Kcc4 associates with septin proteins of *Saccharomyces cerevisiae*

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Received 12 December 2000; accepted 8 January 2001

First published online 17 January 2001

Edited by Horst Feldmann

Abstract Kcc4, a kinase of the budding yeast *Saccharomyces cerevisiae*, is homologous to the bud neck protein kinases Hsl1/Nik1 and Gin4. We report here that a GFP-Kcc4 fusion protein is localized at the bud neck and that the non-kinase domain is required for this localization. We also demonstrate that Kcc4 associates with septin proteins in vitro and in vivo by two-hybrid analysis, GST pull-down experiments, immunoprecipitation, and analysis of direct association with affinity-purified GST-Kcc4 and MBP-Septin proteins. From the results obtained here, we suggest that Cdc11 is the primary association partner of Kcc4.

Key words: Hsl1; Gin4; Septin; Cytokinesis

1. Introduction

Septins are conserved in eukaryotes from yeast to humans [1–5]. Five septin proteins, Cdc3, Cdc10, Cdc11, Cdc12 [6], and Sep7 [7], have been identified in budding yeast. These represent the major structural components of the neck filament system, which forms a 10 nm ring at the mother-bud neck [5,7–9]. Cytokinesis was inhibited when septin functions were disrupted by mutations or by injecting anti-septin antibodies into budding yeast [6], *Drosophila* [10], and mammalian cells [11]. Temperature-sensitive mutations in these septin genes also result in defective cytokinesis at the restrictive temperature, causing cell cycle arrest and altered morphology with elongated buds, which suggests a crucial role for septins in cytokinesis [6,12].

Hsl1 (also known as Nik1 [13]) is also localized to the bud neck, and co-immunoprecipitates with Cdc3. Autophosphorylation and kinase activity of Hsl1 are affected by septin mutations, indicating that Hsl1 may also be a component of the septin complex [12]. Two-hybrid analysis indicated that the bud neck protein Gin4 [14], an Hsl1-like kinase, also associates with Cdc3 [15]. However, the direct interaction between Hsl1/Gin4 and septin proteins has not been examined to date. Kcc4, another Hsl1/Gin4-like kinase, is also reported to be a bud neck protein [12], but its association with septins remains obscure. Therefore, we have examined the association of Kcc4 with septin proteins in detail. Here we report that Kcc4 directly associates with septin proteins, and suggest that Cdc11 is the primary association partner of Kcc4.

2. Materials and methods

2.1. Yeast strains and manipulations

Standard techniques were used for the manipulation of yeast cells [16]. The yeast strains used in this study (gifts from Dr. Araki) are *kcc4* in the *1784* background [14] for the GFP experiment, Y190 [17–19] for two-hybrid analysis and CB018 for protein extraction.

2.2. Disruption of the KCC4 (YCL024W) gene

A 4.5-kb *Xba*I *KCC4* (*YCL024W*) genomic fragment was subcloned into the *Xba*I site of pBluescript KS(+) II (Stratagene, La Jolla, CA, USA). The resulting construct was cleaved with *Hin*dIII and *Eco*RI within the coding region of *KCC4*, and the ends of the plasmid were filled in using the Klenow fragment of polymerase I. A 1.4-kb DNA fragment containing the *ARS1-TRP1* gene was then ligated into the blunt-ended construct, and the resulting construct containing the mutant allele (*kcc4Δ::ARS1-TRP1*) was linearized by digestion with *Mlu*I and *Bcl*I and used to transform haploid yeast strains. Southern blot analysis of the genomic DNA of the yeast transformants was used to verify the successful substitution at the genomic locus (data not shown).

2.3. Fluorescence experiment

To construct a 2-μ *KCC4*-GFP plasmid, a *KCC4* fragment extending from the *Bgl*II site to the stop codon was generated by PCR. This fragment was subcloned into pCR-Script SK(+) vector (Stratagene), excised with *Bgl*II and *Xba*I, and ligated with the *Xba*I–*Bgl*II fragment containing the 5′-upstream region of the *KCC4* gene. The ligated *Xba*I–*Xba*I *KCC4* fragment, in which the stop codon was replaced by an *Asc*I site, was inserted into the *Xba*I site of YEplac181 [20] to yield YEplac181-*KCC4*. Finally, an *Asc*I GFP (green fluorescent protein) fragment [14] was inserted into the *Asc*I site of YEplac181-*KCC4* to yield YEplac181-*KCC4*-GFP.

