Identification of FANCA as a protein interacting with centromere-associated protein E

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This study sought to isolate and identify proteins that interact with centromere-associated protein E (CENP-E), provide new clues for exploring the function of CENP-E in cell cycle control and the pathogenesis of tumor. Yeast two-hybrid screen and regular molecular biologic techniques were undertaken to screen human HeLa cDNA library with the kinetochore binding domain of CENP-E. The bait from the C-terminus of CENP-E was created by subcloning methods to find out optimal candidate proteins that interact with the kinetochore binding domain of CENP-E. Eight novel CENP-E interacting proteins including Homo sapiens Fanconi anemia complementation group A (FANCA) were obtained. In yeast two-hybrid assay, the N-terminal 260 amino acids of FANCA were found to be necessary and sufficient for the interaction with the C-terminus of CENP-E. The interaction was confirmed by in vitro glutathione S-transferase pull-down assay and in vivo co-immunoprecipitation assay. Our finding of the interaction of CENP-E with FANCA demonstrates that CENP-E and FANCA may play important roles in the functional regulation of the mitotic checkpoint signal pathway.

Keywords Fanconi anemia complementation group A (FANCA); centromere-associated protein E; yeast two-hybrid; protein interaction

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Introduction

Cancer is a disease where regulation of the cell cycle goes awry and normal cell growth and behavior is lost. Rigorous quality control steps, termed checkpoints, tightly regulate progression through the cell cycle. Most cancer cells harbor mutations in tumor suppressors and/or oncogenes, which impair certain cell checkpoints. There are at least three DNA damage checkpoints, at G1/S, S, and G2/M as well as a mitotic spindle checkpoint. centromere-associated protein E (CENP-E) is the signal transducing linker responsible for silencing mitotic spindle checkpoint through its capture at kinetochore microtubules [1].

Up to now, there have been reported only four proteins interacting with CENP-E [2–4]. This interaction was initially identified in a yeast two-hybrid screen for proteins that associate with CENP-E. BubR1 and CENP-F were found to bind directly to CENP-E. CENP-E activated BubR1-related checkpoint signaling in a microtubule-dependent manner [5–7], and CENP-E-BubR1 interaction was quite important in controlling the spindle checkpoint pathway [6,7]. Our recent studies revealed that SEP7 formed a link between kinetochore distribution of CENP-E and HuNUF2 provided a link between mitotic motor CENP-E and kinetochore [3,4]. Although our previous studies showed that mitotic kinesin CENP-E formed a link between attachment of spindle microtubules to kinetochores and the mitotic checkpoint [5,7,8], the molecular mechanism underlying kinetochore-microtubule interactions in mammalian cells remains elusive.

To illustrate the molecular mechanisms underlying CENP-E in mitotic checkpoint signaling, we carried out yeast two-hybrid assays to search for CENP-E-binding partners using the kinetochore binding domain of CENP-E as a bait. Among 42 positive clones isolated,
eight CENP-E interacting proteins including Homo sapiens Fanconi anemia complementation group A (FANCA) were obtained. The interaction was confirmed by in vitro glutathione S-transferase pull-down assay and in vivo co-immunoprecipitation assay. Interacting domain analysis revealed that the N-terminal 260 amino acids of FANCA were necessary and sufficient for the interaction with the C-terminus of CENP-E.

Materials and Methods

Plasmid construction
The C-terminus of CENP-E (2131–2701 aa) was digested with BamHI, and then subcloned into pGBK7 vector (BD Clontech, Palo Alto, USA). FANCA cDNA was subcloned into pGADT7 (BD Clontech). All constructs were subjected to sequencing for verification.

Yeast two-hybrid assay
Match-maker GAL4 two-hybrid system 3 (BD Clontech) was used. The following plasmids were used for in vitro and in vivo studies: BD-CENP-E2131–2701aa in pGBK7, AD-FANCA728–1455aa, AD-FANCA1090–1455aa, AD-FANCA1195–1455aa, AD-FANCA1325–1455aa, and AD-FANCA1–728aa in pGADT7. All constructs were sequenced. To test its interaction with CENP-E, the pGBKT7-FANCA plasmids were co-transformed with pGBKT7-CENP-E2131–2701aa into yeast strain AH109. Quantitative measurements of CENP-E 2131–2700aa – as fusion proteins. The purified soluble GST-fused FANCA was pre-bound to glutathione–agarose (Sigma, St. Louis, USA). The column was washed using at least 5 × column volumes of buffer A (10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1 mM PMSF), and then equilibrated with buffer B (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, and 1 mM PMSF). The purified 6 × His-tagged CENP-E2131–2701aa, which was dialyzed against buffer B, was loaded on the column and incubated for 1 h at 4°C, and followed by washing with buffer B. The bound materials were then eluted with buffer C (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, and 1 mM PMSF). GST protein pre-bound column was used as negative control. All fractions collected were subjected to western blotting analysis using His mAb (1:250) (Cell Signal, Beverly, USA).

