UBL1, a Human Ubiquitin-like Protein Associating with Human RAD51/RAD52 Proteins

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The RAD51/RAD52-dependent DNA repair pathway is involved in DNA recombination and DNA double-strand break repair in yeast. Although many proteins in the RAD51/RAD52-dependent DNA repair pathway have been identified in yeast, a novel protein(s) that functions with RAD51/RAD52 may also exist in humans. Using a yeast two-hybrid system, we have identified a 12-kDa protein that associates with the human RAD51 and RAD52 proteins. This protein shares significant amino acid homology with the yeast protein SMT3, which functionally associates with the yeast mitosis fidelity protein MIF2. It also shares moderate homology with ubiquitin and several other proteins, including the N-terminus of the RAD23 protein and a ubiquitin cross reacting protein. Therefore, the gene is tentatively designated UBL1 for ubiquitin-like 1. The UBL1 mRNA is expressed in many human tissues, most highly in testis. The UBL1 gene is mapped to chromosome 2q32.2-q33, and a related sequence may be located on chromosome 1q23-q25.

INTRODUCTION

DNA double-strand break (DSB)1 is one of the most important forms of DNA damage caused by ionizing radiation. Efficient repair of DSB is essential for the cell to recover from radiation damage. In yeast, the RAD52 epistasis group genes encode proteins involved in DSB repair and recombination (Friedberg et al., 1991). Several yeast genes in the RAD52-dependent repair pathway have been cloned. These include RAD50, RAD51, RAD52, RAD53 (SPK1), RAD54, RAD55, and RAD57 (Adzuma et al., 1984; Alani et al., 1989; Basile et al., 1992; Emery et al., 1991; Kans and Mortimer, 1991; Lovett, 1994; Schildd et al., 1983; Shinohara et al., 1992; Zheng et al., 1993). It is known that RAD51 is a RecA-like protein that has DNA-binding, ATP-binding, and DNA strand-exchange activities, while RAD54 contains regions homologous to DNA helicase. Interactions among RAD51, RAD51, RAD55, and RAD57 have been identified (Firmenich et al., 1995; Hays et al., 1995; Shinohara et al., 1992; Smith and Rothstein, 1995). RAD52 and RAD54 are highly expressed during yeast meiosis (Cole et al., 1989). Yeast RAD51 and RAD53 also have cell-cycle-dependent expression (Basile et al., 1992; Zheng et al., 1993), and RAD53 also participates in cell cycle control (Sanchez et al., 1996).

In humans, only RAD51 and RAD52 have been reported (Muris et al., 1994; Shen et al., 1995; Shinohara et al., 1993). The human RAD51 protein has activities similar to those of yeast RAD51, including DNA binding and a specific interaction with RAD52 protein (Benson et al., 1994; Shen et al., 1996a). Also, we have found that human RAD52 protein self-associates (Shen et al., 1996b). More recently, RAD51 has been shown to accumulate in the synaptonemal complex, indicating involvement of RAD51 in meiosis and chromosome recombination (Haaf et al., 1995). Human RAD52 overexpression in monkey cells enhances their resistance to radiation and increases the frequency of homologous recombination (Park, 1995). However, little is known about how these interactions might fit into the context of DSB repair.

The importance of DSB repair factors is not limited to their roles in modulating radiation sensitivity, but includes their roles in DNA recombination, in repair of alkylating and cross-linking agent-induced DNA damage, and in some physiological processes. For example, mutations in the yeast RAD52 group genes result in increased sensitivity to the alkylating agent methyl methanesulfonate (MMS). Indeed, the yeast RAD52 gene was cloned by its ability to complement MMS sensitivity (Adzuma et al., 1984; Schildd et al., 1983). The RAD52 gene has been found to function in plasmid
recombination induced by the cross-linking agent psoralen (Han and Saffran, 1992; Saffran et al., 1992). In mammalian immune systems, various types of antibody genes or antigen receptor genes are generated by V(D)J rejoining, where a DNA strand-break is introduced, and the DSB repair machinery completes. In other cases, integration of viral DNA into the host genome may require DSB repair-associated mechanisms. During meiosis, mechanisms of chromosome exchange (recombination) may overlap with DSB repair. However, the mechanism of the RAD52-associated repair pathway is poorly understood, especially in human cells.

