RECQ5/QE DNA Helicase Interacts with Retrotransposon *mdg3* gag, an HIV Nucleocapsid-Related Protein

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(Received August 18, 2005; Accepted October 6, 2005; Published online October 11, 2005)

Three hereditary human disorders, Werner's syndrome, Bloom's syndrome and a subset of Rothmund-Thomson syndrome, are associated with the loss of function of the respective RecQ homologues BLM, WRN, and RTS. These RecQ homologues are composed of a conserved helicase domain flanking C- and N-terminal domains. In contrast to BLM, WRN, and RTS, another RecQ homologue, RECQ5, possesses only short N-terminal region preceding the helicase domain and a long unique C-terminal domain. Although no disease has yet been genetically linked to a mutation in RECQ5, the prominent roles of RecQ helicase in the maintenance of genome stability suggest that RECQ5 helicase is likely to be important *in vivo*. To acquire a better understanding of RECQ5 function, we investigated protein interaction with the C-terminal domain of *Drosophila melanogaster* RECQ5/QE. A portion of *Drosophila melanogaster* retrotransposon *mdg3*, which corresponds to a nucleocapsid protein (gag-NC), was identified by use of the yeast two-hybrid system as interacting specifically with it. Glutathione S-transferase (GST) pull down experiments indicated that the *mdg3* gag-NC bound mainly to an acidic region in the C-terminal domain of RECQ5/QE, which is adjacent to the RecQ helicase domain. The helicase activity of RECQ5/QE was stimulated by *mdg3* gag-NC protein *in vitro*. These data suggest that RECQ5/QE helicase interacts physically and functionally with *mdg3* gag-NC through the acidic region and that RecQ homologue might be involved in retrotransposition and genomic stability.

Key words —— RecQ, nucleocapsid, retrotransposon, recombination, repair, integration

INTRODUCTION

RecQ helicases are evolutionally conserved enzymes required for the maintenance of genome stability.[1] Mutations in 3 of the 5 known human RecQ helicase genes, BLM, WRN, and RTS, cause distinct clinical disorders, *i.e.*, Bloom's, Werner's, and Rothmund-Thomson syndromes, respectively, which are characterized by genome instability and a predisposition toward cancer.[2–4] As with RecQ1, no human genetic disease has been attributed thus far to a deficiency in the RecQ5 gene. RecQ5 has 3 alternative splicing products, the longest of which, RecQ5β, is localized only in nuclei and corresponds to RECQ5/QE in *Drosophila melanogaster*.[5,6] Previously we showed that RECQ5/QE complements several phenotypes of *sgs1*, a mutant of the sole RecQ in yeast, and suggested that RECQ5/QE has common and unique RecQ functions in comparison with BLM and WRN and that the C-terminal domain has a specific function in the absence of Top3.[7] All RecQ family members contain a catalytic helicase domain that comprises 7 highly conserved motifs found in many DNA and RNA helicases. RECQ5/QE protein (1058 aa) is composed of a short N-terminal region, a helicase domain, and a long unique C-terminal domain (~700 aa). It is likely that these non-conserved sequences flanking to the helicase domain are important in functionally differentiating the roles of the RecQ helicases within the cell by either providing additional enzymatic functions, such as the exonuclease activity depen-

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dent upon the N-terminal domain of WRN,[8] or by mediating interactions with other proteins, for example, binding of p53 by the C-terminal domain of WRN.[9] Since no obvious motif was identified in the C-terminal domain of RECQ5/QE,[5] we decided to investigate its interaction with other proteins.

