Ph.D. thesis

The role of HLTF (*Helicase like transcription factor*) and SHPRH (*SNF2 histone linker PHD RING helicase*) in the replication of damaged DNA

Ildikó Hajdú

Supervisor: Dr. Lajos Haracska

Biological Research Center of the
Hungarian Academy of Sciences
Institute of Genetics

University of Szeged
Faculty of Science and Informatics

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1 Introduction and goals

Genetic and biochemical studies in the yeast *Saccharomyces cerevisiae* have been instrumental in yielding an understanding of the pathways and mechanisms that eukaryotic cells employ to rescue the replication fork stalled at DNA lesion sites. Unless rescued in a timely and orderly fashion, the stalling of replication can lead to DNA strand breaks and result in chromosome rearrangements and enhanced rates of carcinogenesis. The Rad6–Rad18 ubiquitin-conjugating complex of yeast governs at least three alternative pathways that promote replication through DNA lesions: DNA polymerase Polη, Pol ζ and Rev1 dependent translesion synthesis (TLS), and an Mms2–Ubc13–Rad5 dependent error-free postreplicational repair (PRR) pathway. Polη, for example, promotes efficient and relatively error-free synthesis through UV-induced cyclobutane pyrimidine dimers; consequently, inactivation of Polη in both yeast and humans confers enhanced UV mutagenesis and in humans causes the variant form of Xeroderma Pigmentosum (XP-V), a cancer-prone syndrome. The Mms2–Ubc13–Rad5-dependent pathway promotes the repair of discontinuities that form in the newly synthesized strand opposite from DNA lesions. Although the mechanism by which the Rad5 pathway operates is not well known, it likely utilizes a template switching mechanism, wherein the newly synthesized daughter strand of the undamaged complementary sequence is used as the template for bypassing the lesion. Rad5, a member of the SWI/SNF family of ATPases, exhibits a DNA-dependent ATPase activity, and it also has a C3HC4 RING motif, characteristic of ubiqutin ligases. Ubiquitin ligases promote the protein ubiquitylation reaction by binding the cognate ubiqutin-conjugating (UBC) enzyme as well as the protein substrate and by positioning them optimally for efficient ubiqutin conjugation to occur. Rad5 physically associates with the Mms2– Ubc13 complex via Ubc13, and it also interacts with the Rad18 subunit of the Rad6–Rad18 complex. Both the ATPase and ubiqutin ligase activities of Rad5 are essential for PRR, as the repair of discontinuities formed in the newly synthesized DNA in UV irradiated cells becomes as highly impaired in the *rad5* mutants defective in either of these functions as in the *rad5* mutant. In DNA damaged yeast cells, proliferating cell nuclear antigen (PCNA) becomes monoubiquitylated by Rad6–Rad18; subsequently, this lysine residue is polyubiquitylated

via in an Mms2–Ubc13–Rad5- dependent manner. Although the biochemical evidence is lacking, genetic evidence in yeast is consistent with a role of Rad5 acting as a ubiqutin ligase in Mms2–Ubc13-dependent polyubiquitylation of PCNA. All three Rad6–Rad18 dependent lesion bypass pathways are rendered inactive in the absence of PCNA ubiquitylation. Monoubiquitylation of PCNA is necessary for TLS by Polη, Pol ζ and Rev1, and polyubiquitylated PCNA activates the Rad5-dependent PRR pathway. PCNA polyubiquitylation is a necessary prerequisite for the activation of this PRR pathway.

The various elements of the Rad6–Rad18 pathway have been conserved in higher eukaryotes, including in humans. Also, similar to that in yeast, PCNA is monoubiquitylated and then polyubiquitylated in response to treatment with DNA damaging agents. Although for humans the various TLS polymerases that promote lesion bypass in a Rad6–Rad18-dependent manner have been described and the Mms2–Ubc13 complex has been identified, the evidence for a Rad5 counterpart has been lacking thus far.

Our goals were to elucidate whether a homologue yeast Rad5-like damage-bypass system operates in human cells, and to understand how human cells replicate damaged DNA with the following experimental approaches:

1. Database screen to identify the structural homologues of the yeast Rad5

2. Complementation studies

We tested whether the identified structural homologues, HLTF and SHPRH, could complement the UV and RTG sensitivity of yeast strains devoid of Rad5 function.

2. Interaction studies

We examined whether HLTF and SHPRH could associate *in vivo* and *in vitro* with Rad6–Rad18, Mms2–Ubc13, and PCNA.

