Zizimin2: a novel, DOCK180-related Cdc42 guanine nucleotide exchange factor expressed predominantly in lymphocytes

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Abstract A novel superfamily of guanine nucleotide exchange factors for Rho GTPases includes DOCK180 and zizimin1. The zizimin subfamily includes three genes of which only zizimin1 has been cloned. We report here the cloning of zizimin2, identified in a screen for genes enriched in germinal center B cells. Zizimin2 and zizimin1 have similar primary structures and both proteins bound and activated Cdc42 but not the Cdc42-related proteins TC10 or TCL. Their tissue distributions are distinct, however, with zizimin2 expressed predominantly in lymphocytes and an opposite pattern for zizimin1. Zizimin3 was also analyzed and showed distinct GTPase specificity and tissue distribution.

Keywords: GEF; Rho; DOCK9; DOCK10; DOCK11; CZH2

1. Introduction

During versatile adaptive immune responses, germinal centers (GCs) are formed in peripheral lymphoid tissues. GCs play central roles in differentiation of late B cells to antibody-secreting plasma cells or to antigen-specific memory B cells. The differentiation processes include immunoglobulin class-switching, somatic hypermutation and affinity maturation through B cell receptor-dependent signals by encountering the cognate antigens [1,2]. During these processes, the activated B lymphocytes migrate from the central to the marginal zone of the GC. A number of genes were recently shown by gene expression profiling techniques to alter their expression during B cell transition through GCs [3,4].

The Rho family of low molecular weight GTPases are pivotal regulators of the actin cytoskeleton and many other cellular processes [5–7]. Rho proteins include twenty-two human genes that can be divided to six or more subfamilies [8]. The three major subfamilies are the RhoA, Rac1 and Cdc42 proteins. Cdc42 mediates cell polarity, gene expression, cell cycle progression and cell–cell contacts [9,10]. In lymphocytes, Cdc42 was shown to be essential for chemokine-induced T cell migration and to regulate T cell polarization towards antigen-presenting cells [11].

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). The classical GEFs for Rho GTPases comprising a family of over sixty proteins [12], share a common motif, designated the Dbl-homology (DH) domain that mediates nucleotide exchange [13]. Additionally, a second family of GEFs that utilize an alternative Rho-GEF domain was recently discovered. Why two different domains evolved is an open question but the inflation in Rho GEFs may reflect the need for selective activation of Rho proteins by different signaling pathways and under diverse conditions.

This new family of Rho-GEFs was named CZH because it includes CDM (Ced-5, DOCK180, Myoblast city) proteins that activate Rac, and zizimin1 that activates Cdc42 [14–17]. Using information from genomic, protein and transcript databases, the family was predicted to include 11 mammalian genes, many of which have not been cloned [15,16]. Based on sequence homology, CZH proteins can be divided into one 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 [16]. The CZH2 domain, also called DOCKER or DHR2, is a GEF domain that shows no sequence homology to DH domains [14–16]. The function of the CZH1 domain, also named DHR1, remains unknown [15,16]. Based on sequence similarity, domain structure and phylogenetic analysis the zizimin-related proteins can be divided to two groups. The first, named zizimin proteins or DOCK-D, includes three proteins zizimin1/DOCK9, zizimin2/DOCK11 and zizimin3/DOCK10,

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Abbreviations: GC, germinal center; BCR, B cell receptor; GEF, guanine nucleotide exchange factor; CDM, CED-5, DOCK180, Myoblast city; CZH, CDM-zizimin homology; GFP, green fluorescence protein; PNA, peanuts agglutinin; PH, pleckstrin homology; GST, glutathione S-transferase; PAK, p21-activated kinase; PBD, p21 binding domain; PBL, peripheral blood lymphocytes

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of which only zizimin1 had been cloned. The second group, named zir for zizimin-related, or DOCK-C, includes zir1/DOCK6, zir1/DOCK7 and zir3/DOCK8 of which only DOCK8 was cloned [15,16,18].

Using subtractive hybridization, in this study we cloned zizimin2 as a gene expressed at higher levels in GC B cells compared to non-GC B cells. Zizimin2 primary structure is similar to zizimin1, exhibiting 60% identity in amino acid sequence. We show that zizimin2 and zizimin1 bind selectively to Cdc42 but not Rac Rho or the Cdc42-related proteins TC10 and TCL. Overexpression of zizimin2 activates Cdc42 in cells. Zizimin2 is expressed predominantly in lymphocytes in contrast to zizimin1 which is enriched in non-hematopoietic tissues. The GTPase specificity and tissue distribution of zizimin3 were also analyzed.