Here we screened a *Drosophila* cDNA library with a RECQ5/QE unique C-terminal sequence as bait by using the yeast two hybrid method. Retrotransposon *mdg3* protein was identified to have a specific interaction with RECQ5/QE. *mdg3* is a long terminal repeat (LTR)-containing retrotransposon of *Drosophila melanogaster* that is related to human immunodeficiency virus (HIV). The implications of these findings for the retroviral life cycle and genome stability are discussed.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening —— To generate pASRECQE∆N, we cloned a DNA fragment corresponding to amino acids 464–1058 of the RECQ5/QE into pAS-2 (Clontech, California, U.S.A.), as described earlier.[7] Yeast strain CG-1945 expressing the Gal4 DNA binding domain (Gal4BD)-RECQ5/QE∆N fusion protein (pASRECQE∆N) was used as a host strain to screen a 3–21 hr *Drosophila* embryo cDNA library as recommended by the supplier of the plasmids and strain (Clontech). The two-hybrid cDNA library inserted in the plasmid pACT2 was used to transform the host strain containing integrated *lacZ* and *HIS3* reporter genes by using a modification of the lithium acetate method of Gietz *et al.*[10] pACT2 utilizes the constitutive *ADH1* promoter for expression of a cDNA-encoded protein as a fusion protein with the activation domain of Gal4 (Gal4AD). An estimated ~10^7 transformants were plated on medium lacking Trp, Leu, and His in the presence of 6 mM 3-aminotriazole. False positives were eliminated by generating Leu^+ Trp^- transformants and assaying them for *β*-galactosidase activity, by co-transforming pACT2 clones and a control bait, pAS-lamin (pLAM5′-1), and by retransforming pACT2 clones into Y190 harboring pASRECQE∆N and reassaying for *β*-galactosidase activity and growth on Trp^- Leu^- His^- medium with 25 mM 3-aminotriazole. Plasmids were recovered from *HIS3*- and *lacZ*-positive clones by rescue in *Escherichia coli* and used to retransform Y190 expressing GAL4BD-RECQ5/QE∆N or other controls to verify the specificity of the two-hybrid interaction. Assays were performed in triplicate. Those cDNAs that induced the reporter genes in a bait-specific manner were identified by DNA sequencing.

Colony-Lift Filter Assay —— Fresh colonies were lifted onto Whatman #5 filters. After the filters had been frozen completely in liquid nitrogen, they were incubated at 30°C in 100 mM $NaPO_4$ (pH7.0) containing 10 mM KCl, 1 mM $MgSO_4$, 0.3% *β*-mercaptoethanol, and 0.3 mg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside.

Preparation of Glutathione S-Transferase (GST)-Fusion Proteins —— pET42b (Novagen, California, U.S.A.) plasmids were used to prepare GST fusion proteins. *ApaL*I-*Sca*I fragments of RECQ5/QE (8–1058 aa) derived from RECQ5/QE cDNA[5] were blunt ended and ligated into the *Stu*I site of pET42b. To construct the C-terminal domain fusion proteins (464–1058 aa), we removed the *Bgl*II fragments from pET42b-RECQ5/QE (8–1058 aa) plasmids. The acidic region of C-terminal domain (464–840 aa) plasmids was generated by ligating the *Bgl*II-*Xho*I fragments of RECQ5/QE into the *Bgl*II-*Xho*I sites of pET42b. The basic region of C-terminal domain (841–1058 aa) plasmids was generated by ligating the *Xho*I fragments of RECQ5/QE into the *Xho*I site of pET42b. GST fusion proteins were expressed and purified from BL21 Star (DE3) cells (Invitrogen, California, U.S.A.) transformed with the pET42b expression plasmids containing various portions of the RECQ5/QE fragments. Overnight cultures of these transformants were inoculated into LB at a 1 : 100 dilution and grown at 37°C to an A_{600} of 0.4–0.6. Isopropyl-*β*-D-thiogalactopyranoside was then added to a final concentration of 0.4 mM, and the cultures were allowed to grow for a further 3 hr. The cultures were harvested, resuspended in cold lysis buffer (20 mM potassium phosphate pH 7.5 containing 10% sucrose, 0.5 mM dithiothreitol, 1 mM PMSF, 1 mM EDTA, and 1 mg/ml lysozyme), and then chilled on ice for 30 min. Cells were then lysed by sonication, and the lysate was centrifuged at 9500 rpm for 60 min at 4°C. The supernatant was stored at –80°C and used for the GST pull down assay.