To elucidate the DSB repair mechanism in humans, one of the first steps is to identify human proteins involved in DSB repair. It is assumed that some proteins involved in the same repair pathway may associate with each other in the cells. Therefore, one strategy to identify novel proteins participating in the RAD51/RAD52-dependent repair pathway is to identify proteins that actually interact with known proteins, such as RAD51 and RAD52. We have initiated experiments to identify a RAD51/RAD52 interacting protein(s) by using the yeast two-hybrid approach. This approach would also identify a gene(s) involved in meiosis. In this article, we report the cDNA cloning of a ubiquitin-like gene by using the two-hybrid approach. This gene is designated UBL1 for ubiquitin-like 1, as recommended by the Human Gene Nomenclature Committee. Tissue-specific mRNA expression shows that UBL1 expresses highest in testis. By FISH analysis using a cDNA probe and PCR analysis using a panel of mouse hybrid cells that contain a single human chromosome, the UBL1 gene was mapped to chromosome 2q32.2-33; a related sequence may be located on chromosome 1q23-25.

**MATERIALS AND METHODS**

Materials. The yeast strains SFY526 (MATa; ura3-52; his3-200; ade2-101; lys2-801; trp1-901; leu2-3,112; can1; gal4-542; gal80-538; URA3::GAL1-LacZ) and HF7c (MATa, ura3-52; his3-200; lys2-801; ade2-101; trp1-901; leu2-3,112; gal4-542; gal80-538; LYS2::GAL1-HIS3; URA3::GAL4 17mers; CYC1-LacZ) were purchased from Clontech Laboratories (Palo Alto, CA). SFY526 has a LacZ reporter gene fused downstream of the Gal-1 promoter. HF7c has a His reporter gene controlled by a Gal-1 promoter and a LacZ reporter gene controlled by a CYC1 promoter. The Gal-4 DNA activation domain (Gal4-DA) fused cDNA library in pACT vector was also purchased from Clontech. Two hybrid vectors for RAD51 and RAD52 proteins have been previously reported (Shen et al., 1996a,b). SD and YPD media/plates were prepared as described in the two-hybrid system manual (Clontech Laboratories Inc.).

Library screening using the yeast two-hybrid system. The library screening for this yeast two-hybrid system was performed according to the Matchmaker Kit manual (Clontech Laboratory Inc.). Briefly, HF7c yeast was first transfected with pGBT9/RAD51 expressing the Gal-4 DB/RAD51 fusion protein using the polyethylene glycol/lithium acetate method. Yeast HF7c with pGBT9/RAD51 was subsequently transfected with cDNAs isolated from the pACT library. These co-transformed yeast were grown in SD/Try-Leu-His agar plates. A positive clone in SD/Try-Leu-His should contain a pACT plasmid coding for a Gal4-DB/RAD51 interacting protein or a protein that is able to activate the reporter gene (His) without the RAD51 protein. The second case is a false positive and was to be eliminated. To do this, the procedure illustrated in Fig. 1 was used. A clone grown on SD/Trp-Leu-His reporter plasmid, will also not grow in SD/Try-Leu. Therefore, the original clone (No.1) is a true positive. Clones not growing in Trp- (lost pGBT9/RAD51) also do not grow in His-. Therefore, the original clone (No.2) is a false positive.

