Human septin–septin interaction: CDCrel-1 partners with KIAA0202

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Abstract Septins are evolutionary conserved cytoskeletal GTPases forming heteropolymer complexes involved in cytokinesis and other cellular processes. CDCrel-1 (cell division cycle related-1) is a recently cloned and characterized human septin which is highly expressed in non-dividing cells, such as neurons. Using a yeast two-hybrid system we demonstrate that CDCrel-1 partners with another uncharacterized human septin, KIAA0202. The interaction of CDCrel-1 and KIAA0202 was confirmed in the human leukemia cell line K-562 using pull-down assays with a KIAA0202–glutathione *S*-transferase fusion protein and by immunoprecipitation of the CDCrel-1–KIAA0202 complex with an anti-KIAA0202 antibody. Expression studies of the two human septins revealed a concomitant expression of both proteins in certain cells. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Septin; Yeast two-hybrid assay; Protein interaction; KIAA0202

1. Introduction

Septins are a cytoplasmic family of proteins essential for cytokinesis in yeast. A plethora of data has documented yeast mutants and their inability to undergo yeast budding [1–4]. Indeed, it was originally suggested that septin proteins may be restricted to the mechanisms involved in asymmetric cell division and, as such, not relevant for higher eukaryotic organisms [2]. However, a growing appreciation for septin proteins in higher eukaryotic organisms suggests an involvement in other, albeit similar, cellular processes [5,6]. Some recently characterized human septins are highly expressed in cells not undergoing active cell division, such as neurons [7,8]. In these cases, septins have been purified as components of the exocytic cellular machinery [9,10].

Purified septin complexes from yeast appear as filaments when viewed by transmission electron microscopy and have the ability to assemble into long filament complexes in vitro [11]. Purified complexes of multiple septins have been reported for yeast, *Drosophila* and rat, with molecular masses ranging from 340 to 370 kDa [10–12]. Thus, the possibility of septin–septin interaction for normal complex assembly may represent a mechanism whereby cell-specific septin complexes are generated for a specialized role in a particular cell type.

We report for the first time the interaction of two human septins. CDCrel-1 (cell division cycle related-1) associates with KIAA0202 and both display a similar, yet restricted, gene expression pattern.

2. Materials and methods

2.1. Yeast two-hybrid method

The Matchmaker yeast two-hybrid-system II was purchased from Clontech and performed according to the manufacturer's manual [13]. The yeast two-hybrid plasmid pAS2-1 containing CDCrel-1 (accession no. U74628, nucleotides 83–2032) was used as the target in pre-transformed fetal brain cDNA- and heart cDNA-libraries made in pACT2.

Three fragments of CDCrel-1 were generated by PCR and cloned into the vector pAS2-1. The first fragment (550 bp) covered the first two GTP-binding motifs, the second fragment (784 bp) included all three GTP-binding motifs and the third fragment (723 bp) corresponded to the GTP-motif AKAD and the 'coiled-coil' domain. The constructs of the fragments were cotransformed with KIAA0202 in the yeast strain PJ69-2A and interaction was demonstrated by His^+, Ade^+ and $lacZ^+$ transformants.

2.2. Quantitative β-Gal assay

The quantitative β-Gal assay was performed using *o*-nitrophenyl-β-D-galactopyranoside as substrate according to the yeast protocol handbook (Clontech) [13]. Positive controls were performed with p53 and SV40 large T-antigen. Negative controls were performed by cotransformation of CDCrel-1 and SV40 large T-antigen as well as by cotransformation of p53 and KIAA0202.

2.3. Glutathione S-transferase (GST) pull-down

The recombinant GST–KIAA0202 fusion protein (19–2225, accession nos. AF179995 and D86957, pGEX4T-2, BL21/DE3) was bound to glutathione Sepharose (Amersham Pharmacia) [14]. Cells of the human tumor cell line K-562 were collected and lysed with RIPA-buffer (9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, 1% NP-40, 0.5% Na–desoxycholat, 0.1% SDS, pH 7.4; addition of proteinase inhibitors prior to use). The supernatant was diluted with PBS buffer and applied to the KIAA0202 immobilized on glutathione Sepharose. Elution was reached by 20 mM reduced glutathione. CDCrel-1 interacting with the KIAA0202–GST fusion protein was detected by Western analysis using a monoclonal anti-CDCrel-1 antibody. Control pull-down assays with the GST vector lacking the KIAA0202 insert and with glutathione Sepharose were performed to exclude an unspecific association.