***In Vitro* Transcription/Translation** —— The *Nco*I-*Xho*I fragments of pACT*mdg3*gagNC were ligated into the *Nco*I-*Sal*I sites of pSPUTK (STRATAGENE, California, U.S.A.). The plasmids were used to generate [^{35}S]methionine-labeled proteins in the TNT-coupled reticulocyte system (Promega, Wisconsin, U.S.A.). The reaction mixtures were incubated at 30°C for 90 min, and the

labeled proteins were immediately used for the GST pull down assay.

GST Pull Down Assay ——— *In vitro* synthesized [^{35}S]methionine labeled *mdg3* gag-NC protein was incubated with GST fusion proteins (1 μg) bound to glutathione Sepharose 4B beads (10 μl, Amersham Biosciences, Buckinghamshire, U.K.) in 150 μl of GST binding buffer (50 mM potassium phosphate pH 7.5, containing 150 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, and protease inhibitors [Roche, Basel, Switzerland]) for 2 hr at 4°C. The beads were then washed 3 times in GST binding buffer, and the labeled proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[5] The gels were dried and analyzed by using a BAS2500 Imaging plate reader (Fuji, Tokyo, Japan).

Helicase Assay ——— The *Sal*I-*Hind*III fragment of pACT*mdg3*gagNC was cloned into *Xho*I-*Hind*III sites of pRSETA vector[11] to produce His-tagged *mdg3* gag-NC protein. The recombinant protein was purified by Probond column chromatography according to the manufacturer's manual (Invitrogen). Full-length RECQ5/QE protein was expressed in a baculovirus/insect cell system and was purified as described previously.[12] Helicase assays were performed using ^{32}P-labeled 20mer annealed with M13mp18 ssDNA, as described earlier.[12] The reaction was initiated by the addition of RECQ5/QE protein and was incubated at 27°C for 10 min. The reaction products were separated on a 12% polyacrylamide gel. Dried gels were analyzed using the BAS 2500 Imaging plate reader (Fuji).

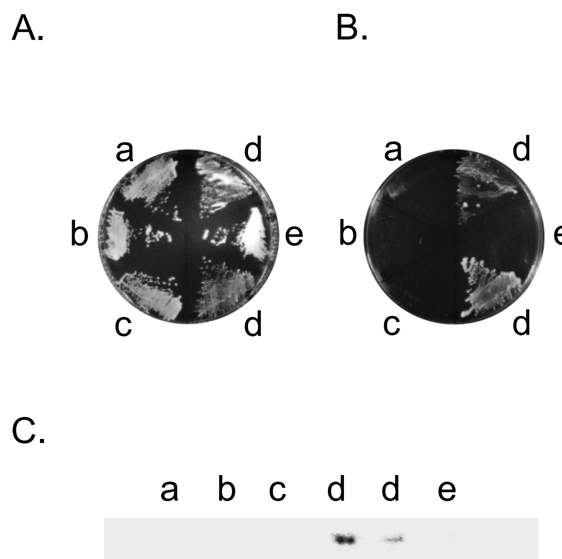

Fig. 1. Retrotransposon *mdg3* gag-NC Interacts Specifically with RECQ5/QE in Yeast Two-Hybrid System

