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Isolation and Characterization of DNA Damaging Agent Sensitivity of *rqh1* mutant from *Schizosaccharomyce pombe*

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The *Rqh1* gene is essential for vegetative growth in fission Yeast. The *rqh1* mutant showed that sensitivity of DNA damaging agent, a wild range of phenotype including abnormal gene expression and cell elongation. This result showed that the *rqh1*-overexpression cell was sensitivity to DNA damaging agent like *rqh1* mutant. When Rqh1 have an over-expression by *nmt1*$^+$ promoter of pREP vector, *rqh1* mutant DNA damaging agent sensitivity could be compensated. We isolated two strong mutant containing complementation gene, *rqh1*56 and *rqh1*72, respectively. This result observed that the DNA damaging agent sensitivity of *rqh1* mutant was complemented by the expression of *rqh1*56 and *rqh1*72. They induced mRNA expression in a dose-dependent manner HU, MMS and UV. The HU sensitivity of the *rqh1* was complemented by the expression of *rqh1*56 and *rqh1*72. The mRNA expression of *rqh1*56 decreased on HU dose dependent but the mRNA expression of *rqh1*72 did not decrease on HU dose dependent. The MMS and UV sensitivity of the *rqh1* was complemented by the expression of *rqh1*56 and *rqh1*72. These results indicate that the isolated *rqh1* gene may play an important role in DNA metabolism.

Key words – RecQ Helicase Family, *rqh1*$^+$, Hydroxyurea, Methyl-Methan Sulfonate, Ultraviolet

Introduction

The RecQ helicase family is highly conserved in evolution from prokaryotes to humans and constitutes a sub-family of helicases that contains a characteristic core domain including seven motifs found in many DNA and RNA helicases. Additionally, this domain is also found in certain proteins that are not bona fide helicases, but which utilize ATP hydrolysis to translocate along DNA. This conserved central domain in RecQ helicases are regions that vary both in size and in sequence between the different family members. In eukaryotes, these domains can be quite large, and in the case of WRN an additional catalytic function in the form of a 3'-5' exonuclease activity is conferred by the extended *N-terminal domain*. In other cases, these domains have been shown to mediate physical associations with heterologous proteins[3,5,6].

Fission yeast *rqh1* is a member of the RecQ helicase family which includes budding yeast *sgs1* and human *BLM*, *WRN* and *REQL4* helicases which are implicated in Bloom's, Werner's and Rothmund-Thomson syndromes, respectively. The RecQ-related family of DNA helicases is required for the maintenance of genomic stability in organisms ranging from bacteria to human. RecQ family helicases possess 3' to 5' helicase activity and are required for the maintenance of genomic stability[10,12,13]. The function of RecQ family helicases is always closely linked to Top3 and associated with recombination. In budding yeast, *S. cerevisiae*, *sgs1* deletion mutants also show genomic instability including hyper- recombination of the rDNA locus and a reduced life span that correlates with the accumulation of extrachromosomal rDNA circles. In humans, mutation of three RecQ-related helicases, *BLM*, *WRN* and *RecQL4*, cause the cancer-prone and premature ageing diseases of Bloom syndrome (BS), Werner's syndrome (WS) and Rothmund-Thompson syndrome (RTS), respectively. Cells derived from BS patients show increases in sensitivity to methyl-methane sulfonate, interchange between homologous chromosomes, and sister chromatid exchange (SCE)[8,9,11]. Cells derived from WS patients show chromosome instability, a shorter life span in culture, and accelerated telomere shortening[1,14,25]. RTS cells show chromosome instability, which becomes manifest as chro-

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mosome mosaicism and trisomy[4,14,26].

