**HRAD1** and **MRAD1** encode mammalian homologues of the fission yeast rad1+ cell cycle checkpoint control gene

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**ABSTRACT**

Eukaryotic cells arrest at the G₂ checkpoint in the presence of DNA damage or incompletely replicated DNA. This cell cycle checkpoint prevents the development and propagation of genomic instability. In the fission yeast, this process requires the action of a number of genes, including rad1+. We report here the identification of human and mouse cDNAs that exhibit extensive sequence homology to rad1+. The human gene, called HRAD1, encodes a 282 amino acid protein that is 27% identical and 53% similar to yeast Rad1p. The human homologue maintains its sequence similarity over the full length of the protein, including the three proposed 3′→5′ exonuclease domains, and the leucine rich repeat region. The mouse gene, called MRAD1, encodes a 280 amino acid protein that is 90% identical and 96% similar to HRAD1 at the amino acid level. Expression of HRAD1 in yeast rad1 mutants partially restores radiation resistance and G₂ checkpoint proficiency to these mutants. Evolutionary conservation of structure between HRAD1, MRAD1, rad1+, Saccharomyces cerevisiae RAD17 and the Ustilago maydis REC1 checkpoint genes suggests that the function of the encoded proteins is conserved as well. The ability of HRAD1 to partially complement yeast rad1 mutants suggests that this gene is required for G₂ checkpoint control in human cells.

**INTRODUCTION**

Cell cycle checkpoints are regulatory mechanisms that ensure prerequisite events are completed before subsequent cell cycle transitions occur. For example, mitotic entry is dependent on the prior completion of DNA replication. Checkpoints also exist to prevent the propagation of damaged chromosomes that can result from radiation or radiomimetic drugs. These DNA damage checkpoints operate predominantly at the G₁/S and G₂/M transition points (1).

Even in the absence of exogenous DNA damage or blocked DNA replication, checkpoint mutants are known to exhibit genomic instability, as seen in RAD9 mutants of *Saccharomyces cerevisiae* at the G₂/M checkpoint (2), and in *p53*−/− mammalian cell lines at the G₁/S checkpoint (3,4). The accumulation of mutations in cells exhibiting genomic instability has been suggested to be the driving force behind tumour formation and metastasis (5,6). This is supported by studies on individuals with inherited chromosome instability diseases which include ataxia telangiectasia, Li-Fraumeni syndrome and Bloom’s syndrome (7–10). In all three cases, genomic instability and cancer predisposition are seen, with the former operating at the cellular level and the latter at the level of the individual. The genes mutated in these diseases are *ATM*, *p53* and *BLM*, respectively. The *ATM* protein is a member of the PI-3 kinase family (11,12) and *p53* is a transcription factor (13–15), and both are known to have checkpoint functions (16–23). The *BLM* protein has structural homology with known helicases and is also thought to function in checkpoint control (24,25).

A recent report has shown a strong correlation between loss of the G₂ checkpoint and the appearance of chromosomal abnormalities (26), suggesting that the G₂ checkpoint is a major protective factor against the development of genomic instability and cancer. Despite its apparent importance, only two presumptive components of the mammalian G₂ checkpoint have been identified to date (27–29). By contrast, the G₂ checkpoint has been well characterised in the fission yeast *Schizosaccharomyces pombe*. Fission yeast undergo a dose dependent G₂ delay following exposure to radiation and the resultant DNA damage that occurs (30,31). The yeast remain arrested at G₂ while the damage is repaired, then enter mitosis and resume progression through the cell cycle. This dose dependent response to radiation is absent from mutants of any one of the six checkpoint rad genes rad1*, rad3*, rad9*, rad17*, rad26* and *hus1* (30–33). Mutants of any one of these genes have similar phenotypes; they are hypersensitive to radiation and to transiently inhibited DNA replication, such as occurs in the presence of hydroxyurea (HU). The sensitivity of these mutants to radiation and HU results from loss of the G₂ DNA damage checkpoint and the S phase checkpoint monitoring completion of DNA synthesis, respectively (30–33).