3. Stimulation of the poliubiquitination of PCNA

We examined whether HLTF and SHPRH could stimulate the poliubiquitilation of PCNA *in vivo* and *in vitro.*

4. DNA fiber studies

We investigated if HLTF has a role in the replication of damaged DNA

2 Methods

Recombinant DNS technics

-Polymerase chain reaction (PCR) and PCR based mutagenesis

-Restrictin enzyme digestions of DNA

-Ligation of DNA fragments

-Agarose gel electrophoresis

Proteins

-Expression and purification of proteins from yeast

-GST pull down

-*In vitro* ubiquitilation

Cell culture

-coimmunoprecipitation

-*In vivo* ubiqutilation

-Analysis of the DNA replication with DNA fiber method

Yeast genetics

-Complementation experiments

Computer based methods

-Multiple alignement os protein sequences

-Protein database filtering

3 Results and discussion

3.1 Identification of the human homologues of the yeast Rad5

Human HLTF shows 43% similarity to *Saccharomyces cerevisiae* Rad5 protein at the amino acid level, while human SHPRH and yeast Rad5 proteins reveals 37% similarity. Both proteins contain the seven motifs found in helicases and the SNF2 family of ATP-hydrolyzing proteins, a RING domain characteristic of ubiquitin ligases, and they share the unusual domain structure in which the RING domain is inserted between helicase domains III and IV. SHPRH has two additional protein domains: a histone H1 and H5 linker sequence and a PHD domain that are not present in Rad5. HLTF and Rad5 have the HIRAN domain in the N terminus that has been proposed to function as a DNA-binding domain for recognizing damaged DNA or a stalled replication fork, followed by a leucine heptad repeat motif. Thus, throughout the entire sequence, HLTF shows a much higher degree of homology to yeast Rad5 than does SHPRH.

3.2 HLTF is able to partially complement the UV sensitivity of a yeast Rad5 deletion mutant

Prompted by the high degree of sequence conservation, we tested whether HLTF and SHPRH could complement the UV sensitivity of yeast strains devoid of Rad5 function. Because we were not able to observe a considerable increase in UV resistance when we expressed HLTF or SHPEH in a *rad5*Δ yeast strain, to increase the sensitivity of the assay further, we examined the complementation of the UV sensitivity of the *rad5*Δ*rad30*Δ strain by HLTF and SHPRH. Because of the simultaneous inactivation of two lesion bypass pathways, the *rad5*Δ*rad30*Δ strain exhibits a much higher level of UV sensitivity than the *rad5*Δ strain. Interestingly, we found that HLTF greatly increases the UV resistance of *rad5*Δ*rad30*Δ mutant cells but SHPRH did not show any complementing effect.

3.3 Complex formation of HLTF and SHPRH with Rad6–Rad18 and Mms2–Ubc13 and PCNA

To establish that HLTF and SHPRH function in human cells in a Rad5-like manner, we next examined whether they could associate *in vivo* and *in vitro* with Rad6–Rad18, Mms2–Ubc13, and PCNA.

In the in vivo experiments we examined these interactions in human embryonic kidney 293FT cells. To facilitate the detection of the interactions, plasmids expressing HA-tagged Rad18, Mms2, Ubc13, or PCNA were cotransfected into cells together with FLAG-tagged HLTF or FLAG-tagged SHPRH. Next, we immunoprecipitated FLAG-HLTF or FLAG-SHPRH and the associated proteins from cell lysates by using anti-FLAG beads, and the precipitated proteins were analyzed for the presence of HA-tagged proteins with anti-HA antibody. We observed a coprecipitation of HLTF with Rad18, Mms2, Ubc13 and PCNA, while only Ub13 coprecipitated with SHPRH.

To provide further evidence for the complex formation of HLTF and SHPRH with Rad6–Rad18 and Mms2–Ubc13, we purified these proteins and tested them for direct physical interaction with GST pull down. We observed that in this *in vitro* experimental condition both HLTF and SHPRH are able to associate with the Rad6-Rad18 and with the Mms2-Ubc13 complex.

3.4 HLTF and SHPRH stimulate the poliubiquitilation of PCNA

In yeast, upon DNA damage Rad6–Rad18 monoubiquitinates PCNA, and the monoubiquitinated form of PCNA is further polyubiquitinated by the combined action of Rad5 and Mms2–Ubc13. Similar to Rad5, complex formation of HLTF and SHPRH with the Rad6–Rad18 and Mms2–Ubc13 enzymes indicated that they might act together as a multisubunit ubiquitin-conjugating complex for PCNA ubiquitination. To explore this possibility, first we examined whether HLTF and SHPRH could promote the polyubiquitination of PCNA.