Rqh1 (also called Rad12 and Hus2) is a member of the RecQ subfamily of DNA helicases[12,15,18,27]. A single allele of *hus2*, *hus2-22* (*rqh1-h2*), was also identified in the screen for *hus* mutants[17,18,24]. For reasons that will become clear below, we have renamed this gene *rqh1*$^{+}$. Defects in RecQ helicases typically result in elevated levels of recombination, and problems with DNA replication and chromosome segregation[24,28]. Like the other *hus* mutants, *rqh1* cells undergo an aberrant 'cut'-like mitosis in HU and are also radiation sensitivity. In the case of *rqh1*$^{-}$ mutants, hyper-recombination is particularly marked following exposure to UV and depletion of deoxynucleotides by HU, which both perturb replication fork progression and result in S-phase arrest. *rqh1*$^{-}$ cells appear to recover normally from S-phase arrest and continue to complete bulk DNA synthesis[18,20]. *S. pombe* RecQ-related helicase, causes cells to display reduced viability and elevated levels of chromosome loss. After S-phase arrest or DNA damage, cells lacking *rqh1*$^{+}$ function display elevated levels of homologous recombination and defective chromosome segregation. In the absence of Rqh1, HJs (Holliday junctions) either remain as a physical link between sister chromatids, preventing their segregation, or are resolved to generate a DSB (double strand break) that is repaired by recombination[1,20,23].

The present study intends to characterize the functional roles of *S. pombe* Rqh1. The *rqh1* mutants are sensitive to DNA damaging agents, how increased levels of 'cut' cells and elevated rates of minichromosome loss following HU treatment. To do this, *rqh1* mutants was isolated. The fission yeast, *S. pombe*, which displays efficient DNA repair systems, was used in this study as a model system for higher eukaryotes. When the Rqh1 have an over-expressed in *rqh1* mutant, we tried to find the complementation gene of sensitivity of DNA damage agent. It would be helpful to define the function of *Rqh1*.

Material and Methods

Strains, cell culture, and genetic methods

E. coli strains were grown on LB media (1% tryptone, 1% sodium chloride, 0.5% yeast extract). Complete (YES) and minimal (SD) growth media were used for growth of *S. pombe*[1]. Complete and minimal growth media for fission yeast was YES and SD with no leucine. YES was 30 g/L glucose, 5 g/L yeast extract, 0.225 g/L adenine, 0.225 g/L histidine, 0.225 g/L lysine, 0.225 g/L uracil, and 0.225 g/L leucine. Minimal medium was 20 g/L glucose, 6.7 g/L yeast nitrogen base w/o amino acid, 0.225 g/L adenine, 0.225 g/L histidine, 0.225 g/L lysine, and 0.225 g/L uracil. Plasmids were constructed by standard techniques[11] and *E. coli DH5a* was used as a host for propagation of plasmids. Transformation of *S. pombe* was performed by the dimethyl sulfoxide (DMSO)-enhanced lithium method[7,22].

The *S. pombe* (*Schizosaccharomyce pombe*) and *E. coli* strains used in this study are listed in Table 1. To make a mutant, pET436 and many strains used (a gift from Tarmar Enoch at Harvard Medical Center). The correct transformants were confirmed by southern blot.

Construction of the rqh1 deletion mutant

To creat *rqh1 deleted mutrants by plasmid shuffling*, the 3.6 kb *NheI-AgeI* fragment of *rqh1*B were deleted. This mutant was unable to complement the HU sensitivity of the *rqh1-h2* allele. An insert bearing the *rqh1* deletion was removed from the pUR19 vector by digestion with *Sac*I and *Sph*I and ligated into *Sac*I and *Sph*I digested pUC19. The 1.7 kb *ura4*$^{+}$ gene was then inserted into the *Not*I digested *rqh1* deletion construct as described previously[11,20]. This plasmid (pTE436, see Table 1: Strains provided by T. Enoch and T. Cech) was digested with *Sac*I and *Sph*I and the linear disruption construct, consisting of the *ura4*$^{+}$ gene flanked by *rqh1*$^{+}$sequences, was isolated. This DNA was used to disrupt one copy of the *rqh1*$^{+}$ gene by the one-step disruption method in an *h*$^{+}$/*h*$^{+}$ *ura4-D18/ ura4-D18* stable