2.4. Immunoprecipitation

Protein A Sepharose was washed with PBS buffer (9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, pH 7.4). K-562 lysate was incubated with Protein A Sepharose (1 h at 4°C) in the presence of proteinase inhibitors. After centrifugation, the supernatant was subjected to anti-KIAA0202 antibody bound to Protein A Sepharose (1 h at 4°C). The Protein A Sepharose was subsequently washed for several times with PBS buffer. Bound proteins were eluted with lithium dodecyl sulfate (LDS)-sample buffer (1.09 M glycerol, 1.41 mM Tris base, 106 mM Tris–HCl, 73 mM LDS, 0.51 mM EDTA, 0.22 mM

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Abbreviations: CDCrel-1, cell division cycle related-1; G3PDH, glyceraldehyde-3-phosphate dehydrogenase

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Serva Blue G250, 0.175 mM phenol red, pH 8.5) for 5 min at 95°C. Control experiments were carried out with rabbit serum or without any antibody.

2.5. Western analysis

Protein medleys of brain and heart were purchased from Clontech (#7800-1, #7801-1). Human platelets were lysed with RIPA buffer. Proteins were separated by gradient SDS–PAGE (4–12%) after denaturation and reduction. After electrophoresis, proteins were transferred to PVDF membranes to react with the corresponding antibody. Positive signals were detected using a peroxidase-conjugated Affini-Pure goat anti-mouse (1:50 000) (Dianova) or peroxidase-conjugated goat anti-rabbit antibody IgG(H-L) (1:50 000) (Pierce) and the ECL-system (Amersham Pharmacia).

2.6. RNA isolation and Northern transfer

Total RNA from the human tumor cell line K-562 and from human platelets was isolated by ultracentrifugation through cesium chloride cushions. Poly(A)$^+$RNA was prepared from K-562 total RNA by affinity chromatography using oligo(dT) cellulose columns (Life Technologies). Gel electrophoretic separation of K-562 poly(A)$^+$RNA and platelet total RNA was performed through denaturing formaldehyde gels prepared as described by Lehrach et al. [15]. Then, the RNA was transferred from the gel onto nitrocellulose by capillary forces. In addition, mRNA blots were purchased from Clontech containing mRNA isolated from brain and heart tissues (#7755-1, #7791-1) (Fig. 4A).

The hybridization probe for CDCrel-1 was a 600 bp 5′-fragment and the hybridization probe for KIAA0202 was a 590 bp 5′-fragment [16]. After hybridization, the nitrocellulose filter was washed three times with 0.3 M sodium chloride/0.03 M sodium citrate/0.1% SDS (42°C) and two times with 0.15 M sodium chloride/0.015 M sodium citrate/0.1% SDS (55°C). Clontech blots were washed according to the supplier′s instructions. Then the nitrocellulose or the nylon membrane was analyzed by autoradiography using Kodak Biomax MR film. A final hybridization with a human β-actin or with a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe confirmed the presence of mRNA on the membrane.

2.7. Antibodies against KIAA0202

The 5′-end of KIAA0202 was cloned into the pET30c vector. KIAA0202 (amino acids 8–113) was expressed as His-tagged protein in the *Escherichia coli* strain BL21/DE3. The recombinant protein was purified with a His-bind-resin (Novagen). Polyclonal antibodies were raised in rabbits (Eurogentec).