2. Materials and methods

2.1. Vectors and bacterial expression

pHis full-length (FL) zizimin2 was generated by inserting the PCR-amplified full length zizimin2 cDNA into pcDNA-4-His-max (Invitrogen) using the *Not*I site. To construct a vector for expression of green fluorescence protein (GFP) fusion proteins with additional tags (pGT), a fragment coding for myc-flag-HA tags was cloned into *Xho*I/*Eco*RI site of the pEGFP-C1 vector (Clontech). Zizimin2 (669-E), Zizimin2 (1516-E) and Zizimin1 (1512-E) were amplified by PCR and cloned into pGT. pEF-oncoVav1 was described elsewhere [19]. HA-tagged human zizimin1 CZH2 (Zizimin1 1512-E) was described before [16]. For HA-tagged human zizimin2 CZH2 construction, EST Genbank accession number H15608 was amplified by PCR using the primers 5′-ATA GGT ACC ACC AAA AGG AAA ACC TTT TTG A-3′ (sense) and 5′-AAA GAA TTC TCA CAC TTC AGC GTA TCT TGG G-3′ (antisense), the product was digested with *Kpn*I and *Eco*RI and cloned into *Kpn*I/*Eco*RI site of pEF4-HA-Kpn [16]. For HA-tagged human zizimin3 CZH2, EST Genbank accession number BM905401 was amplified by PCR using the primers 5′-ATA GGT ACC AAC AAG CAG AAG TCA ATT GTC CG-3′ (sense) and 5′-AAG GAT CCT CAG ACT TCA GCA CTA GAT GAG-3′ (antisense). The product was digested with *Kpn*I and *Bam*HI and cloned into appropriate sites of the modified pEF4-HA-Kpn vector which possessed an additional *Bam*HI site just prior to *Sfu*I site. Glutathione S-transferase (GST)-TC10 in the bacterial expression vector pGEX2T was expressed and purified as described [20]. For GST-TCL protein, human TCL (Genbank Accession No. AJ276567) in pcDNA3.1, obtained from UMR cDNA resource center at the university of Missouri-Rolla (Rolla, MO), was subcloned into pGEX4T1 using *Eco*RI/*Xho*I sites, expressed and purified as described for TC10 [20].

2.2. Cell culture and gene introduction

BAF0/3 cells were maintained as described previously [21]. A20, EL-4, WEHI-231 cell lines and mouse primary splenic cells were cultured in RPMI 1640 with 50 μM 2-mercaptoethanol. Other cell lines including COS-1 and COS-7 cells were cultured in DMEM. All culture media was supplemented with 10% FCS, 50 units/ml penicillin and 50 μg/ml streptomycin. Transient transfection of COS-1 cells was performed with Fugene6 reagent (Roche Diagnostics). COS-7 cells were transfected using the Effectene Reagent (Qiagen). Cells were harvested at 24–48 h post transfection.

2.3. Generation of subtracted cDNA library

For GC and non-GC B cells preparation, C57bl/6J mice were immunized with 4-hydroxy-3-nitrophenyl acetyl coupled to chicken globulin (NP-CG) and single splenocyte suspensions were prepared from spleens 7 days after priming. $CD19^+$ B cells were isolated by auto MACS (Miltenyi Biotec) by incubation with FITC-conjugated anti-CD19 antibody and anti-FITC magnetic beads, followed by removal of the beads by anti-FITC isolation kit. The isolated $CD19^+$ cells were found to be positive for B220 as well by FACS analysis (Becton Dickinson). The cells were then incubated with biotinylated peanut agglutinin (PNA; Vector Laboratories), followed by streptavidin magnetic beads. PNA^{high} or PNA^{low} B cells were obtained by auto MACS sorting. Total RNA preparation, cDNA synthesis and PCR-select cDNA subtraction were carried out as described previously [22], the PNA^{high} B cells cDNA was used as tester and the PNA^{low} B cells cDNA was used as driver.

