-dimerize. Thus, the fragments and the full-length protein appear to preferentially associate with themselves.

In other systems, protein dimerization can enhance signal transduction by increasing binding affinity for membrane-binding sites, facilitating formation of larger protein complexes that can include upstream regulators, effectors, and anchoring proteins, and by increasing localized concentration. Interestingly, the DH domains of Tiam1, RasGRF1, RasGRF2, Dbs, and Dbl also form oligomers (23–26). OncoDbl monomers and oligomers have similar in vitro activity, but activation of Rho GTPases in vivo by monomers was significantly reduced, and βPIX loss of activity was correlated with mislocalization of the monomers (27).

The affinity measured for the zizimin1 interaction with ND-Cdc42 was around 15 nM (Fig. 6A). This value is comparable with the affinity of the DH protein p115-RhoGEF for ND-Rho. We are not aware of any other studies measuring the affinity of DH proteins to ND-Rho proteins.

Based on sequence similarity and domain architecture, CZH proteins can be divided to zizimin-related and DOCK180-related proteins (9, 13). The amino acid sequence homology within the dimerization domain between zizimin1 and the other zizimin-related CZH proteins is high (39–66% identity and 56–82% similarity). This result argues that dimerization is probably a general feature of the zizimin-related proteins. Sequence homology to the DOCK180-related proteins within the dimerization domain is much lower. However, structure prediction programs suggest overall similar structures for zizimin1 and DOCK180 CZH2 domains, and indeed both domains interact with Rho proteins (9, 13, 14). The result that DOCK180 self-associates suggests that this property may be conserved despite the limited homology.

The results reveal that zizimin1 dimers have two individual Cdc42-binding sites and that the affinity of zizimin1 for Cdc42 is increased at higher Cdc42 concentrations. This suggests that Cdc42 activation by zizimin1 may be cooperative. This behavior may represent a mechanism to amplify or threshold an initial signal. Further work will be required to identify upstream signals that regulate zizimin1 activity and test these concepts.

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