**FIG. 1.** Procedures to eliminate false positives in a yeast two-hybrid screen. See text for details.

In vivo assay of protein interaction using the yeast two-hybrid system. A description of the protein interaction assay using the yeast two-hybrid system can be found in the manufacturer's Matchmaker manual (Clontech Laboratories) and previous publications (Shen et al., 1996a,b). Briefly, plasmids for the two fusion constructs (one fused with the Gal4-DB, the other fused with Gal4-DA) were co-transformed into the genetically constructed yeast cells SFY526. Transformed yeast cells were grown on Trp-Leu-synthetic agar plates for 3 days to select yeast clones bearing both fusion vectors. To measure the expression of the β-galactosidase (LacZ) reporter gene, which correlates with the interaction of two fusion proteins expressed from these two vectors, LacZ activity in three independent transformants was measured by filter assay (see Matchmaker Manual, and Shen et al., 1996a,b). Quantitative LacZ activity in Miller’s unit (Miller, 1972) was assayed according to the Matchmaker kit manual (Clontech Laboratories). Briefly, yeast from a single clone were grown...
overnight in synthetic media lacking Trp/Leu. The density of yeast was determined by measuring the absorbance at 600 nm. Then, 0.1 ml of culture was mixed with 0.7 ml of Z-buffer (16.1 g/liter Na2HPO4•7H2O; 5.5 g/liter NaH2PO4•H2O; 0.75 g/liter KCl; 0.246 g/liter MgSO4•7H2O; pH 7.0), 50 μl of CHCl3, and 50 μl of 0.1% SDS. O-Nitrophenylgalactoside (4 mg/ml) was used as substrate for LacZ. After 2 h of 30°C incubation, the reaction mix was centrifuged, and the absorbance of the supernatant was read at 420 nm. The LacZ in Miller’s unit was calculated as 1000 [OD 420/ (t + V + OD 600)], where t is time of incubation, V is volume of yeast culture, and OD 600 is the absorbance of yeast culture at 600 nm.

Chromosome localization by cDNA FISH mapping. To map the chromosome, the protocol described by Heng et al. was used (Heng et al., 1992). Briefly, human lymphocytes were synchronized with phytohemagglutinin, and metaphase spreads were made by standard procedures. cDNA probe was biotinylated with dATP using the BRL BioNick labeling kit and hybridized to the chromosome slides. The FISH and DAPI signals were photographed separately. The DAPI chromosome pictures were superimposed on the FISH signal pictures to localize the region better.

DNA sequencing. A primer-walking strategy was used to sequence the cloned plasmid cDNA inserts. Sequencing was performed with the Taq DyeDeoxy Terminator Cycle Sequencing Kit and the ABI 373A automatic DNA sequencer (Applied Biosystems Inc., Foster City, CA) according to the manufacturer’s protocols. Both cDNA strands were sequenced at least once. Sequence editing was performed with the SeqEd software (Applied Biosystems) on a Macintosh computer. Further sequence analysis was performed using the GCG sequence analysis software package (University of Wisconsin, Madison, WI).

Northern hybridization. A multiple-tissue Northern blot containing mRNA from leukocyte, colon, small intestine, ovary, testis, prostate, thymus, and spleen was purchased from Clontech Laboratories. This membrane was sequentially hybridized to the yeast SMT3 protein (GenBank Accession No. U33057), ubiquitin, human RAD23A (HHR23A), human RAD23B (HHR23B), and a ubiquitin cross reacting protein (UCRP). The alignment was performed with the BESTFIT program in the GCG sequence analysis software package (University of Wisconsin, Madison, WI).

The DNA sequence of the cloned fragment was determined by PCR using primers synthesized with an Applied Biosystems Model 394 synthesizer. The DNA sequence of cDNA PL1 was determined by the dideoxy chain termination method using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and the ABI 373A automated DNA sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocols. Both cDNA strands were sequenced at least once. Sequence editing was performed with the SeqEd software (Applied Biosystems) on a Macintosh computer. Further sequence analysis was performed using the GCG sequence analysis software package (University of Wisconsin, Madison, WI).