We examined overexpressed HA–PCNA for ubiquitination in the presence of overexpressed 5His-ubiquitin in HEK293FT cells. In this experimental system, we first enriched His-ubiquitinated proteins on nickel beads followed by detection of

ubiquitinated HA–PCNA by HA antibody and observed that overexpression of HLTF markedly stimulated PCNA polyubiquitination

Next, we investigated whether HLTF and SHPRH can catalyze the polyubiquitination of PCNA *in vitro* by using highly purified proteins. We have already shown that for PCNA monoubiquitination to occur by Rad6–Rad18, PCNA first has to be loaded onto DNA by replication factor C (RFC). Therefore, first we preincubated PCNA with RFC and DNA followed by the addition of ubiquitin, Uba1, and various combinations of HLTF or SHPRH, Rad6–Rad18, and Mms2–Ubc13. As expected, Rad6–Rad18 monoubiquitinated PCNA and the addition of Mms2-Ubc13 to this reaction did not have any effect on PCNA ubiquitination. Importantly, however, the addition of HLTF or SHPRH to the Rad6–Rad18 and Mms2–Ubc13-containing reaction resulted in the polyubiquitination of PCNA, where almost all of the monoubiquitinated form of PCNA was converted to polyubiquitinated species

3.5 HLTF has a role in the replication of damaged DNA

Our findings promted us to investigate whether HLTF promotes the replication of damaged DNA *in vivo.* We examined the effect of knocking down the expression of HLTF by siRNA on the progress of individual replication-forks, on damaged DNA using the chromosomal fiber technique.

We found that the recovery of the progression of the replication fork after DNA damage was impaired in cells in which HLTF level was reduced compared to control cells. Considering those individual replication forks which were active even on the damaged DNA we also compared the rate of replication before and after MMS treatment. These studies revealed that after DNA damaging treatment, the progression of replication forks is slowed down to a much greater extent in HLTF knocked down cells than in control cells. These results indicate that HLTF affects the resumption of DNA synthesis at replication forks stalled at DNA lesion sites.

We have provided biochemical *in vivo* evidences, that two putative human tumor suppressor proteins, HLTF and SHPRH are functional homologues of yeast Rad5 and that they act as a ubiquitin ligase for promoting Mms2–Ubc13-dependent polyubiquitylation of PCNA.

In yeast, the Rad5–Mms2–Ubc13-dependent PRR pathway promotes lesion bypass in an error-free manner. For UV lesion, in addition to this pathway, Polη also contributes to error-free bypass via its role in promoting error-free TLS through cyclobutane pyrimidine dimers. Consequently, the frequency of UV induced mutations rises dramatically when both the Rad5-dependent PRR and Polη-dependent TLS pathways are inactivated. The inactivation of Polη in humans also leads to an increase in the frequency of UV induced mutations and causes the cancer-prone syndrome, the variant form of xeroderma pigmentosum. Also, there is evidence in human cells that inactivation of Mms2 leads to an enhancement of mutation frequencies, and that PCNA polyubiquitylation protects human cells against mutations caused by TLS. Overall, the evidence for both yeast and human cells is consistent with a role for the Rad5–Mms2–Ubc13 complex in promoting error-free lesion bypass.

We expect HLTF and SHPRH to resemble Rad5 in promoting error-free lesion bypass. Such a role predicts that inactivation of HLTF or SHPRH would confer elevated levels of mutagenesis, resulting from the increased dependence on the TLS polymerases for lesion bypass. In this regard, it is of interest to note that loss of heterozygosity at the human chromosomal band region 6q24-q27, where the *SHPRH* gene is located, has been observed in multiple human malignancies, particularly in melanomas and breast and ovarian cancers. Moreover, sequence analysis of 44 melanoma and ovarian tumor cell lines has identified four mutations in the *SHPRH* gene, whereas none of these mutations are seen in normal cell lines; three of these mutations were observed in the hemizygous or homozygous state, indicating a complete lack of functional SHPRH protein in these cell lines. A cancer suppressor role for HLTF is indicated from the observations that loss of HLTF expression accompanied by HLTF promoter methylation occurs in as many as 50% of colon and gastric cancers.