To examine the localization of a series of six GFP-Kcc4 constructs (GFP-Kcc4.1∼6) driven by the *GAL1* promoter in live cells, the *KCC4.1∼6* fragments were generated by PCR with *Asc*I and *Not*I sites at each end of the fragments. They were inserted into the SY3B vector to yield SY3B-*KCC4.1∼6*. Then the *Asc*I GFP fragment described above was inserted into the *Asc*I site of SY3B-*KCC4.1∼6* constructs to express Kcc4 constructs with N-terminally fused GFP (*SY3BGFP-KCC4.1∼6*). The *kcc4*Δ cells were transformed with the GFP-Kcc4.1∼6 plasmids, and cells were grown in synthetic medium lacking appropriate amino acids. Cells were collected and washed with the same medium and spotted onto coverslips. GFP fluorescence was detected using a Zeiss Axiophot microscope as described previously [14].

2.4. Pull-down experiments

To express Kcc4.1 and Cdc11 under the *GAL1* promoter, the *KCC4.1* and *CDC11* fragments described above were inserted into the SY3B-based SYM2 [13] and SYG3 (unpublished) vectors, respectively. The resulting constructs were used in order to express Kcc4 protein tagged with Myc epitopes (SYM2-*KCC4.1*) and Cdc11 protein fused with GST (SYG3-*CDC11*) in CB018, a protease deficient strain. The co-expressed cells were mixed with lysis buffer [21]. An aliquot of about 3 mg of total cell lysate was constantly mixed with glutathione-Sepharose beads (50 μl of 50% slurry; Pharmacia) at 4°C for 2 h by

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0014-5793/01/$20.00
PII: S0014-5793(01)02104-4

rotating the tubes. The beads were washed four times with 1 ml of wash buffer [21]. Lysates and beads were separated on a 10% SDS–polyacrylamide gel, followed by immunoblotting as described previously [14].

2.5. Purification of fusion proteins and in vitro binding assays

A series of *KCC4.2∼6* fragments and septins (*CDC3*, *CDC10*, *CDC11* and *CDC12*) amplified by PCR fragments were subcloned into the *Asc*I and *Not*I sites in the multicloning sites of the modified pGEX6P (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and pMAL-cri (New England Biolabs, Beverly, MA, USA) vectors, respectively. Expression of the resulting constructs in *Escherichia coli* (AD202) produced GST-Kcc4.2∼6 and MBP-septin fusion proteins, which were purified by glutathione-Sepharose and amylose resin chromatography. MBP-septin-bound amylose resin was eluted with PBS plus 10 mM maltose. For the MBP-septin binding assay, each fusion protein produced from the cell lysates (GST-Kcc4.2∼6) was incubated with 50 μl of glutathione-Sepharose beads (50% slurry) in order to saturate the binding capacity of the beads, and washed with phosphate-buffered saline (PBS). The beads bound to the GST fusion proteins were then coated with the purified MBP protein to avoid a non-specific interaction between MBP and GST. After washing with PBS, the MBP-septin proteins were added to the GST-Kcc4.2∼6 protein beads and the beads were washed with PBS. SDS sample buffer was used to release the MBP-septin proteins bound to the beads.

3. Results and discussion

3.1. Kcc4-GFP is localized to the bud neck

To characterize the physiological role of Kcc4, we generated *kcc4*Δ cells and found that the mutation does not lead to any apparent phenotype. The *kcc4*Δ cells were transformed with the YEplac181-*KCC4*-GFP plasmids, and the expressed Kcc4-GFP fusion protein was observed at the bud neck in live cells irrespective of the size of the buds (Fig. 1A; denoted by arrowheads). This result is consistent with the recent observation made by immunostaining with an HA-tagged Kcc4 protein [12]. GFP fusion proteins of Gin4 or HA-tagged Hsl1 are reported to be localized at the bud neck in a septin-dependent manner, and the detection of an interaction between these proteins suggests that they may be present in the same complex at the bud neck [12,14,15]. Thus, Kcc4 appears to be a bud neck protein similar to Gin4 and Hsl1. Both Kcc4-GFP (Fig. 1A) and Gin4-GFP [14] are distributed in the form of a collar or a ring around the bud neck.