Cell culture
HeLa and 293T cells, from American Type Culture Collection (Rockville, USA), were maintained as sub-confluent monolayers in DMEM (Invitrogen, Carlsbad, USA) with 10% FBS (Gibco, Gaithersburg, USA) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen, Carlsbad, USA).

Transient transfection and immunoprecipitation
293T cells were grown to about 50–60% confluency in DMEM with 10% FBS at 37°C in 5% CO2. Cells were co-transfected with GFP fused CENP-E2131–2701aa and Flag fused FANCA by 2 μg/ml of Lipofectamine 2000 (Invitrogen), according to manufacturer’s protocols. Cells were collected 24–36 h after transfection, and proteins were solubilized in lysis buffer (50 mM Tris–HCl, pH 7.2, 100 mM NaCl, 2 mM EGTA, 5 mM MgSO4, 0.05% Triton X-100, 1 mM PMSF, 2 mM benzamidine, 10 μg/ml pepstatin A, 0.1 mM leupeptin, and 10 μg/ml aprotonin). The lysates were passed through a 25 G needle 10 times. The lysates were diluted 1:1 with lysis buffer and clarified by centrifugation at 16,000 g for 10 min at 4°C. The lysates were incubated with anti-Flag mAb (about 20 μl beads) (Sigma) bound to protein A/G beads (Pierce Chemical, Rockford, USA) and anti-GFP mAb (about 20 μl beads) (BD Clontech) bound to protein A/G beads, respectively. Beads were washed five times with lysis buffer and then boiled in SDS–PAGE sample buffer for 2 min. Then proteins were transferred to a nitrocellulose membrane. The membrane was incubated with antibody against the GFP epitope (1:2000), Flag epitope (1:1000), and tubulin (1:5000), respectively, and detected with ECL kit (Pierce Chemical).
Results

Human CENP-E used as a bait to screen interacting proteins
Mitotic kinesin CENP-E was highly expressed in human cancerous cell. Therefore an adult human HeLa cDNA library was chosen to search for its putative substrates. The C-terminal 2131–2701 aa of CENP-E was fused and inserted into pGBKTK7 vector to generate pGBKTK7-CENP-E2131–2701aa. This plasmid will be inserted in it a segment of GAL4BD DNA next to the site of bait DNA insertion. Therefore, when the DNA from the plasmid is transcribed and converted to protein, the bait will now have a BD attached to its end. Yeast transformed with BD-CENP-E2131–2701aa was plated on SD/-Trp/-His and SD/-Trp, respectively. After 3 days, no yeast can grow on SD/-Trp/-His; while on SD/-Trp plate, apparent clones can be seen. This phenomenon indicates that BD-CENP-E2131–2701aa cannot activate reporter genes itself. Results indicated that the BD-CENP-E2131–2701aa protein could be used as bait for two-hybrid screening. Western blotting analysis using c-Myc-tag antibody can give the verification of recombinant protein expression in yeast (strain AH109) (Fig. 1).

Identification of FANCA from screening
Yeast strain AH109 transformed with BD-CENP-E2131–2701aa was subsequently transformed with human GAL4AD fusion HeLa cDNA library. Among 1 × 10^6 colonies screened, over 400 big colonies were chosen to grow on the minimal medium (SD/-Trp/-Leu/-His/-Ade/-α-gal plates), and 78 of them showed positive response in the β-galactosidase assay. The 78 plasmids isolated from primary positive yeast clones were transformed into Escherichia coli by electroporation. The pGADT7-specific inserts of plasmids were then amplified by PCR, and the PCR products were characterized by digestion with HaeIII. By agarose gel electrophoresis, the clones having identical size between PCR products and HaeIII digesting products were identified as repetitive clones and discarded. Approximately 36 clones were discarded for this reason. Pseudo-positive clones were detected and eliminated using three criteria. First, isolated clones that could independently activate the reporter gene were discarded. Second, isolated clones that could interact with a non-specific bait plasmid, in this case a GAL4-lamin C fusion protein that did not interact with other proteins, were discarded. Third, isolated clones that could interact with the protein translated from pGBKTK7, which has only the DNA-binding domain, were discarded. A total of 28 auto-activating clones were discarded in this manner.