Other molecular methods. Primers were synthesized with an Applied Biosystems Model 394 synthesizer. pGAD424/UBL1 was constructed by fusing bases 67-375 (Fig. 2) to the Gal4 DNA activation domain in vector pGAD424, and pGBT9/hRAD12 was constructed by fusing bases 67-375 (Fig. 2) to the Gal4 DNA binding domain in pGBT9 vector by PCR using BamHI- and SalI-tagged primers. Detailed procedures and construction of other two-hybrid vectors have been described previously (Shen et al., 1996a).

FIG. 2. cDNA and predicted amino acid sequences. The cDNA clone was isolated by screening a pACT cDNA library (Clontech Laboratories) with pGBT9/RAD51 according to the manufacturer’s instructions and previously described procedures (see Materials and Methods). His-selective positive clones were LacZ assayed in HF7c cells using a different promoter. Yeast clone plasmids were isolated and electroporated into HB101 cells. Growth in M9 (Leu -) minimal media resulted in selection of bacteria containing only the pACT vector. Purified pACT plasmids were sequenced with an ABI 373A automatic DNA sequencer (ABI Biosystems) by using a primer-walking strategy. Bidirectional sequencing and at least two independent sequencing reactions were performed to obtain the cDNA sequence. A potential poly(A) addition signal in the cDNA is underlined. The sequence has been deposited with GenBank under Accession No. U38784.

TABLE 1

Homology between UBL1 and SMT3, Ubiquitin, and Other Ubiquitin-like Proteins

| UBL1 | SMT3 | Ubiquitin | HHR23A | HHR23B | UCRP |
|------|------|-----------|--------|--------|------|
| 100% | 100% | 100%      |        |        |      |
| 72%  | 100% | 100%      |        |        |      |
| 45%  | 43%  | 100%      |        |        |      |
| 52%  | 38%  | 67%       | 100%   |        |      |
| 48%  | 44%  | 70%       | 82%    | 100%   |      |
| 43%  | 43%  | 60%       | 41%    | 41%    | 100% |

Note: The BESTFIT program in the GCG package was used for sequence analysis. Only the N-terminal 85 amino acids were compared for HHR23A and HHR23B.
FIG. 4. Interaction of UBL1 with RAD51 and RAD52 in a yeast two-hybrid system. (A) Interaction of UBL1 with other proteins by LacZ filter assay. (B) Quantitative LacZ assay for UBL1's interaction with other proteins. The numbers in parentheses are the average LacZ activity of at least three independent colonies. Error bars indicate the standard errors. Experimental procedures for two-hybrid liquid assay and LacZ unit definition can be found under Materials and Methods. **LacZ activity is statistically higher than that in the controls as tested by Student's t test (P < 0.001).

RESULTS

cDNA Cloning and Sequence Analysis

UBL1 was identified from a pACT vector-based library through its interaction with the human RAD51 (hRAD51) protein in a two-hybrid system utilizing His and LacZ as selection genes in the HF7c yeast strain (Clontech Laboratories). Subsequently, a 1017-bp cDNA was isolated from 0.3 x 10^6 independent clones (Fig. 2). The cDNA contains an open reading frame starting at base 67 and ending at base 369 (Fig. 2). Because of the characteristic base A at position 64 (Ko-zak, 1984), bases 67–69 were assigned as the translation start codon. This open reading frame codes for a protein of 101 amino acids with a molecular mass of 12 kDa.

Comparison of the amino acid sequence with nonredundant protein databases (including GenBank, Swiss-Port, PIR, etc.) showed no direct match. However, a yeast protein, SMT3 (GenBank Accession No. U33057), has 72% similarity and 52% identity with UBL1 (Fig. 3). Also, many proteins in the ubiquitin family showed moderate homology (40–55% similarity, ~20% identity) to UBL1. Figure 3 shows the alignment of the UBL1 protein sequence to human ubiquitin (Callis et al., 1989) and several ubiquitin-like proteins, including the N-terminal 85 amino acids of the human RAD23 proteins (Masutani et al., 1994; van der Spek et al., 1994) and an interferon-inducible protein (UCRP) (Loeb and Haas, 1992, 1994). Table 1 summarizes the homology between the above-mentioned ubiquitin-like proteins.
UBL1 Interacts with Human RAD52 as well as with RAD51