The fission yeast rad1* gene has previously been shown to be conserved among lower eukaryotes. *Saccharomyces cerevisiae*
RAD17 (34) and Ustilaginoidea virens RECI (35,36) are functional homologues of rad1+. RAD17 and RECI were shown independently to be required for checkpoint function, and both exhibit moderate sequence conservation with rad1+ (25–30% at the amino acid level). We report here the cloning of human and mouse homologues of the S. pombe rad1+ gene, called HRAD1 and MRAD1, respectively. Expression of HRAD1 in yeast rad1 mutants results in partial restoration of the G2 checkpoint response to radiation. Expression of HRAD1 in these yeast does not restore resistance to HU. We propose that HRAD1 and MRAD1 are components of the G2 checkpoint mechanism in humans and mice, respectively.

MATERIALS AND METHODS

DBJ/EMBL/GenBank accession numbers

The accession numbers for the HRAD1 and MRAD1 cDNA sequences are AF011905 and AF038841, respectively.

cDNA libraries, screening and sequencing

The HaCaT cDNA library in λ ZAP II was a gift of D.Beach, and the CB7 mouse erythroleukemia cDNA library was a gift from P.A.Greer. A probe for screening the HaCaT cDNA library was generated by amplification of a 399 bp portion of the EST sequence (DBJ/EMBL/GenBank accession no. AA029300) using primers A (GGTACATGACCTGGCTCTAT) and B (AGTTCCCACTGGACTATCC), and HaCaT cDNA as template. The full-length HRAD1 cDNA was used as a probe to screen the mouse cDNA library. Library screens were performed using standard techniques (37). Sequencing of both strands of the HRAD1 and MRAD1 cDNAs was performed on an ABI 377 automated sequencer after subcloning into pHBluescript KS-.

Amino acid sequence alignments were generated using the CLUSTAL W program (38). In the amino acid alignment, similar amino acids are defined as I/L/V/M, D/E, S/T, A/G, N/Q, R/K/H and W/F/Y.

Plasmid constructions

The rad1+ cDNA was excised from pGR4-rad1+ (gift of S.E.Sorensen) with BamHI and XhoI, the 3′ recessed ends were filled in with Klenow polymerase, and the cDNA was blunt end ligated into the SmaI site of the S. pombe expression vector pART1 (39), to generate pART1-rad1+. The HRAD1 open reading frame (ORF) was amplified with primers HRAD1-5 (GGACGTCGACATGCCCTCTTGACCACAA) and HRAD1-3 (ACGGATCTCAAGACTCAGATTCAGG), and blunt end ligated into the SmaI site of pART1, to generate pART1–HRAD1. Orientation of the inserts within pART1 was determined by restriction enzyme digestion.

Schizosaccharomyces pombe culture and manipulations

Schizosaccharomyces pombe was cultured using standard techniques (40). The strains used in this study were Sp337, h+N rad1::ura4+ leu1-32, and Sp199, h+N cdc25-22 rad1-1 leu1-32. Sp337 was generated by crossing 975 (40) with Sp267 (41), and Sp199 was generated by crossing SP32 (41) with SP1202 (41). Schizosaccharomyces pombe transformations were performed using the method of Okazaki et al. (42).

Radiation sensitivity and radiation-induced cell cycle delay

X-irradiation was delivered using a Clinac 2100 C/D with a 6 MV beam, at a dose rate of 0.24 Gy/s. UV radiation treatments were performed at 254 nm, with a dose rate of 1.8 J/m²/s. For viability assays, S. pombe was cultured to mid-logarithmic phase (5 × 10⁶ cells/ml) at 25°C, plated on minimal selective media at a density of 1000 cells per plate, and irradiated with the indicated dose of radiation. The plates were incubated at 30°C until colonies were easily visible. Relative viability was expressed as the number of treated versus untreated cells that were able to form colonies.

To assess radiation-induced checkpoint control, plasmids were transformed into a cdc25-22 rad1-1 strain background. These cells were cultured to mid-logarithmic phase at 25°C, plated on pre-warmed minimal selective plates, and incubated at 36°C for 3 h to synchronize the cells in G2. Immediately prior to release from 36°C, the plated cells were irradiated with the indicated dose of UV radiation, transferred to liquid minimal selective media, and incubated at 25°C. Samples were removed at the indicated times and fixed in 3.7% formaldehyde. Fixed cells were washed once with phosphate buffered saline (PBS), once with PBS containing 1% Triton X-100, and resuspended in PBS. The cells were then stained with 0.2 μg/ml 4′6-diamidino-2-phenylindole (DAPI) and viewed under a fluorescence microscope. Binucleate cells were scored as having passed mitosis.