Interaction in the yeast two-hybrid system between retrotransposon *mdg3* gag-NC and C-terminal domain of RECQ5/QE (d). Also shown are 2 negative controls: lamin C (pLAM5′-1, e), which does not interact with *mdg3* gag-NC, and an empty vector control, demonstrating that the C-terminal domain of RECQ5/QE (b) or the *mdg3* gag-NC (c) does not alone activate the reporter genes. Y190 yeast cells containing integrated *lacZ* and *HIS3* reporter genes were co-transformed with plasmids pAS2-1 and pACT2 (a), pASRECQEΔN and pACT2 (b), pAS2-1 and pACT2-*mdg3* gag-NC (c), pASRECQEΔN and pACT2-*mdg3* gag-NC (d, duplicate) or pLAM5′-1 and pACT2-*mdg3* gag-NC (e). The transformed clones were incubated on S.D./-Trp/-Leu (A) and S.D./-HIS (B) plates. The β-galactosidase activities were measured by the colony-lift filter assay (C) as described in MATERIALS AND METHODS.

RESULTS

The yeast two-hybrid screening method was used to identify proteins that interacted with the C-terminal domain of RECQ5/QE. Since RECQ5/QE is expressed most preferentially in early embryos,[5] *Drosophila* embryo cDNA Gal4AD fusion library DNA was introduced into CG1945 harboring Gal4BD fused to the C-terminal domain of RECQ5/QE and screened for being a His^+ Leu^+ Trp^+ auxotroph as described in MATERIALS AND METHODS. Expression of the C-terminal domain of RECQ5/QE as a Gal4BD fusion protein was confirmed by immunoblot analysis of the yeast cell lysates with anti-RECQ5/QE and anti-Gal4BD antibodies (data not shown). Several colonies were obtained whose *HIS3* and *lacZ* reporter genes had been activated. These colonies were considered to contain partners interacting with RECQ5/QE. To further verify this interaction, we isolated the Gal4AD-cDNA plasmids from these colonies and used them for simultaneous co-transformation with pASRECQ5/QEΔN, empty vector, or pAS-lamin as a negative control in Y190 strain. Together with Gal4BD-RECQ5/QE C-terminal domain, a Gal4AD-cDNA clone, pACT*mdg3*gagNC, specifically activated the *HIS3* reporter gene (Fig. 1A and 1B), and also the *LacZ* reporter gene (Fig. 1C), indicating that the activation depends on both the C-terminal domain of RECQ5/QE and the cDNA-encoded proteins. The cDNA sequence was 100% identical to the Gag nucleocapsid portion of *mdg3* DNA (*mdg3* gag-NC, 1091–1739 nt, Fig. 2).[13] Thus, retrotransposon *mdg3* gag-NC and the C-terminal domain of RECQ5/QE specifically activated *HIS3* reporter gene and *lacZ* reporter gene in the yeast two hybrid system, suggesting specific interaction between them (Fig. 1).

A.

LTR gag pol LTR

B.

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      83                                                 132
mdg3  TDYKDAIRCANCNSRGHKADICKKPKREPGSCYACGQLGHLVAQCPTRKS
HIV2  AQQRKVIRCWNCGKEGHSARQCRAPRRQ--GCWKCGKTGHVMAKCPERQA
HIV1  RNQRKIIKCFNCGKEGHIAKNCRAPRKR--GCWKCGKEGHQMKDCTERQA
             C  C    H    C          C  C    H    C
           [   CCHC-BOX1     ]     [   CCHC-BOX2     ]
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Fig. 2. Organization of the Retrotransposon *mdg3* Genome

A. Diagram of retrotransposon *mdg3*. The box shaded in gray shows the gag-coding region containing 2 zinc fingers (gray region is shown in B). The underlined region was cloned in this study (*mdg3* gag-NC). The open box is the pol-coding region. Hatched boxes represent LTRs. B. Amino acid sequence alignments of gag nucleocapsid protein around the 2 zinc fingers in *mdg3*, HIV-2 and HIV-1. Residues indicated by dark or light shading represent identical or similar amino acids, respectively. Numbers on the top represent positions of amino acids from the first Met of *mdg3* gag.

mdg3 is an LTR containing retrotransposon of *Drosophila melanogaster* that is related to HIV. *mdg3* gag-NC was most related to HIV-2 nucleocapsid protein as well as HIV-1 nucleocapsid protein in having the characteristic 2 zinc fingers domains of the CCHC type (Fig. 2B).[14)]