To confirm its interaction with hRAD51, only the coding region of UBL1 was fused to the Gal4 DNA activation domain in vector pGAD424, which contains a weaker promoter than the pACT vector (Clontech Laboratories). This fusion also eliminated the noncoding region at the 5’-end of UBL1 cDNA. UBL1’s interaction with hRAD51 was further tested in another yeast strain, SFY526 (Clontech Laboratories), using LacZ as the reporter gene. As shown in Fig. 4, neither the vector alone nor UBL1 itself activated the expression of LacZ. Cotransfection of pVD3 (amino acids 72–390 of p53 fused to the Gal4 DNA binding domain in the pGBT9 vector) or pGBT9/UBL1 with pGAD424/UBL1 did not activate the LacZ gene, indicating no association of UBL1 with the truncated p53 nor UBL1 itself. However, when pGBT9/RAD51 or pGBT9/hRAD52 was co-transfected with pGAD424/UBL1, the LacZ gene was activated, indicating an association of UBL1 with hRAD51 as well as hRAD52.

Expression of UBL1 and Human RAD51 mRNA in Testis

Since it has been shown that the human RAD51 mRNA is highly expressed in testis where meiosis and mitosis are active (Shinohara et al., 1993), we further examined the UBL1 mRNA level in several tissues, including testis, by Northern blot (Fig. 5A). It is evident that, although UBL1 mRNA is expressed in all the tissues tested, testis exhibits the highest level of mRNA, which is consistent with human RAD51.

Northern hybridization to human and mouse total RNA showed a single mRNA species of 1.3–1.4 kb (Fig. 5B), indicating that transcripts of this gene exist in human and mouse cells. Since the average poly(A) tail in an mRNA is about 250 bases (Birnstiel et al., 1985) and a putative poly(A) addition site (AAATAA) is identified in the 1017-bp cDNA clone (Fig. 2), this cDNA clone (Fig. 2) is at least close to full length.

Chromosome Localization of UBL1

The chromosome localization of UBL1 was determined by FISH analysis using the full-length cDNA as a probe (Heng et al., 1992; Heng and Tsui, 1993, 1994). Among 100 cells examined, 50 showed paired chromatid signals from chromosome 2q only (Figs. 6A and 6B), 7 showed paired chromatid signals from chromosome 1q only, 32 showed signals from both chromosomes 1q and 2q (Figs. 6C and 6D), and 4 showed paired chromatid signals from chromosome 1q only. Therefore, 82% of the cells showed signals from chromosome 2q, and 39% of the cells showed signals from chromosome 1q. To define the regional localization further, 10

*Fig. 5. mRNA analysis of UBL1. (A) Multiple tissue mRNA blot (Clontech Laboratories) with 2 μg of mRNA on each lane was sequentially hybridized with UBL1 cDNA probe, human RAD51 cDNA probe, and β-actin probe according to the manufacturer's protocol, with stripping between each hybridization. (B) Northern analysis of UBL1 in human skin fibroblast (HSF) and C3H mouse 10½ embryo fibroblast (C3H 10½ cells). The procedure is as described previously (Shen et al., 1995).*
FIG. 6. Chromosome localization of the UBL1 gene. (A and B) cDNA FISH (A) and DAPI banding (B) analysis of UBL1 showing the localization on chromosome 2q. (C and D) Signals can be seen from both chromosome 1q and chromosome 2q. See Materials and Methods for technical details.
partial human chromosome 2 (Chen et al., 1994) were used for regional mapping by PCR analysis (Fig. 8B). As shown in Fig. 8B, while DNA from cell lines containing the region 2q32.3–q33 (cell lines 6CS-5, XHB-78, 6x(neo2)-18, and XRV15b(neo2)-11) showed a positive PCR result, cell lines not containing this region (cell lines 6CS-7 and XHB-104) showed negative PCR results. These data confirmed the localization to 2q32.3–q33.