Sensitivity to HU

Schizosaccharomyces pombe was cultured to mid-logarithmic phase at 32°C, and then HU was added to a final concentration of 12 mM. At the indicated times after the addition of HU aliquots of cells were removed, and plated on PM media at a density of 1000 cells per plate. The plates were incubated at 30°C until colonies had reached a suitable size for counting, and relative viability was assessed as described above for radiation sensitivity.

RESULTS

Isolation of the HRAD1 and MRAD1 genes

A search of the dBEST data base revealed an EST of interest obtained from a normalized and directionally cloned human cDNA library (43). The complementary strand of the EST appears to encode a predicted protein similar to the S. pombe rad1+ gene product. This ORF predicted a protein that is 30% identical and 57% similar over an 80 amino acid stretch, which represents approximately one quarter of the Rad1p protein. It is aligned closer to the C-terminal portion of the protein which is a moderately conserved region in the S. pombe rad1+, S.cerevisiae RAD17 and U.maydis RECI gene products. The extent of homology in the region that the EST is aligned with S.pombe rad1+ is comparable to that of rad1+ and RAD17 (44). This same region contains nine identical residues between Rad1p, RAD17p and REC1p, of which seven are also present in the human EST. Based on the alignment and extent of sequence identity, this was evidence for the existence of a possible human homologue of S.pombe rad1+.

Because a positive orientation clone had not been identified in the original library, we chose to search other cDNA libraries for the bona fide human rad1+ homologue. A HaCaT (spontaneously transformed human keratinocyte) cDNA library in λ ZAP II was amplified by PCR using oligonucleotide primers directed against
The full-length HRAD1-7 clone was used to probe a mouse CB7 erythroleukemia cDNA library by low stringency hybridization. Five positives were identified, four of which were the same length, and one was slightly shorter than the others. Clone MRAD1-2.1 was chosen for further analysis.

**Sequence analyses of the HRAD1 and MRAD1 genes**

Full DNA sequences of both strands of the insert of clone HRAD1-7 showed that the cDNA was 1300 bp long with a 214 bp 5′ untranslated region (UTR), an 846 bp coding region and a 240 bp 3′ UTR (Fig. 1A). The 3′ UTR contains a consensus AATAAA polyadenylation signal sequence. The ORF of HRAD1 encodes a 282 amino acid polypeptide with 27% identity and 53% similarity to Rad1p. This is 41 amino acids shorter than the S. pombe rad1+ gene product.

Complete sequencing of both strands of clone MRAD1-2.1 identified a cDNA that was 1380 bp long with a 218 bp 5′ UTR, an 840 bp coding region and a 322 bp 3′ UTR (Fig. 1B). The 3′ UTR contains a common variant of the consensus polyadenylation signal sequence (ATTAAA). However, no poly A tail is observed to Rad1p. The sequence similarity of HRAD1p and MRAD1p to the other structural homologues of the fission yeast S. pombe rad1+ gene product.

**HRAD1 partially rescues the G2 DNA damage checkpoint defects of rad1 yeast mutants**

The HRAD1 ORF was subcloned into the S. pombe expression vector pART1 under control of the strong, constitutive adh1+ promoter. Expression of HRAD1 in a rad1::ura4+ strain background increased the survival of these mutants following UV irradiation, to levels above that of the vector transformed control. Cells expressing wild type HRAD1p also undergo a dose dependent delay in entry into mitosis (Fig. 4B). Yeast expressing HRAD1p also undergo a dose dependent delay in entry into mitosis (Fig. 4C). The dose dependence is not equal to that of cells expressing Rad1p, however, this is what one would expect for partial rescue.

**Expression of HRAD1 restores minimal resistance to HU in rad1::ura4+ yeast**

Expression of HRAD1 in Sp337 confers weak, but statistically significant resistance to the transient DNA synthesis inhibitor HU. However, this rescue is not nearly as high as that observed in other instances, such as HRAD9 rescue of rad9 S. pombe mutants (27). As shown in Figure 5, HRAD1 expressing cells lose viability with kinetics similar to that of the vector transformed control. Cells expressing wild type rad1+ remain viable for at least 6 h in HU (Fig. 5).