Table 1. Strains and plasmids used in this study

Strain	Genotype
CF199	*h*$^{-}$*leu1-32 his3-D1 ura4-D18 ade6-M210* (wild type)
1589	*h*$^{-}$*leu1-32 his3-D1 ura4-D18 rqh1::ura4*$^{+}$
TE480	diploid *ade-M216/ade-M216 leu1-32/leu1-32 ura4-D18/ ura4-D18 h*$^{+}$/*h*$^{+}$
TE558	diploid *ade6-M210/ade6-M210 leu1-32/leu1-32 ura4-D18/ ura4-D18 h*$^{-}$/*h*$^{-}$
TE786	*rqh1::ura4*$^{+}$ *ura4-D18 ade6-M210* containing Ch^{16}
TE788	*ade6-M210* containing Ch^{16}
TE767	*rqh1::ura4*$^{+}$ *ura4*$^{-}$*D18 h*$^{-}$
Plasmid	**Constructions**
pTE151	rqh1B
pTE152	rqh1A
pTE436	*rqh1*$^{+}$ disruption construct

diploid (TE480, see Table 1 ; a generous gift from G. Cottarel and MS O). The homologous integration event was confirmed by Southern blotting. This strain was crossed to an *h⁻/h⁻ura4-D18/ura4-D18* stable diploid (TE558, see Table 1) to generate a sporulating diploid heterozygous for the *rqh1* deletion. These diploids were sporulated, tetrads were dissected and haploids that were HU sensitive and *ura*$^+$ were identified. The HU-sensitive and *ura*$^+$ phenotypes were found to co-segregate and to segregate 2:2 in all of the tetrads analyzed. One such haploid was picked for further analysis and southern blot analysis was used to confirm deletion of the *rqh1*$^+$ gene (TE767, see Table 1). Further crosses established that *rqh1Δ* was allelic to *rqh1-h2*.

The preparation of Rqh1 overexpression strains

The pREP41-HAN vector was used for Rqh1 overexpression. Cells bearing *rqh1*-overproducing plasmid were first grown in EMM medium containing 4 mM thiamine (transcription repressed), washed three times with EMM medium, and further grown in EMM lacking thiamine for 20 hour (transcription induced at 14-16 hour). The overexpression of HA-Rqh1 was confirmed by western blot using anti-HA antibody (Boehringer Mannheim).

Sensitivity test to hydroxyurea (HU), Methyl-Methan sulfonate (MMS) and ultraviolet (UV)

Cells were grown to mid-log phage, washed with fresh SD medium, and then resuspended at a concentration of 1 × 10^7 cell/㎖. In duplicate, serial 10-fold dilutions were spotted onto SD agar plates containing the damage agent and onto a control plate (none damage agent). HU plates contained 4 mM/㎖ HU. MMS plates contained 0.004 % MMS. UV plates exposed 50 J/㎡ UV. As before, each plate was incubated at 30℃ for 5 days.

Three days cultures of wild type CF199 and 1589 mutants, 1589#56, 1589#72 growing at 30℃ were diluted to 1 × 10^4 cell/㎖. Appropriate dilutions containing 1000 cells were plated onto SD agar plates contained HU, MMS and onto SD agar plates exposed UV which were incubated at 30℃ for 5 days, and then numbers of the colonies were counted.

Results and Discussion

Identification of *rqh1*$^-$(1589) mutant

Goodwin *et al.*[12] examined the phenotypic consequennces of germinating top3 deleted spores and observed abnomal nuclei, multiple septa and elogated cells. However, they were not able to directly examine the role of Top3 in DNA damage repair or chromosome stability because the only viable top3 defective cells examined were, by necessity, also deleted for the rqh1 gene. In our study, we have examined the phenotype of top3 deficient cells by creating top3-ts mutants. Following a temperature shift to the restrictive temperature, we see morphological phenotypes the are very similar to those reported by Goodwin *et al.*[12]. These above results indicate that Rqh1 might play a role in DNA replication step to exert to propagate accurate DNA structures and information to daughter cells. Rqh1 gene was constructed using Dr. MS O's method[20] (Fig. 1). However, their exact roles needed to elucidate in detail. For these purpose, we tried to search the suppressor gene. To explore the cellular functions of rqh1 mutant, the recepient yeast host strain was constructed. The *rqh1::ura4*$^+$ diploid strain was transformed with *rqh1*$^+$ plasmid and sporulated. The plasmid containing *rqh1* deletion allele by *in vitro* mutageneses was inserted into host strain and the