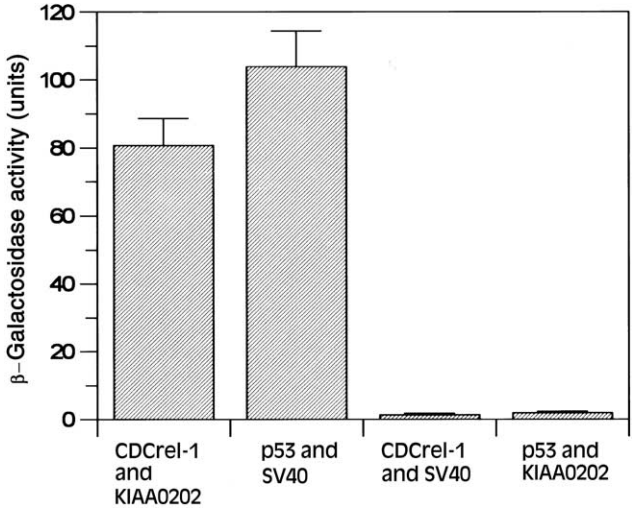

Fig. 1. Quantitative analysis of the β-galactosidase activity generated in the presence of DBD–CDCrel-1 and AD–KIAA0202 is shown along with control constructs coding for either the DBD from c-Myc-p53 or AD from the SV40 large T-antigen. Demonstrated is: (i) the strong interaction of CDCrel-1 with KIAA0202, (ii) the positive control c-Myc-p53 with SV40 large T-antigen, (iii) the negative control CDCrel-1 with SV40 large T-antigen and (iv) the negative control c-Myc-p53 with KIAA0202.

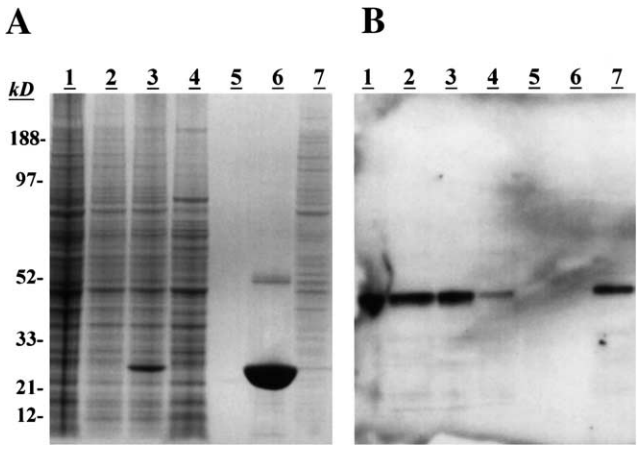

Fig. 2. GST pull-down assay using a KIAA0202–GST fusion protein. Coomassie stain of SDS–PAGE (A) and Western analysis (B) using an anti-CDCrel-1 monoclonal antibody were performed. A strong signal (B) was observed corresponding to the migration position of CDCrel-1 (45 kDa). Lane 1: K-562 lysate; lane 2: K-562 lysate that did not bind to glutathione Sepharose (negative control); lane 3: K-562 lysate that did not bind to GST–glutathione Sepharose (negative control); lane 4: K-562 lysate that did not bind to GST–KIAA0202–glutathione Sepharose; lane 5: proteins of K-562 lysate that bind to glutathione Sepharose (negative control); lane 6: proteins of K-562 that bind to GST–glutathione Sepharose (negative control); lane 7: proteins of K-562 lysate that bind to GST–KIAA0202–glutathione Sepharose.

3. Results

To identify proteins interacting with human CDCrel-1, we constructed a recombinant plasmid containing the in-frame coding sequence for the DNA binding domain (DBD) of Gal4 and the coding sequence for the residues 83–2032 of CDCrel-1 (accession no. U74628). A fetal brain cDNA library expressing fusions of the activation domain (AD) of Gal4 was screened using a yeast two-hybrid system. Sixteen positive clones were identified from a screening of 9.4×10^8 transformants. Sequence analyses of these clones revealed that 12 clones were unique and contained the coding sequence for another human septin, KIAA0202 (accession nos. AF179995 and D86957). The KIAA0202 cDNA sequence is present in GenBank but no further characterization of the gene/protein has been reported.