To confirm physical interaction of *mdg3* gag-NC with RECQ5/QE, we performed a GST pull down assay by using various fragments of RECQ5/QE. The *mdg3* gag-NC bound the C-terminal domain of RECQ5/QE but not GST alone in the GST-pull down assay (Fig. 3), thus providing independent confirmation of the *mdg3* gag-NC interaction with RECQ5/QE identified by the yeast two-hybrid system. The *mdg3* gag-NC bound whole RECQ5/QE and the acidic region within the C-terminal domain, but not the basic region. These data suggest that the intact RECQ5/QE molecule interacts with *mdg3* gag-NC through the acidic region.

Since *mdg3* gag-NC binds to the RECQ5QE acidic region, which is adjacent to the helicase domain, this binding may have effects on helicase activity of RECQ5/QE. To investigate functional relationship between RECQ5QE and *mdg3* gag-NC, we purified *mdg3* gag-NC protein by tagging it with His, as described in MATERIALS AND METHODS, and examined its effect on the RECQ5/QE helicase reaction (Fig. 4, lanes 3 and 5). The *mdg3* gag-NC protein enhanced the helicase activity of RECQ5/QE (Fig. 4, lanes 4 and 6). In this condition, the *mdg3* gag-NC itself did not cause any change in strand displacement (data not shown).

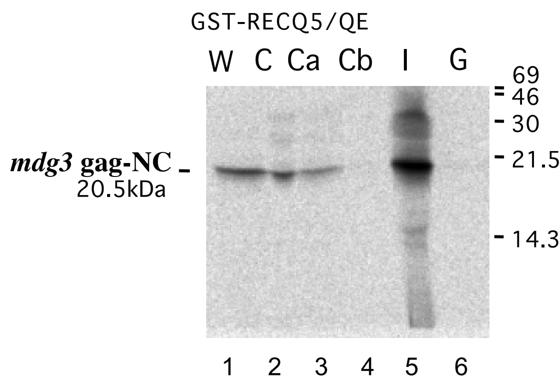
DISCUSSION

In this present study, we demonstrated that RECQ5/QE specifically bound to retrotransposon *mdg3* gag-NC through its acidic region. In addition, RECQ5/QE helicase activity *in vitro* was enhanced by *mdg3* gag-NC protein.

The *mdg3* element is localized at 15–17 sites on different chromosomes of *Drosophila melanogaster*, with its location varying from one animal to another; and it move around mostly to the sites of intercalary heterochromatin.[15)] The ORF of the *mdg3* sequence encodes a putative retrotransposon polyprotein of 1449 aa and a portion of gag-NC was most related to HIV nucleocapsid protein having 2 zinc fingers of the CCHC type (Fig. 2B). Most of what is known about the function of nucleocapsid proteins has resulted from the study of retroviruses.

HIV-1 nucleocapsid protein NCp7, is a small protein with 2 zinc fingers (Fig. 2B), is found in the virion core where several hundred molecules of it coat the viral RNA. Virion NCp7 enters cell with the viral genomic RNA and accumulates in the nucleus after entry.[16)] NCp7 has nucleic acid chaperone properties that guide reverse transcriptase to synthesize the proviral DNA. *In vitro*, NCp7 can

A.



B.