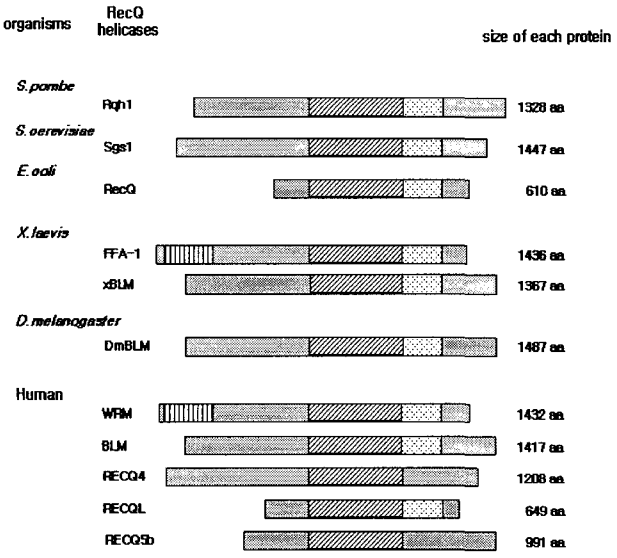

Fig. 1. Schematic representation of RecQ helicases found in various organisms. The size of each protein in amino acid residues is indicated on the right. The conserved helicase and non-conserved terminal domains are depicted by a oblique box and grey boxes, respectively. The region of extended homology C-terminal to the helicase domain is indicated as a spotting box. This domain may be present in all family members, but may have diverged in sequence in RECQ4 and RECQ5. The exonuclease domains of WRN and FFA-1 are shown as striped boxes.

wild type gene was removed by 5-FOA treatments. The *rqh1* deletion mutant alleles were identified by comparison for growth at 30℃. Which its over-expression could compensate the DNA damage sensitivity of *rqh1*1589 mutant, which their HU, MMS and UV sensitivity could be diminished, when they have an over-expressed protein by *nmt1*$^+$ promoter of pREP vector[20] (Fig. 2). To select mutant containing complementation gene, the *S. pombe* cDNA library with *nmt1*$^+$ promoter of pREP vector was transformed into the *rqh1*1589 mutant. the transformants were then selected at 30℃ incubation. Approximately 600 transformants were spotted and screened on selective media for the complementation of DNA damage agent sensitivity (Fig. 3). We identified two potentially mutant containing complementation gene by this procedure. These isolated mutant were renamed, *rqh156*, *rqh172* mutant cell that DNA damaging sensitivity of *rqh1*1589 could be complemented. The complementation of sensitivity in HU, MMS and UV were compared in a *rqh1*1589 and a *rqh156*, *rqh172*. All they were viable to control plate (Fig. 3). However *rqh1*1589 mutant could not grow to plates treated HU, MMS and UV, but similar to control sensitivity, the *rqh156* and *rqh172* were able to grow to HU, MMS and UV at 30℃ (Fig. 3C, 3D, 3E) result indicates that the expression of *rqh156* and *rqh172* functionally complemented the sensitivity of *rqh1*1589 mutant DNA damat.