In order to further characterize the domain that is required for the bud neck localization of Kcc4-GFP, we constructed six truncated *KCC4-GFP* constructs driven by the *GAL1* promoter (Fig. 1B), which were used to transform *kcc4*Δ cells. In the absence of galactose, the GFP signals of Kcc4.1 and Kcc4.4 were found to be localized to the bud neck (Fig. 1C; denoted by arrowheads). The result suggests that the N-terminal kinase domain is irrelevant to the bud neck localization. These bud neck localizations were not observed with the other forms of Kcc4 (Fig. 1C), suggesting that the internal domain (amino acids 280–750) is essential for Kcc4 localization to the bud neck. Successful expression of these GFP-Kcc4 fusion proteins in the transformed *kcc4*Δ cells was confirmed by Western blot analysis with anti-GFP antibody (Fig. 1D).

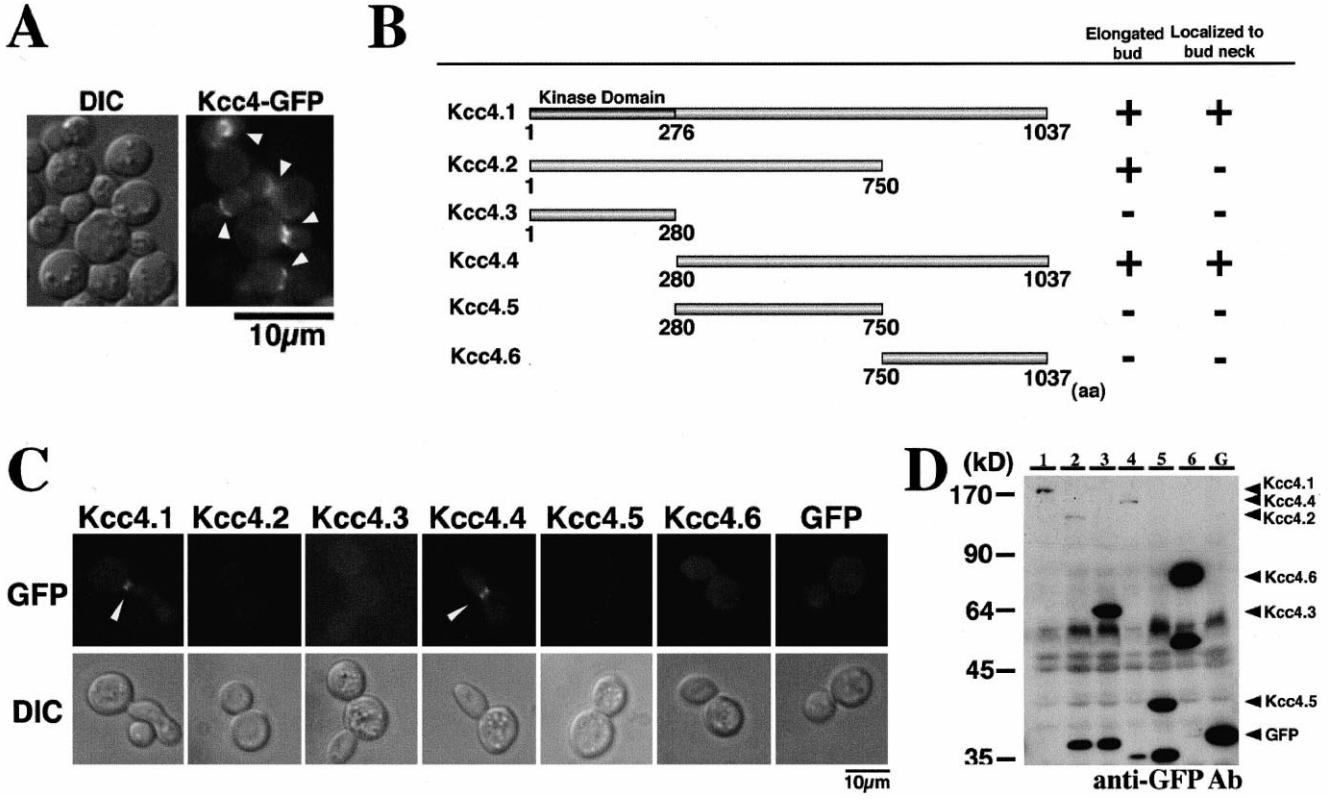

Fig. 1. Localization of Kcc4-GFP fusion proteins to the bud neck in live cells. A: Kcc4-GFP localization at the bud neck (arrowheads). Kcc4-GFP was expressed from a high copy vector (2-μ plasmid) in *kcc4*Δ cells. B: Schematic representation of the full-length and truncated forms of the Kcc4 protein. Plus signs at the right panels indicate an elongated bud phenotype or the bud neck localization of GFP signals caused by the expression of each construct. These *KCC4* genes were expressed from the *GAL1* promoter. C: Presence (arrowheads) or absence of GFP signals at the bud neck for each of the GFP-Kcc4.1∼6 fusion proteins constitutively expressed from the *GAL1* promoter in *kcc4*Δ cells. D: Confirmation of successful GFP-Kcc4.1∼6 protein expression as detected by immunoblotting with anti-GFP polyclonal antibody.

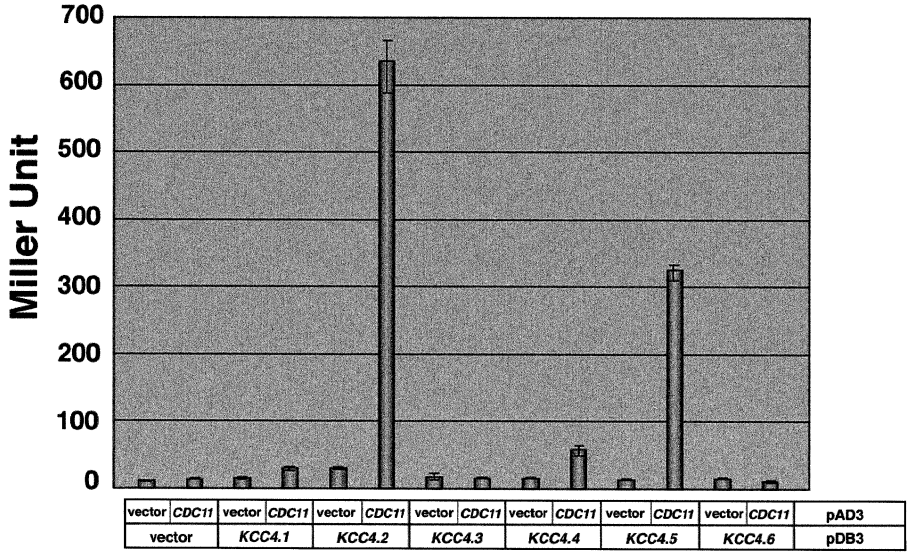