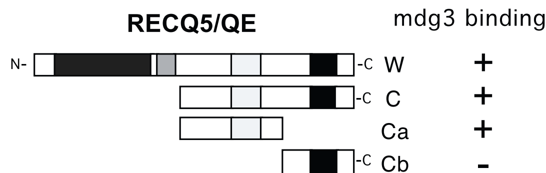


Fig. 3. The *mdg3* gag-NC Binds Whole RECQ5/QE, the C-Terminal Domain, and the Acidic Region, but Not the Basic Region, in the GST-Pull Down Assay

A. Physical interaction between *mdg3* gag-NC protein and RECQ5/QE. [^{35}S]-methionine-labeled *mdg3* gag-NC protein was pulled down by a series of GST-fused RECQ5/QE proteins. Lane 1, whole RECQ5/QE (W); lane 2, C-terminal domain of RECQ5/QE (C); lane 3, acidic region of C-terminal domain (Ca); lane 4, basic region of C-terminal domain (Cb); lane 5, 10% of input (I); lane 6, GST alone (G). Bars at the right side of the panel indicate molecular weight markers (kDa). B. Schematic representation of truncated RECQ5/QE molecules and ability to bind the *mdg3* gag-NC protein. Interaction in the GST pull-down system between *mdg3* gag-NC and various fragments of RECQ5/QE is indicated.

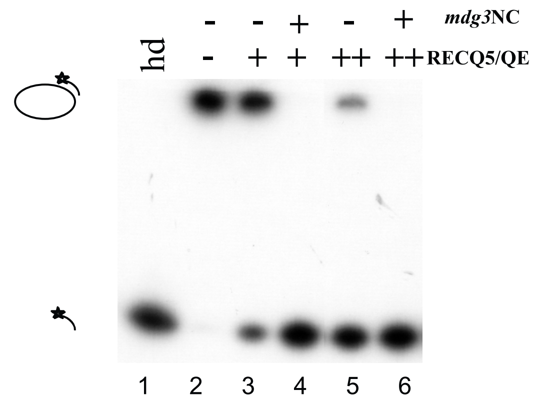


Fig. 4. The *mdg3* gag-NC Enhances the RECQ5/QE Helicase Activity

Helicase assays measure strand displacement activity, whereby a partially double-strand substrate (upper band) is converted to its component single-strand products (lower band, ^{32}P-labeled 20-mer). The helicase activity of RECQ5/QE (0.2 pmol [lanes 3 and 4] and 0.8 pmol [lanes 5 and 6]) was measured in the absence (lanes 2, 3, and 5) and the presence of *mdg3* gag-NC protein (1 pmol, [lanes 4 and 6]) as described in MATERIALS AND METHODS. Lane 1, heat-denatured substrate; lane 2, no RECQ5/QE.

strongly activate magnesium-dependent LTR-DNA strand transfer by integrase (IN).[17] IN activation relies on the zinc fingers of NCp7. It was proposed that these zinc fingers function to stabilize IN at the LTR ends and to promote the formation of a nucleoprotein complex competent for integration.[18] These properties of NCp7 are believed to be conserved among nucleocapsid proteins of retrotransposon and retrovirus origins. RECQ5/QE is mainly localized in the nucleus,[6,19] and the integration process of retrotransposons, like retrovirus, occurs there. Although no *in vitro* system is yet available to study *mdg3* integration, when one becomes available it will be interesting to see if RECQ5/QE affects *mdg3* integration. Furthermore, we should soon be able to determine whether HIV nucleocapsid and human RECQ5β can interact physically and functionally because HIV *in vitro* studies are progressing well.

It is also impressed whether the nucleocapsid protein and RECQ5/QE are involved in the choice of integration sites on chromosomes. HIV-1 preferentially integrates into some sites of chromatin.[20–22] The possible influence of host factors has been implicated in the significant bias among potential integration sites *in vivo*.[23–25] RECQ5/QE helicase is a structure-specific DNA helicase,[12,26] is capable of recognizing specific DNA structures and might recruit the integration machinery to certain regions of the genome. Therefore, RECQ5/QE might be involved in the preference of *mdg3* integration sites. This study would provide new insight into retrotransposition.

Acknowledgements We thank Yasue Ichikawa and Rie Nakazawa for DNA sequencing, and Boris Syomin for providing plasmids. This work was supported in part by the Ministry of Education, Science, Sports, and Culture of Japan, and by the Bioarchitect project of RIKEN.

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