A second AD library containing cDNA prepared from human adult heart mRNA was also screened (3.5×10^9 clones) and provided similar results with the isolation of more clones containing KIAA0202 coding sequence. Isolated KIAA0202 cDNA fragments contained unique 5′-ends corresponding to nucleotides 19, 23, 75, 83, 305, 371, 407 and 662 of GenBank accession no. D86957. To examine the interaction specificity between the DBD–CDCrel-1 and AD–KIAA0202 fusion proteins, the KIAA0202 recombinant plasmids were cotransformed with other yeast two-hybrid vectors. No positive signals were generated in the absence of the human CDCrel-1 coding sequence. Quantitative analysis of the β-galactosidase activity generated in the presence of DBD–CDCrel-1 and AD–KIAA0202 is shown in Fig. 1 along with control con-

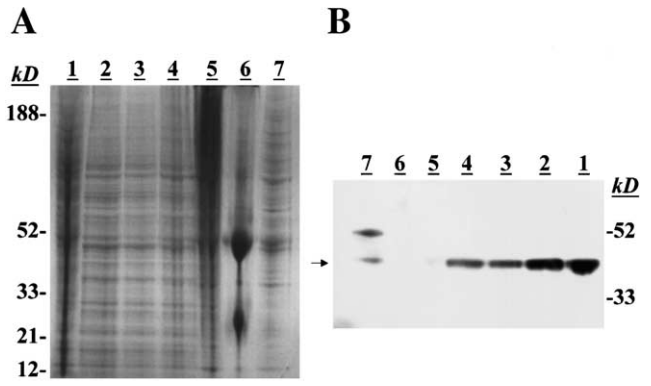

Fig. 3. Immunoprecipitation of CDCrel-1 from the human leukemia cell line K-562 by an anti-KIAA0202 polyclonal antibody. Coomassie stain of SDS–PAGE (A) and Western blot analysis (B) using an anti-CDCrel-1 monoclonal antibody were performed. Lane 1: K-562 lysate; lane 2: K-562 lysate that did not bind to Protein A Sepharose (negative control); lane 3: K-562 lysate that did not bind to rabbit serum immobilized on Protein A Sepharose (negative control); lane 4: K-562 lysate that did not bind to anti-KIAA0202 antibody immobilized on Protein A Sepharose; lane 5: elution fraction of proteins (K-562 lysate) that bind to Protein A Sepharose (negative control); lane 6: elution fraction of proteins (K-562 lysate) that bind to rabbit serum immobilized on Protein A Sepharose (negative control); lane 7: elution fraction of proteins (K-562 lysate) that bind to anti-KIAA0202 antibody immobilized on Protein A Sepharose. The CDCrel-1 protein is marked with an arrow (45 kDa).

structs coding for either the DBD from c-Myc-p53 or AD from the SV40 large T-antigen. The interaction between CDCrel-1 and KIAA0202 is very strong and comparable to that of c-Myc-p53 and SV40 large T-antigen.

To verify the interaction observed in the yeast two-hybrid system, GST pull-down experiments were carried out. We have previously reported that the human leukemia cell line K-562 expresses CDCrel-l [17]. Thus, we used K-562 cell extracts to determine if an interaction occurs between CDCrel-1 and KIAA0202. An expressed GST–KIAA0202 fusion protein was immobilized on glutathione Sepharose beads and mixed with a K-562 cell lysate. The bound proteins were eluted and analyzed by SDS–PAGE and Western blot analysis using an anti-CDCrel-1 monoclonal antibody (Fig. 2). A strong signal was observed corresponding to the migration position of CDCrel-1 (45 kDa). Mixture of a GST fusion protein lacking the KIAA0202 sequence (KIAA0202 protein) or glutathione Sepharose with K-562 lysate did not result in the pull-down of the human CDCrel-1 protein. Additionally, we performed an immunoprecipitation of the CDCrel-1–KIAA0202 complex with the anti-KIAA0202 antibody in K-562 lysate. The antibody was immobilized on Protein A Sepharose and mixed with a K-562 cell lysate. The bound proteins were eluted and analyzed by SDS–PAGE and Western blot analysis using an anti-CDCrel-1 monoclonal antibody. A protein with the migration position of CDCrel-1 (45 kDa) could be detected (Fig. 3). The 45 kDa CDCrel-1 band could not be detected using rabbit serum or Protein A Sepharose. The results of the GST pull-down and the immunoprecipitation are thus consistent with those of the yeast two-hybrid experiments and demonstrate an intermolecular association between human CDCrel-1 and KIAA0202 in cells expressing both proteins.