**DISCUSSION**

We have identified novel human and mouse genes that are structural homologues of the fission yeast rad1+ checkpoint control gene. The sequence similarity extends over the entire
coding regions, indicating that the isolated cDNAs are full length. Particularly high levels of conservation were seen in two of three putative exonuclease domains, as well as in the leucine rich region that have been previously defined (35). The extent of amino acid conservation between HRAD1p and Rad1p, 27% identity and 53% similarity, is comparable to that observed between Rad1p and RAD17p (23% identity, 50% similarity). Rad1p and RAD17p have been shown by independent means to be involved in checkpoint control in fission and budding yeast, respectively (44). In different regions, HRAD1p and MRAD1p appear more like each of Rad1p, RAD17p and REC1p. Together with the functional complementation of rad1 mutants by HRAD1, and the extent and pattern of structural similarity within this family, HRAD1 and MRAD1 are highly likely to be involved in mammalian G2 checkpoint regulation.

While it has been clearly demonstrated that REC1p is a 3′→5′ exonuclease, it has also been demonstrated that this function is not required for checkpoint control by this protein (35). The sequence similarity between HRAD1p, MRAD1p and other members of the family over the exo II and exo III domains is high, but less so in the exo I domain. The role of the putative 3′→5′ exonuclease in HRAD1p function is questionable at this point.

We were able to show that HRAD1 can partially rescue radiation sensitivity in rad1 mutant yeast. This rescue is due to partial restoration of the G2 checkpoint defect of these mutants, which is shown by the radiation-dose dependent delay experiment (Fig. 4).Checkpoint deficient vector transformed yeast begin to transit mitosis within 40 min of being released to the permissive temperature, regardless of the dose received. The checkpoint proficient yeast overexpressing Rad1p undergo a dose dependent delay in entry into mitosis. The unirradiated cells do not begin to transit mitosis until 60 min after release to the permissive temperature, which is 20 min later than the vector transformed cells. This difference is due to the additive effect of two cell cycle delaying influences, the overexpression of Rad1p and the cdc25-22 mutation, which is not completely wild type even at the permissive temperature. Neither overexpression of Rad1p nor the cdc25-22 allele alone is sufficient to cause the observed delay. Yeast rad1 mutants overexpressing HRAD1p also undergo a dose dependent delay in entry into mitosis. The observed delay is not equivalent to that of the Rad1p expressing cells, but this is what one would expect for partial rescue. The maximal percentage of cells passing mitosis in both Rad1p and HRAD1p expressing yeast is lower than yeast carrying empty vector. This is due to the quality of the synchrony of cells passing mitosis. As the delay increases, the synchrony of the cells begins to diminish. Therefore, the highest percentage of cells passing mitosis is observed in the checkpoint deficient cells, where release from the block is quick. Checkpoint proficient cells will gradually lose synchrony over time and the maximal percentage of cells passing mitosis is lower.

This partial complementation suggests that HRAD1 is the human homologue of fission yeast rad1*. Cross species complementation by checkpoint genes has been demonstrated in other cases, but full complementation of all the defects of any particular mutant has not been observed. HRAD9, the human homologue of S.pombe rad9*, restores resistance to HU in rad9 null mutants, but fails to rescue UV sensitivity (27). FRP1/ATR is the human homologue of S.cerevisiae MEC1/ESR1 and S.pombe rad3* (28,29,45). While FRP1/ATR will rescue some of the checkpoint defects of MEC1/ESR1 mutants, it will not restore checkpoint proficiency to rad3 mutants (29). Further analysis of HRAD1 will be necessary to clearly define its role in human cell cycle checkpoint control.

In mammalian cells, the G1 checkpoint is regulated in part by the p53 and ATM genes, and defects in these genes have been associated with a variety of human cancers (3,4,8–11,16,18,19,21,46,47). By contrast, very little is known about the...
molecular control of the G2 checkpoint in mammalian cells. Like yeast, mammalian cells will respond to DNA damage or incompletely replicated DNA by arresting the cell cycle in G2, prior to entry into mitosis. The presence of such a G2 checkpoint has been shown to correlate with viability after exposure to radiation (48–52).