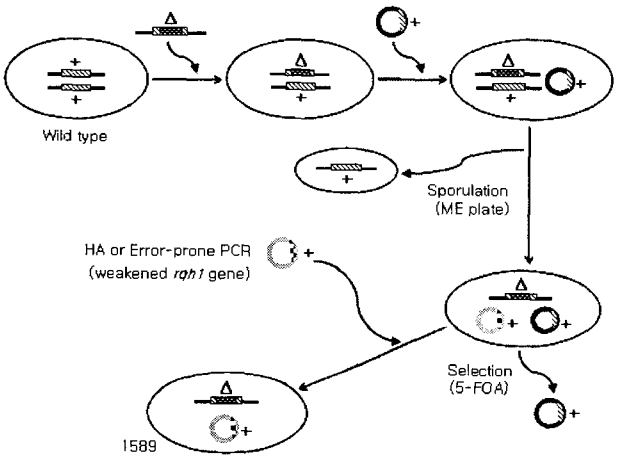

Fig. 2. Schematic diagram of steps involved in preparetion of *rqh1* deletion mutant by plasmid shuffling method. The recipient yeast host strain was constructed. The *rqh1::ura4*$^+$ diploid strain was transformed with *rqh1*$^+$ plasmid and sporulated. The isolation of eposomal *rqh1* deletion mutant. The plasmid containing *rqh1* deletion allele by *in vitro* mutageneses was inserted into host strain and the wild type gene was removed by 5-FOA treatments. The *rqh1* deletion mutant alleles were identified by comparison for growth at 30℃. The (+) symbol refers to wild type and (△) refers to replacement of specific gene with selectable marker gene.

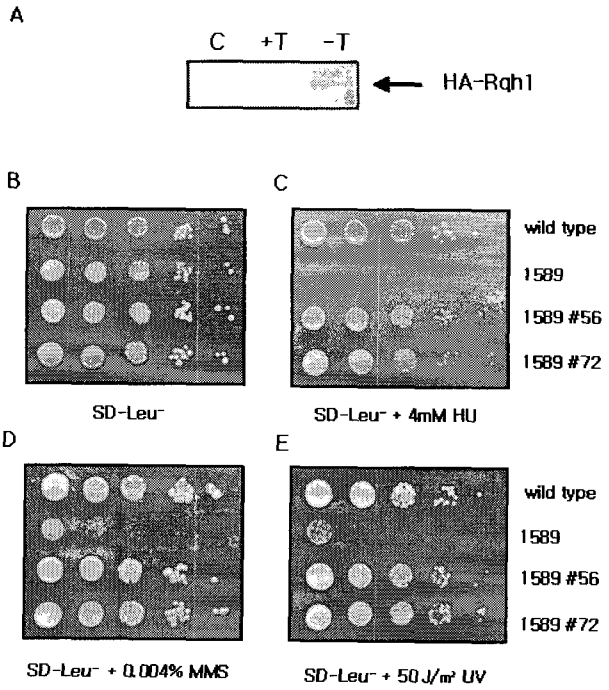

Fig. 3. The complementation of the sensitivity of *rqh1* mutant cell. Rqh1 was fused to three HA epitopes in the pREP41-HAN vector. After induction for 20 hour by the removal of thiamine, HA-Rqh1 was detected by western blot analysis using anti-HA antibody (A). Wild type (CF199), the *rqh1* deletion mutant (1589), compleneted tow mutants (56 and 72) were serially diluted and spotted onto plate treated 4 mM HU or 0.004 % MMS or 50 J/㎡ UV and onto control plate untreated nothing. Each plate incubated at 30℃ for 4 days (B~E).

A viability identification of the cell after the addition of DNA damage agent

We found a mutants survival rate regarding DNA damage agents (HU, MMS, UV). As shown in Fig. 4, it indicated survival rate of wild type (CF199), *rqh1*1589 mutant, *rqh156* and *rqh172* in HU in a dose-dependent manner from 0 to 4 mM HU. The survival of wild type maintained from 0 mM HU to 4 mM HU. The survival of *rqh1*1589 mutant decreased rapidly on HU dose dependent and there is hardly survival rate on 4 mM HU. On the other hand, survival of *rqh156* and *rqh172* maintained like a wild type. The survival of wild type maintained from 0 % MMS to 0.004 % MMS. The survival of *rqh1*1589 mutant decreased rapidly to 0.002 % MMS dose dependent and there is hardly survival rate since (Fig. 4). On the other hand,