We previously reported that another septin, human CDCrel-2, shares a remarkable similarity to the human CDCrel-1 cDNA and protein sequence [17]. However, CDCrel-1 and CDCrel-2 are different in their expression patterns. This observation was most apparent in the analysis of tumor cell lines in which the expression analysis for CDCrel-1 and CDCrel-2 was examined and no overlap in the expression pattern was seen. A similar analysis of KIAA0202 mRNA and CDCrel-1 mRNA was performed in the K-562 tumor cell line,

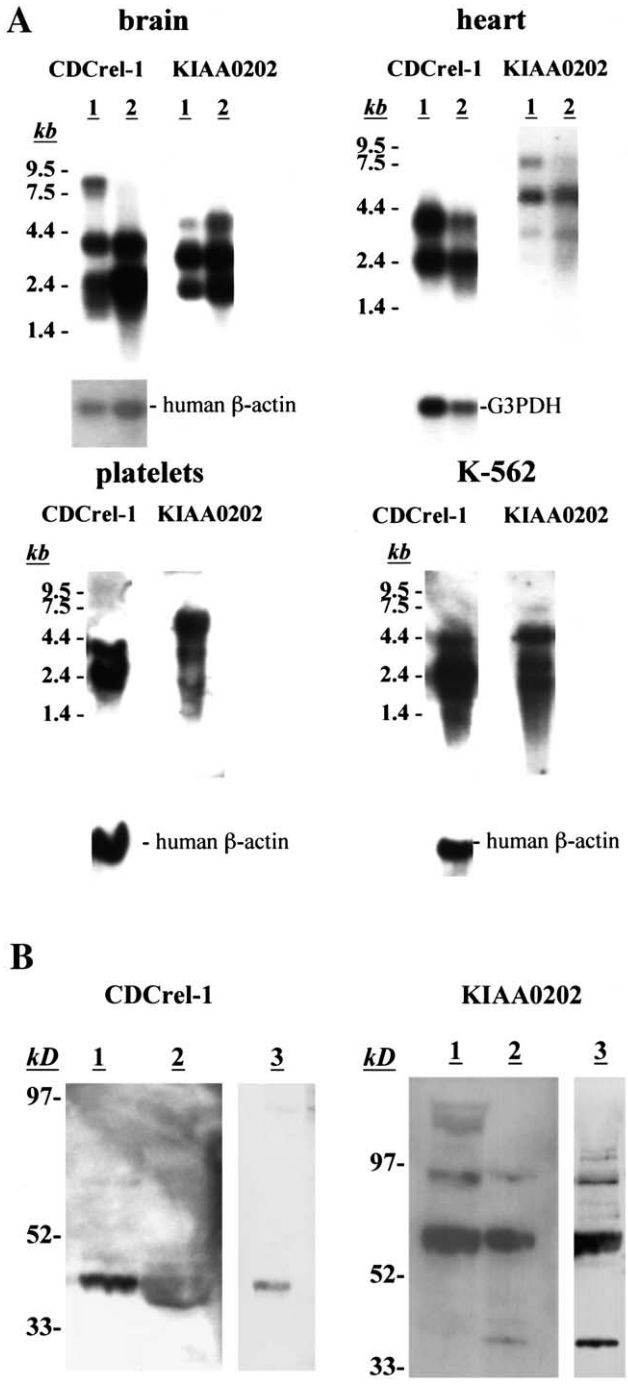

Fig. 4. A: Northern analyses for CDCrel-1 (3.5, 2.4 kb) and KIAA0202 (4.4, 2.8, 2.2 kb) in poly(A^+)RNA from human brain (lane 1: cerebellum, lane 2: cerebral cortex), human heart (lane 1: fetal heart, lane 2: adult heart), total RNA from platelets and poly-(A^+)RNA from K-562 (chronic myelogenous leukemia). Shown below is the autoradiograph from a hybridization of the filter with a human β-actin or G3PDH probe. B: Western analyses of CDCrel-1 and KIAA0202. Lane 1: brain, lane 2: platelets, lane 3: heart.