2.4. Cloning of zizimin2

Sequences obtained from the subtracted library were used for Genbank searches. A 99 bp clone completely matched sequences of the putative zizimin2/DOCK11 protein. The hypothetical FL cDNA sequence of zizimin2 was generated by combining sequences of overlapping Genbank expressed sequence tag (EST) clones. To obtain the authentic sequence of zizinin2 as expressed in B cells, primers from the putative beginning and end of zizimin2 open reading frame (5′-GAA AGT GCG CAA ATT CAC C-3′ and 5′-TCA TAC TTC CGC GTA TCT TG-3′) were designed and PCR was performed using murine splenic B cells cDNA as template. Consequently, a single product 6.2 kb long was amplified. Three independently amplified clones were sequenced to be verified. Finally the untranslated 5′ region of Zizimin2 was determined using the GeneRacer rapid amplification of cDNA end (RACE) kit (Invitrogen) with the following gene specific primers, 5′-TGG CAA CAT CC GAA AGT CCC CAG A-3′ and 5′-TGG GCC CTC TTT TCA CAT CCT CTG G-3′. The sequence of zizimin2 was deposited at the GenBank database with the accession number AB116935.

2.5. GTPases binding and activation assays

Transfected cells were washed twice in cold PBS and lysed 10 min on ice in 50 mM Tris, pH 7.5, 500 mM NaCl, 1% Triton X-100, 5 mM EDTA and 1% protein inhibitors mixture (Sigma # P8340). The EDTA serves to deplete GTPases from nucleotides by chelating Mg^{2+} ions that are essential cofactor for nucleotide binding. Lysates were cleared by 10 min centrifugation at $16\,000 \times g$. A portion of this total lysate was reserved and the rest incubated for 1 h at 4 °C with 25 μg GST fused GTPases bound to glutathione agarose beads (Sigma). The beads were washed 3 times in lysis buffer (LB) and either eluted directly in SDS–PAGE loading buffer (Fig. 2B) or eluted first in LB lacking EDTA and containing 5 mM $MgCl_2$ and 100 μM GTPγS followed by a elution of the beads in SDS–PAGE loading buffer (Fig. 3). For assaying Cdc42 activation, we used the Cdc42 activation assay kit (Cytoskeleton) according to the manufacturer's instructions.

2.6. RNA analysis

For Northern blotting, total RNA from various mouse tissues was isolated with TriZol (Invitrogen) and then poly-A^+ RNA was purified with μMACS mRNA isolation kit (Miltenyi Biotec). Poly-A^+ RNA (0.5 μg) was separated by electrophoresis on 1.5% agarose, 6% formaldehyde gels and transferred onto Hybond-N^+ membrane (Amersham), which was hybridized with [^{32}P]-labeled PCR amplified murine zizimin2, zizimin1, zizimin3 or β-actin cDNA probes. The following primers were used for probe amplification: zizimin2, 5′-GAC AAA ATA TTG TTA TCC-3′ (sense) and 5′-GCT GGC AAT GAT GGA AGA AT-3′ (antisense); zizimin1, 5′-AAG GTC AAC CCG AAG GAT CT-3′ (sense) and 5′-GGC TCC TTC TCT CTC CCA TT-3′ (antisense); zizimin3, 5′-TGG CCA AAG TCG AAA AAG TC-3′ (sense) and 5′-GGC TCC ATC GTC ATC TGA AT-3′ (antisense). β-actin probe was purchased from Clontech. Probe hybridization was performed with Perfecthyb™ (Sigma) as recommended by the manufacturer.

Quantitative real-time RT-PCR was carried out as described [22]. Each RNA sample was analyzed in parallel for β-actin to control for differences in starting RNA amounts. The following gene specific primers were used: human β-actin, 5′-CCA AGG CCA ACC GCG AGA AG-3′ (sense) and 5′-GCC AGA GGC GTA CAG GGA TA-3′ (antisense); mouse and human zizimin2, 5′-TTG CCT TTT ATG GCC AGT CT-3′ (sense) and 5′-GAG CGA ATT TTG GAT CAA GC-3′ (antisense); mouse zizimin1, 5′-CCA AAT TCC CTG CAC ATC TT-3′ (sense) and 5′-GGC TCC TTC TCT CTC CCA TT-3′ (antisense); mouse β-actin, 5′-CTA AGG CCA ACC GTG AAA AG-3′ (sense) and 5′-ACC AGA GGC ATA CAG GGA CA-3′ (antisense). Especially, for GC and non-GC B cell cDNA preparation in Fig. 4A, four-color sorting (B220, CD19, PNA, and GL-7) was performed