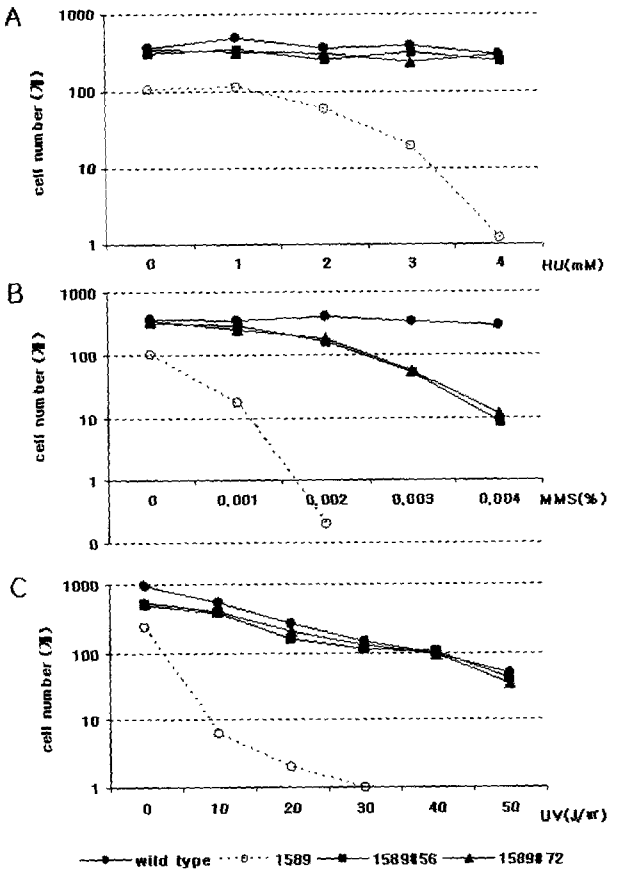

Fig. 4. The relative to survival of *rqh1* mutant cell. The wild-type and mutant cells were incubated onto SD-Leu$^-$ plates treated with HU (A), MMS (B) and UV (C). Each plate incubated at 30℃ for 4 days.

survival of *rqh156* and *rqh172* were lower than a wild type but were complemeted more than *rqh11589* mutant. As shown in Fig. 4C, it indicated survival rate of wild type, *rqh11589* mutant, *rqh156* and *rqh172* in UV in a dose-dependent manner from 0 to 50 J/㎡ UV. The survival of wild type maintained from 0 J/㎡ UV to 50 J/㎡ UV. The survival of *rqh11589* mutant decreased rapidly to 30 J/㎡ UV dose dependent and there is hardly survival rate. On the other hand, survival of *rqh156* and *rqh172* maintained like a wild type.

These results indicated that the *rqh156* and *rqh172* are to have the survival rate such as wild type ulike *rqh11589* mutant about DNA damage agent. Therefore we are regarded that they are complemented according DNA damage agent.

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초록 : 분열형 효모인 *Schizosaccharomyces pombe* 로부터 *rqh1* 돌연변이의 DNA damaging agent sensitivity를 보상하는 유전자의 특성 연구

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분열형 효모에서 Rqh1은 Top3과 함께 vegetative growth에 필수적이다. *rqh*$^-$ 돌연변이는 DNA damaging agent에 민감성을 보이는데 이 때, 부적절한 유전자 발현, 세포 신장, 염색체의 불안전성, 비정상적인 다중격막, 발아의 결핍을 포함한 넓은 범위의 표현형을 보인다. rqh1-overexpression cell 역시 *rqh1* deletion mutant에서 보이는 DNA damaging agent 민감성을 관찰할 수 있다. 논문은 *nmt1* promoter를 가지는 pREP vector에 Rqh1이 과발현 할 때 나타나는 DNA damaging agent 민감성를 보상하는 유전자를 찾아 *rqh1*$^+$의 기능을 알아보는 것이다. 여기서 보상능이 보이는 *rqh156*, *rqh172* 두 개의 돌연변이를 골라냈다. *rqh1* deletion mutant의 DNA damaging agent 민감성은 *rqh156*, *rqh172*의 발현에 의해 보상 되어지는 것을 확인하였다.