**DISCUSSION**

Similar to ubiquitin, ubiquitin-like proteins seem to be involved in many cellular processes. For example, a UCRP has been shown to be conjugated to many cellular proteins that are distributed in a cytoskeleton pattern. The N-terminal amino acids homologous to ubiquitin in RAD23 proteins are also essential for RAD23’s DNA repair function (Pejovic, 1995). The location of UBL1 to 2q32.3–q33 and a related gene on 1q23–q24 is worthy of discussion. The 2q33 region has been identified as an aphidicolin-inducible fragile site (Tedeschi et al., 1992), and several cancer cells, such as human small-cell lung carcinoma (Kohno et al., 1994) and ependymomas (Rogatto et al., 1993), have chromosome changes within the region 2q32–q33. A potential tumor suppressor gene has also been mapped to 1q23–q24 (Horikawa et al., 1995). Genetic changes on 1q23–q25 have been observed in ovarian cancers and in Burkitt lymphoma-derived cell lines (Polito et al., 1995). RAD51 shares a moderate sequence homology and some functional similarity (such as DNA binding and filament formation along DNA strands) with the bacterial recombination protein RecA (Benson et al., 1994; Ogawa et al., 1993; Shinoara et al., 1992, 1993). The human RAD52 protein shares significant homology with the yeast RAD52 protein only at the N-terminus (Muris et al., 1994; Shen et al., 1995). Two independent functional domains involved in self-interaction and interaction with RAD51 have been identified (Shen et al., 1996a,b). In addition, expression of human RAD52 in monkey cells enhances radiation resistance (Park, 1995). UBL1’s yeast homolog, SMT3, is a suppressor of the yeast gene MIF2 mutation (see GenBank Accession No. U33057). MIF2 protein is a yeast centromere protein with homology to the mammalian centromere protein CENP-C (Brown, 1995; Meluh and Koshland, 1995). MIF2 is also involved in yeast chromosome segregation (Brown et al., 1993). These results may therefore suggest that UBL1 is also involved in mitosis. Coincidentally, human RAD51 is highly expressed during the S/G2/M phase of the cell cycle in mammalian cells, and human RAD52 in the G2/M phase (unpublished data). We have cloned a human homolog of yeast ubiquitin-conjugation enzyme UBC9, which is involved in S- and M-phase cyclin degradation (Seufert et al., 1995) and mitosis control (Al-Khodairy et al., 1995). This hUBC9-like protein also interacts with UBL1, hRAD52, hRAD51, and p53 proteins (Shen et al., 1996a,b).
FIG. 8. PCR analysis of human chromosome hybrids in mouse cells as described before. (A) PCR from chromosomes 1, 2, 3, 4, 5, 11, 12, 13, and 14 are shown. “Buffer” denotes negative control using buffer alone as the PCR template, “mouse A9” indicates DNA from mouse A9 cells from which the hybrid panel was constructed, and “HSF” denotes total DNA from human skin fibroblast. Other lanes are molecular size markers. PCR results from other human chromosomes are negative and not shown. The primers used are 5′GGTGATCAAGCCTCAGTC (positions 552–569 in Fig. 2) and 5′CCACAGTTCAGTTCTCTG (positions 791–774 in Fig. 2). (B) A few X-ray hybrids, containing partial human chromosome 2, were further analyzed by PCR. Labels at the top denote the cell lines used; other labels are the same as in A. While DNA from cell lines containing the region 2q32.3–q33 (cell lines 6CS-5, XHB-78, 6x(neo2)-18, and XRV15b(neo2)-11) showed a positive PCR result, cell lines not containing this region (cell lines 6CS-7 and XHB-104) showed negative PCR results. For detailed information about these cell lines, please refer to Chen et al. (1994).
ASSOCIATION OF UBL1 WITH HUMAN RAD51/RAD52

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