Fig. 2. Two-hybrid analysis reveals associations between certain Kcc4.1∼6 constructs and Cdc11 septin protein. pAD3-*CDC11* expressed the AD-Cdc11 fusion protein and pDB3-*KCC4.1∼6* constructs expressed DB-Kcc4.1∼6 fusion proteins. Cells transformed with each plasmid combination were examined by the liquid culture assay using ONPG as a substrate. Miller units and standard deviation (S.D.: error bars) were obtained from three separate experiments.

3.2. Two-hybrid analysis demonstrates the interaction of Kcc4 with septin proteins

To examine the possible interaction of Kcc4 with septin proteins, we performed two-hybrid analysis using a series of truncated *KCC4* constructs (Fig. 1B) fused to the Gal4 DNA binding domain of a modified pDB3 vector, and the open reading frame of Cdc11 fused to the Gal4 activation domain of a modified pAD3 vector [17]. These constructs were introduced into the Y190 strain, and the expression of the *LacZ* reporter gene was examined [19]. When pAD3-*CDC11* was co-transfected with pDB3-*KCC4.2* or pDB3-*KCC4.5*, the transfected cells showed prominently high Miller unit values (Fig. 2). On the other hand, the transfected cells carrying pDB3-*KCC4.4* showed a value that was low but not negligible (Fig. 2). The Kcc4.2, Kcc4.4 and Kcc4.5 constructs all contain the internal domain (amino acids 280–750), suggesting that this domain plays a role in the interactions between Kcc4 and Cdc11. In addition, both the Kcc4.2 and Kcc4.5 constructs harbor the domain corresponding to the growth inhibitory domain of Gin4 [14], in which there is a high degree of sequence similarity (39% amino acid identity) between Kcc4 and Gin4. Considering that neither full-length Kcc4, nor the truncated versions (Kcc4.3 and Kcc4.6) associated with Cdc11, it appears that the kinase domain and the C-terminal region of Kcc4 are unnecessary for association with Cdc11. These results suggest that the internal domain (amino acids 280–750) interacts with Cdc11 either directly or indirectly. We also examined the interaction of Kcc4 with other septin proteins (Cdc3, Cdc10, and Cdc12), and detected an interaction only between Kcc4.5 and Cdc12 (data not shown).