There are now three candidates for human G2 checkpoint control genes: HRAD1, HRAD9 and FRP1/ATR, homologues of the rad1+, rad9+ and rad3+ genes of S.pombe. To date, none of these has been shown to function in human G2 checkpoint control, though HRAD1, HRAD9 and FRP1/ATR have been shown to rescue some of the defects in checkpoint deficient fission or budding yeast. Interestingly, BRCA1p co-localizes with the repair protein RAD51p, and both are found in regions of meiotic chromosomes similar to where FRP1p/ATRp is located (53,54). This spatial association with RAD51p and FRP1p/ATRp, and evidence that developmental arrest in Brca1 null mice is partially rescued by a p53 mutation indicates a role for BRCA1p in DNA damage repair (55). Genetic evidence from yeast indicates that rad1+, rad3+ and rad9+ are part of the same G2 checkpoint control pathway, and may form a physical complex. This suggests that HRAD1p, as the homologue of Rad1p, may be part of a multisubunit complex that includes other checkpoint proteins including HRAD9p, FRP1p/ATRp, RAD51p and BRCA1p.

Figure 3. HRAD1 expression restores resistance to DNA damage in rad1::ura4+ mutants of S.pombe. Sp337 was grown to mid-logarithmic phase in PM media, plated on PM plates, and irradiated with the indicated doses of radiation. Colonies were counted after 6 days and relative viability is expressed as the number of irradiated cells relative to unirradiated cells that were able to form colonies. (A) The UV dose versus survival curve for Sp337 carrying pART1 (●), pART1-rad1+ (■) or pART1-HRAD1 (▲). (B) The ionizing radiation dose versus survival curve. The symbols are the same as in (A). Both panels are the average of two independent experiments, each performed in duplicate. Error bars indicate standard error of the mean.

Figure 4. HRAD1 expression restores dose dependent radiation-induced cell cycle delay to rad1-1 mutants of S.pombe. Sp199 was grown to mid-logarithmic phase at 25°C, synchronized at the G2/M transition by a 3 h incubation at 36°C, irradiated with the indicated doses of radiation (time zero), and released back to 25°C. At the indicated time points after irradiation, cells were removed, fixed, stained with DAPI and viewed under the fluorescence microscope. The % cells passing mitosis for each sample is the number of binucleate cells expressed as a percentage of the total number observed. Greater than 100 cells were scored for each timepoint. (A–C) Sp199 carrying either pART1 (A), pART1-rad1+ (B) or pART1-HRAD1 (C). In each panel the doses were 0 J/m² (●), 10 J/m² (■) and 30 J/m² (▲). This figure is a representative example of three independent experiments.

Figure 5. HRAD1 expression does not restore resistance to transient DNA synthesis inhibition in rad1::ura4+ mutants of S.pombe. Sp337 was cultured to mid-logarithmic phase at 32°C, HU was added to 12 mM (time zero), and aliquots of cells were removed at the indicated times and plated on PM media. Relative viability is expressed as the number of drug-treated versus untreated cells that were able to form colonies. The symbols represent Sp337 carrying either pART1 (●), pART1-rad1+ (■) or pART1-HRAD1 (▲). The experiment was performed in duplicate and the error bars represent standard error of the mean.
It has been shown that caffeine treatment partially restores sensitivity to radiation in cell lines which have lost G1 checkpoint control through the loss of p53 (56,57). Presumably, the loss of the ability to undergo apoptosis in response to radiation in p53 mutant cells leads to radiation resistance. Caffeine is presumed to eliminate the G2 checkpoint in these cells, leading to radiation-induced death by premature mitosis, typical of checkpoint defective cells. Directly targeting HRAD1 or HRAD1p could be an efficient way of targeting human G2 checkpoint control. If elimination of G2 checkpoint function would restore sensitivity to radiation or chemotherapeutic drugs to cells which have lost G1 checkpoint defective cells. Directly targeting HRAD1 or other G2 checkpoint control genes and protein functions, in conjunction with radio- or chemotherapies.

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