After the above exclusions, the eight remaining clones including Homo sapiens FANCA (GenBank accession No. NM_000135) were sequenced and their open reading frames were analyzed using BLAST software available on the internet (Table 1).
overlapping bipartite nuclear localization signal, located at aa 18–34 and 19–35. In addition, a partial leucine zipper consensus sequence was located in the region 1069–1090 aa [Fig. 2(A)]. Five deletion constructs corresponding to each domain were constructed and tested for their ability to interact with CENP-E [Fig. 2(B)]. All of these deletions were co-transformed with C-terminus of CENP-E into AH109 cells, respectively. Those positive colonies were selected and β-galactosidase activity (LacZ reporter) was used to measure the protein–protein interaction. As shown in Fig. 2(B), yeast genetic assay indicated that the N-terminal 260 aa of FANCA bind to CENP-E C-terminal tail. Thus, our results from the yeast two-hybrid assay clearly demonstrated that the C-terminal CENP-E (2131–2701 aa) interacted directly with the N-terminal 260 aa of FANCA.

Confirmation of the interaction of FANCA with CENP-E in vivo and in vitro

To validate the interaction between FANCA and CENP-E observed in our yeast two-hybrid assay and test if FANCA forms a complex with CENP-E in vivo, we carried out immunoprecipitation in Flag-FANCA and GFP-CENP-E2131–2701aa co-transfected 293T cells using epitope tag antibodies. Western blotting analysis confirmed that GFP-CENP-E2131–2701aa was pulled down by Flag antibody via Flag-FANCA. Conversely, western blotting analysis using Flag antibody verifies that FANCA was indeed pulled down by GFP-CENP-E2131–2701aa. No Flag-tagged FANCA or GFP-CENP-E2131–2701aa was precipitated with control IgG (Fig. 3). Thus, we conclude that interaction between FANCA and CENP-E is specific.

To test if CENP-E directly binds to FANCA in vitro, we employed GST-FANCA as an affinity matrix and loaded an equal amount of purified 6 × His-tagged CENP-E2131–2701aa fusion protein. GST protein pre-bound column was set as a negative control (Fig. 4). The western blotting analysis probed with GST pAb validates that CENP-E binds to FANCA via its C-terminus. Thus, we conclude that FANCA interacts directly with CENP-E in vitro.

Discussion

CENP-E is a kinesin-related microtubule motor protein that is essential for chromosome congression during
mitosis. The mitotic kinesin CENP-E forms a link between attachment of spindle microtubule to the kinetochore and the mitotic spindle checkpoint signal cascade \[1,5,8\]. CENP-E is a central component in the mitotic spindle checkpoint that modulates signaling activity in a microtubule-dependent manner \[6\]. FA (FA means mutation in Fanconi anemia) is a highly heterogeneous genetic disorder: it consists of at least 13 complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, M, and N), each of which is associated with a distinct disease gene \[9\]. The FA proteins function as signal transducers in a DNA-damage response network. The FA pathway contains two main complexes, the nuclear E3 monoubiquitin ligase core complex (referred to as complex 1) and the chromatin-associated FANCD2/BRCA2 DNA repair complex (referred to as complex 2) \[10\]. Protein complex 1 contains the A, B, C, E, F, G, L, M proteins, and two FAAP proteins (Fanconi anemia associated proteins). Protein complex 2 contains FANCD2-Ub, BRCA2/ FANCD1, PALB2/FANCN, and possibly FANCJ.

During S phase, when a replication fork encounters a DNA cross-link, the FA core complex (complex 1) is activated by phosphorylation \[9\]. This activation leads to the monoubiquitination of FANCD2 and FANCI, which are then targeted to chromatin containing the cross-link \[9\]. FANCD2 is deubiquitinated by USP1, thereby inactivating the pathway \[9,10\]. The FA pathway may function to coordinate several aspects of the DNA damage response including \[1\] direct enzymatic DNA processing \[2\], recruitment of DNA repair proteins involved in HR and TLS, and \[3\] cell cycle checkpoint activation \[11–14\].

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. There are at least three DNA damage checkpoints at G1/S, S, and G2/M as well as a mitotic spindle checkpoint. Many phenotypes have been reported to characterize cells from patients with FA, such as increased chromosome breakage, radial chromosomes, and other cytogenetic abnormalities that occur during metaphase. Another phenotype of FA is an increase in the proportion of cells with 4N DNA content \[15,16\]. This indicates that a G2/M or late S-phase delay occurs in the cell cycle \[17\]. The FA proteins could therefore be involved in the cell cycle checkpoint and DNA-repair pathways. Published evidence suggests that the FA pathway may have roles in the DNA replication, intra-S phase, and G2/M DNA damage checkpoints \[18–24\]. However, FA-associated proteins that are involved in the mitotic spindle
checkpoint pathways have not been identified. Our finding of FANCA-CENP-E interaction provides direct evidence that FA-proteins are involved in the mitotic spindle checkpoint pathway. FANCA and other FA-proteins may thus participate in a novel signal process which must play an important role in the regulation of mitotic progression and maintenance of genomic integrity. A better understanding of the relationship between the FA-proteins and CENP-E may provide a new direction for studies elucidating the mechanisms of cell cycle control, and further development of strategies in FA patients or those carrying a heterozygous mutation.

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