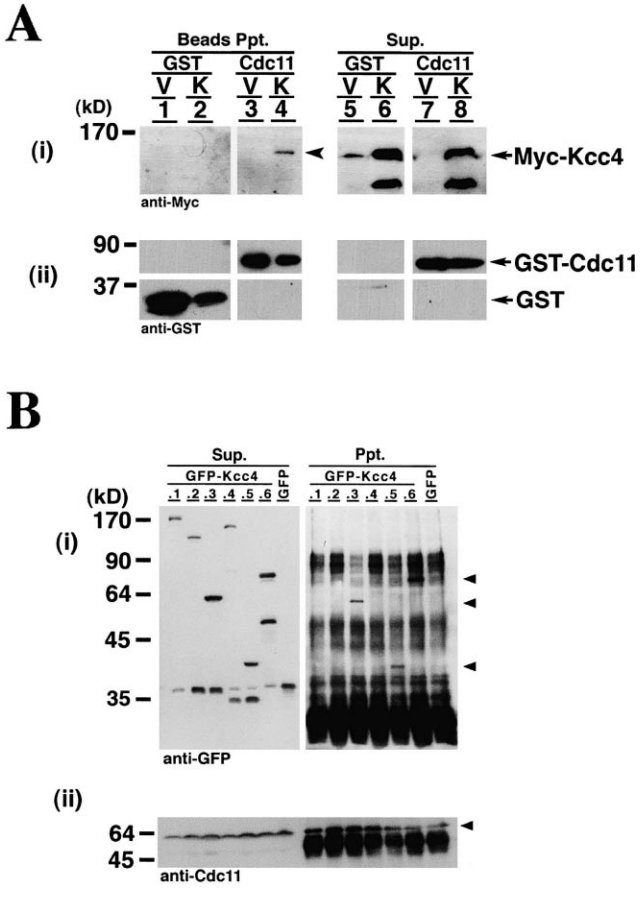

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Fig. 3. Association of Kcc4 with Cdc11 in vitro and in vivo. A: Whole cell lysates were prepared from strains expressing different combinations of each of the constructs, including GST (GST), GST-Cdc11 fusion protein (Cdc11), *myc* vector (V) and Myc-Kcc4 fusion protein (K) under the control of *GAL1* promoter. Each lysate was mixed with glutathione-Sepharose beads and precipitated by centrifugation. Proteins coprecipitated with the beads were analyzed by immunoblotting using anti-Myc monoclonal antibody (i) or anti-GST polyclonal antibody (ii). B: Immunoprecipitates pulled down by anti-Cdc11 antibody from cell lysates that expressed the GFP-Kcc4.1∼6 fusion proteins separately from the *GAL1* promoter were analyzed by immunoblotting using either an anti-GFP monoclonal antibody (i) or an anti-Cdc11 polyclonal antibody (ii).