These observations, taken together with our evidence that HLTF and SHPRH are a ubiquitin ligase for mediating Mms2–Ubc13-dependent PCNA polyubiquitylation, and the fact that this pathway promotes error-free replication through the DNA lesions, raise the strong possibility that HLTF and SHPRH function provides an important deterrent to mutagenesis and carcinogenesis in human cells.

3.6 Acknowledgement

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3.7 Publications:

Research papers

Publications:

Unk I*, **Hajdu I*,** Fatyol K, Bermudez V, Hurwitz J, Yoon Jung Hoon, Prakash L, Prakash S, Haracska L.
Human HLTF functions as a ubiquitin ligase for PCNA poliubiquitination.
PNAS, 2008 Mar 11;105(10):3768-73.

* Contributed equally

András Blastyák*, **Ildikó Hajdú***, Ildikó Unk, Lajos Haracska
Role of double stranded DNA translocase activity of human HLTF in DNA damage bypass

* Contributed equally

Under submission

Unk I, **Hajdu I,** Fatyol K, Szakal B, Blastyak A, Bermudez V, Hurwitz J, Prakash L, Prakash S, Haracska L.
Human SHPRH is a ubiquitin ligase for Mms2-Ubc13-dependent polyubiquitylation of proliferating cell nuclear antigen.
PNAS, 2006 Nov 28;103(48):18107-12

Péter Burkovics, **Ildikó Hajdú**, Valéria Szukacsov, Ildikó Unk and Lajos Haracska
Role of PCNA-dependent stimulation of 3'-phosphodiesterase and 3'-5' exonuclease activities of Ape2 in repair of oxidative DNA damage

Under submission

Posters :

International meetings

EMBO conference on DNA recombination
May 19-23 May, 2008, Il Ciocco, Italy
Ildikó Hajdú, Ildikó Unk, Barnabás Szakál, Károly Fátyol, Jerald Hurwitz, Jung-Hoon Yoon, Louise Prakash, Satya Prakash, Lajos Haracska
Indication for a role of HLTF in the replication of damaged DNA

EMBO workshop
„Invasive Growth: A Genetic Program for Stem Cells, Cancer and Cancer Stem Cells"
Third IRCC International Cancer Conference
May 26-29, 2005 Candiolo, Torino-Italy
Kornélia Szabó, Éva Bálint, Tamás Lukacsovich, **Ildikó Hajdú**, Enikő Molnár, Adrienn Hossó, Klarissza Domokos, István Török, Bernard Mechler and Istvan Kiss:
Novel screens for cancer-related genes in Drosophila and *in vitro* cell line models**.**

National meetings

VII. Hungarian Congress in Genetics, 15-17, 2007 Balatonfüred
Hajdú Ildikó, Apolinar Maya-Mendoza, Dean Alan Jackson, Lajos Haracska
Role of Rad18 in the replication of damaged DNA

VII. Hungarian Congress in Genetics, 15-17, 2007 Balatonfüred
Barbabás Szakál, **Ildikó Hajdú,** Szilvia Juhász, Lajos Haracska
Interaction of TLS polymerases with the human homologue of yRAd5

VII. Hungarian Congress in Genetics, 15-17, 2007 Balatonfüred
Péter Burkovics, **Ildikó Hajdú**, Valéria Szukacsov, Ildikó Unk, Lajos Haracska
Ape2 is an anti-mutagene proofreader exonuclease in the replicational machinery

VI. Hungarian Congress in Genetics Április 10-12, 2005 Eger
Ildikó Hajdú, Lajos Pintér, Ildikó Unk, Lajos Haracska:
Indications for a role of HLTF in the replication of damaged DNA

8th Symposium of the Hungarian Biochemical Society
May 12-15, 2003 Tihany
Ildikó Hajdú, Éva Bálint, Kornélia Szabó, Imre Boros, István Kiss:
Identification of novel *dp53* interacting genes using activating transposon mutagenesis in *Drosophila melanogaster*

Presentations:

Straub days, 2004, Szeged
Ildikó Hajdú, Lajos Pintér, Ildikó Unk, Lajos Haracska
Indications for a function of HLTF in the replication of damaged DNA

HHMI 2006, Meeting of International Research Scholars
September 26-29, Janelia Farm Research Campus in Virginia, USA
Lajos Haracska, **Ildikó Hajdú**, András Blastyák, Péter Burkovics, Ildikó Unk
Replication of damaged DNA in yeast and human cells