brain, heart tissue and platelets (Fig. 4A). The autoradiograph documents two hybridizing mRNA species of 3.5 and 2.4 kb for CDCrel-1 [8,17]. We observed a strong correlation between the presence of the human CDCrel-1 mRNA and the KIAA0202 mRNA (Fig. 4A). In addition, CDCrel-1 protein and KIAA0202 protein were detected in brain, heart tissue and in platelets (Fig. 4B).

As for CDCrel-1 [8] several transcripts of KIAA0202 (4.4, 2.8, 2.2 kb) could be detected in Northern analysis. By screening three different cDNA libraries we identified these transcripts as different splice variants of KIAA0202.

4. Discussion

The septin family of proteins was originally identified in yeast through the analysis of temperature-sensitive mutants showing impaired cytokinesis and budding [3]. The loss of yeast septins coincided with a loss of neck filaments in budding yeast. Subsequent studies established that septins contribute to the formation of these filamentous structures and that multiple septins assemble into a large protein complex [18]. Indeed, septins and their association with these filamentous structures have led to the hypothesis that septins may represent a novel class of cytoskeletal filaments [19]. More recently, work has demonstrated that septin homologs extend well-beyond yeast and are present in a wide variety of eukaryotic cells [5,20–22]. Again, protein complexes composed of multiple septin proteins have been purified from mammalian cell extracts [23,24]. Intriguingly, mammalian septins can be purified with components of the exocytic or t-SNARE complex involved in regulated cellular secretion [9,10]. Thus, for mammalian cells there is speculation that septins form a scaffolding matrix upon which other proteins assemble and perform a unique job for a particular cell type [18].

An intriguing aspect of the septin gene family is the restricted expression pattern for individual septin genes. This leads to the possibility that the septin–septin interaction may generate cell-specific complexes with a unique function for a given cell type. In the current study we used one of the best-characterized human septin genes, CDCrel-1 to probe a library of cDNAs looking for potential interaction partners. The results from screening two different human cDNA libraries illustrate an interaction with the septin KIAA0202. A cDNA sequence of KIAA0202 is present in GenBank (accession nos. D86957 and AF179995) but no further characterization of this sequence has been reported. Moreover, Northern analysis for the KIAA0202 mRNA transcript revealed a strikingly similar pattern to that observed for the CDCrel-1 mRNA. In addition, the presence of CDCrel-1 protein coincides with the presence of the KIAA0202 protein and further supports a hypothesis that septin complexes containing different polypeptides may assemble to accomplish biological processes unique to the particular cell type.

Beyond the yeast two-hybrid system, we documented in a pull-down assay the ability of recombinant GST–KIAA0202 to interact with native CDCrel-1 present in extracts from the cell line K-562. Furthermore, we showed the interaction of native KIAA0202 and CDCrel-1 in the extracts from the cell line K-562 by immunoprecipitation. Quantitative analysis demonstrated that the interaction between CDCrel-1 and KIAA0202 is as strong as one of the well-characterized positive controls for the two-hybrid system; namely, the interaction between SV40 large T-antigen and the p53 AD (Fig. 1). We attempted to further localize the interaction domain of CDCrel-1 with KIAA0202 by making deletions within the coding sequence of CDCrel-1. In all cases, any removal of CDCrel-1 sequence resulted in lack of interaction with KIAA0202 (data not shown). Thus, the interaction between CDCrel-1 and KIAA0202 seems to be dependent upon the intact protein and what is presumed to be some native folding of the CDCrel-1 polypeptide.

Our study validates the yeast two-hybrid system as a tool to identify potential septin interaction partners. Given the presence of at least eight individual human septin genes, the use of a yeast two-hybrid system coupled with expression analysis of individual cell types is useful in defining the constituent polypeptides comprising the larger septin complexes within an individual cell.

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