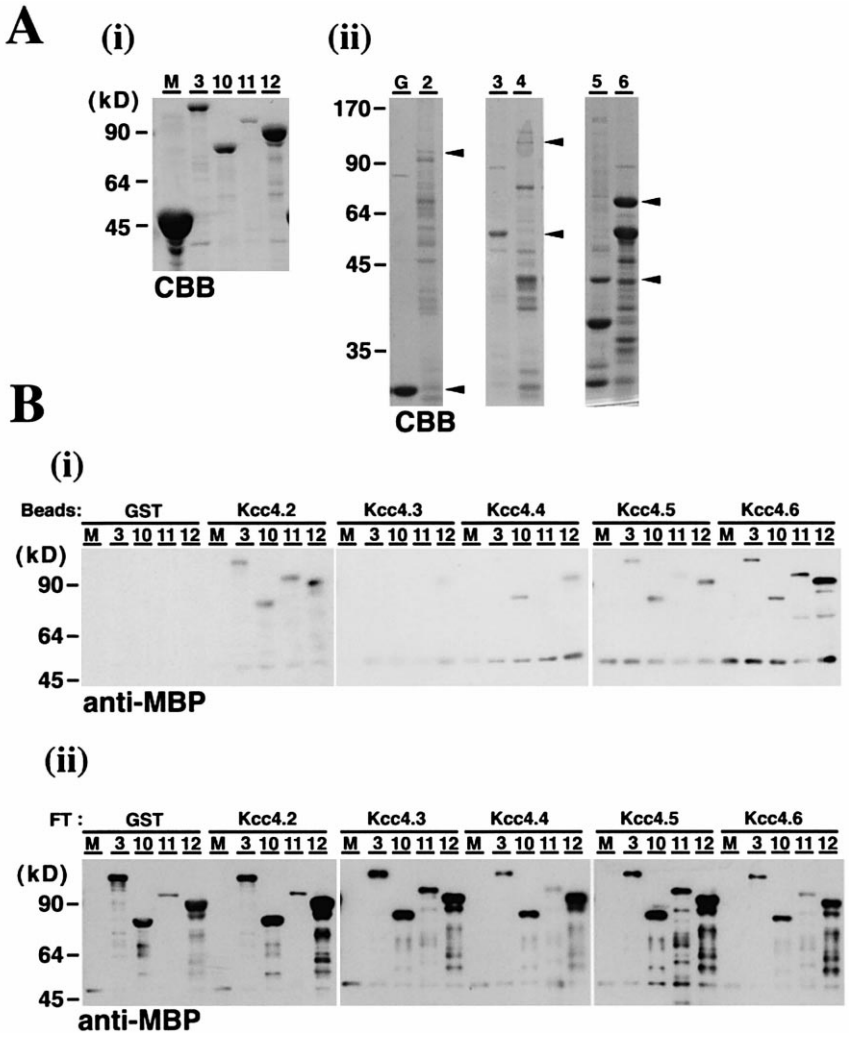

Fig. 4. A GST pull-down assay reveals direct interactions of Kcc4 with septin proteins. A: (i) MBP and MBP-septin proteins that were eluted from amylose resin by PBS containing 10 mM maltose were separated by SDS–PAGE and stained with Coomassie blue (CBB) to show the intactness of the purified MBP-septin proteins. MBP, MBP-Cdc3, Cdc10, Cdc11 and Cdc12 are denoted by M, 3, 10, 11, and 12, respectively. (ii) GST or GST-Kcc4.2∼6 fusion proteins that were bound to glutathione-Sepharose beads were separated by SDS–PAGE and stained with Coomassie blue (CBB). GST and GST-Kcc4.2∼6 are denoted by G, 2, 3, 4, 5 and 6, respectively. B: (i) Interaction between GST-Kcc4.2∼6 beads and purified MBP-septin proteins. Purified MBP-septin proteins were incubated with GST beads alone or with GST-Kcc4.2∼6 protein beads. GST fusion proteins and interacting MBP fusion proteins were isolated using glutathione-Sepharose beads, and analyzed by immunoblotting with anti-MBP polyclonal antibody. (ii) Immunoblotting of the flow-through fractions with anti-MBP antibody to show that an equal amount of each MBP-septin protein was mixed with the GST-Kcc4.2∼6 beads.

3.3. Kcc4 interacts with Cdc11 in vitro and in vivo

Next we performed a GST pull-down experiment to confirm the association of Kcc4 with Cdc11 in cell extracts in vitro. The plasmid constructs expressing Kcc4.1 tagged with a Myc epitope at the N-terminus (SYM2-*KCC4.1*) and Cdc11 tagged with GST at the N-terminus (SYG3-*CDC11*) were placed under the control of the *GAL1* promoter and co-transfected into CB018 cells, and pull-down assays were performed on whole cell extracts using glutathione-Sepharose beads. Proteins released from the beads were analyzed by immunoblotting with anti-Myc antibody. A specific band was detected when GST-Cdc11 was co-expressed with Myc-Kcc4 (Fig. 4A, i). The levels of Myc-Kcc4 (Fig. 4A, i) and GST-Cdc11 (Fig. 4A, ii) in the supernatants, and the amount of Cdc11 precipitated by the beads in each lane (Fig. 4A, ii), were almost equal as judged by the similar intensities of the bands. This result suggests that Kcc4 associates in vitro with the septin complex containing Cdc11.

We then performed immunoprecipitation with the anti-Cdc11 antibody to confirm that Kcc4 associates with the endogenous Cdc11 protein in vivo. The six GFP-Kcc4 constructs (1∼6) were expressed in *kcc4*Δ cells, Cdc11 was immunoprecipitated using the antibody against Cdc11, and the immunoprecipitates were analyzed for the presence of GFP-Kcc4.1∼6 or GFP (a negative control) by immunoblotting with the anti-GFP antibody. The bands for GFP-Kcc4.3, Kcc4.5 and Kcc4.6 specifically coprecipitated with endogenous Cdc11 proteins were observed (Fig. 3B, i, arrowheads in right panel), indicating that Kcc4 (even the partial Kcc4 domain) can associate either directly or indirectly with Cdc11 in yeast cells. The reason why the kinase domain associated with Cdc11 in vivo may be due to some modifications of this

domain or via interaction with other components of the septin complex in yeast cells. The bands for GFP-Kcc4.1, Kcc4.2 and Kcc4.4 could not be detected probably because of the low efficiency of immunoblotting for proteins with high molecular weights as judged by the low intensity of their bands in the supernatant (Fig. 3B, i, left panel) when a similar amount of the cell extract was loaded for electrophoresis (Fig. 3B, ii, an arrowhead).

3.4. Direct association of Kcc4 with septin proteins

To examine whether the Kcc4 protein could bind directly to septin proteins, we performed a GST pull-down assay with affinity-purified MBP-septin proteins (Fig. 4A, i) and affinity-purified truncated versions (Fig. 1B) of GST-Kcc4.2∼6 (Fig. 4A, ii). While little or no MBP-septin protein bound to GST alone (Fig. 4B, i, left panel), significant amounts of GST-Kcc4.2, 5, and 6 bound to all the MBP-septin proteins (Fig. 4B, i, left and right panels). GST-Kcc4.4 associated with MBP-Cdc10 and Cdc12 but its association with MBP-Cdc3 and Cdc11 was very weak (Fig. 4B, i, middle panel). GST-Kcc4.3, containing the N-terminal kinase domain alone, failed to bind to any of the MBP-septin proteins added in equivalent amounts. Immunoblotting of the flow-through fractions with anti-MBP antibody demonstrated that an equal level of septin proteins was mixed with the GST-Kcc4.2∼6 beads (Fig. 4B, ii). These results indicate that the interaction of Kcc4 with septin proteins was direct and that a domain other than the kinase domain of Kcc4 protein was responsible for direct Kcc4 binding to septins.

Localization of Kcc4 at the bud neck has been demonstrated by immunostaining [12] and by expression of GFP-Kcc4 fusion protein in live cells (Fig. 1A), suggesting that Kcc4 is also a component of the septin complex. Indeed, we have shown here that Kcc4 physically interacts with Cdc11 by two-hybrid analysis (Fig. 2), and we confirmed this association in vitro using GST pull-down experiments (Fig. 3A), and in vivo using immunoprecipitation (Fig. 3B). It is of note that Kcc4 associates with Cdc11 through an internal domain, which shares significant structural homology with the growth inhibitory domain of Gin4 [14]. Gin4 is also reported to associate with Cdc3 through its N-terminus, but not to associate with other septins [15]. We propose that this difference in the association partners of Kcc4 and Gin4 may explain the distinct functions of these two closely related kinases, namely, Gin4 receives the bud neck signal from Cdc3, whereas Kcc4 mainly senses the state of septin organization through interaction with Cdc11. Thus, the distinct functions of Kcc4 and Gin4 are reflected in the distinct roles of the septin components with which they interact.

Acknowledgements: We thank Dr. Hiroyuki Araki (NIG, Mishima, Japan) for providing the yeast strains. This work was supported by a Grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan, and grants from the Osaka Cancer Society and Uehara Foundation.

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