Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1

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Fission yeast Cut5/Rad4 plays a unique role in the genome maintenance as it is required for replication, replication checkpoint, and normal UV sensitivity. It is unknown, however, how Cut5 protein is linked to other checkpoint proteins, and what part it plays in replication and UV sensitivity. Here we report that Cut5 interacts with a novel checkpoint protein Crb2 and that this interaction is needed for normal genome maintenance. The carboxyl terminus of Crb2 resembles yeast Rad9 and human 53BP1 and BRCA1. Crb2 is required for checkpoint arrests induced by irradiation and polymerase mutations, but not for those induced by inhibited nucleotide supply. Upon UV damage, Crb2 is transiently modified, probably phosphorylated, with a similar timing of phosphorylation in Chk1 kinase, which is reported to restrain Cdc2 activation. Crb2 modification requires other damage-sensing checkpoint proteins but not Chk1, suggesting that Crb2 acts at the upstream of Chk1. The modified Crb2 exists as a slowly sedimenting form, whereas Crb2 in undamaged cells is in a rapidly sedimenting structure. Cut5 and Crb2 interact with Chk1 in a two-hybrid system. Moreover, moderate overexpression of Chk1 suppresses the phenotypes of cut5 and crb2 mutants. Cut5, Crb2, and Chk1 thus may form a checkpoint sensor-transmitter pathway to arrest the cell cycle.

[Key Words: UV damage; hydroxyurea; DNA polymerase; phosphorylation; two-hybrid screen]

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The eukaryotic genome is maintained by a number of controlling elements. Weinert and Hartwell (1988) identified a checkpoint gene RAD9 in the budding yeast Saccharomyces cerevisiae, which monitors the damage of DNA on UV irradiation, and delays the progression of cell cycle. In the absence of the RAD9 function, cells containing the damaged DNAs enter fatal cell division without a delay. A number of checkpoint genes involved in DNA damage, replication, and spindle defects have been identified (e.g., Elledge 1996; Kitazono and Matsu moto 1997). The word checkpoint in the present paper is used as synonymous to a surveillance mechanism that blocks or delays cell-cycle transitions (Nasmyth 1996). Cell-cycle regulators are also important elements in maintenance of the genome. When the mitotic cyclin gene, cdc13+, is lost in the fission yeast Schizosaccharomyces pombe, cells are unable to enter mitosis but perform repeated DNA replications, leading to the formation of polyploidy giant nucleus (Hayles et al. 1994). Similar phenotypes can be produced by overproducing Rum1, the inhibitor for CDK/cyclin B (Moreno and Nurse 1994) or constitutively activating Cdc18, an essential replication initiation factor (Kelly et al. 1993; Nishitani and Nurse 1995). In mammalian cells, the genome is maintained by tumor-suppressing proteins such as p53 and RB. p53 is a transcription factor, activating transcription of certain inhibitors against CDK kinase on DNA damage, leading to the arrest of cells in the G1 phase (e.g., El-Deiry et al. 1993). If p53 is lost, the damage checkpoint in the G1 phase no longer seems to be functional. These various gene functions contribute to the quality control of genome.

The fission yeast cut5+ gene is uniquely implicated in the genome maintenance as it is essential for DNA replication, replication checkpoint control, and normal UV sensitivity (Saka et al. 1994a,b). Temperature-sensitive cut5 mutants at the restrictive temperature (36°C) block DNA replication but enter mitosis, producing the cut phenotype (Saka and Yanagida 1993), which strikingly differs from the arrest phenotype of temperature-sensitive cdc mutations in DNA polymerase or ligase, or ribonucleotide reductase (RN R) (Nurse et al. 1976; Gordon and Fantus 1986). The cell-cycle arrest of these replica-
tion-defective cdc mutants is caused by the replication checkpoint control that also operates in the presence of hydroxyurea (HU), an inhibitor of RNR. The double-mutants between cut5 and polymerase or ligase mutants also displayed no retardation in cell division at 36°C (Saka et al. 1994a,b). These results showed that Cut5 was required for the replication checkpoint arrest induced by HU or mutations in the replication enzymes. In S. pombe, the HU-induced replication checkpoint arrest is also abolished in hus and certain rad mutants, rad1, rad3, rad9, rad17, and rad26 (Al-Khodairy and Carr 1992; Enoch et al. 1992; Rowley et al. 1992; Shedrick and Carr 1993; Ford et al. 1994; Griffiths et al. 1995; Bentley et al. 1996; Lieberman et al. 1996; Kostrub et al. 1997).

In addition to the phenotypes described above, cut5 mutants are sensitive to UV irradiation at the permissive temperature. Consistent with the phenotype, isolation of the cut5 gene (Saka and Yanagida 1993) indicated that it was identical to rad4+ (Duck et al. 1976; Fenech et al. 1991). All cut5+rad4 mutants were found to be UV sensitive (Duck et al. 1976; Saka and Yanagida 1993; Saka et al. 1994a). Intriguingly, however, the UV- or 4NQO-induced checkpoint control was retained in cut5 mutants even at 36°C (Saka et al. 1994a,b; F. Esashi, Y. Saka, and M. Yanagida, unpubl.).

Cut5 may sense the replication defect and generate a signal to delay the cell cycle. Multiple phenotypes of cut5 in replication and damage, however, are intriguing, and the actual molecular role of Cut5 in maintaining the genome is unknown. In this study, we addressed the question of what kind of proteins interact with Cut5. Identification of the interacting proteins should shed light on how Cut5 protein functions within cells. Cut5 is a 74-kD nuclear protein consisting of several regions (Saka et al. 1994a). The amino-terminal 100 amino acids long motif repeats twice, and is similar to the regions found in the human repair protein XRCC1 (Thompson et al. 1990), the oncoprotein Ect1 (Miki et al. 1993) and the yeast protein Rev1 (Larimer et al. 1989). This region may be involved in replication or damage, however, is intriguing, and so on. More than 1 × 10^6 S. pombe cut5 cDNA clones were screened for each plasmid. Two identical clones (designated crb2+, cut5-repeat binding) were obtained by the bait of R1R2, whereas eight identical clones (crb3+) were isolated by the bait of R3. Combinations of R1R2 and Crb2, and of R3 and Crb3, produced β-galactosidase activities (Fig. 2A), respectively, as strong as the control interaction (p53 and T-antigen). Other control combinations showed low activities. The interaction of Cut5 with Crb2 or Crb3 was specific as seen by pairwise combinations (Fig. 2B; β-galactosidase positives are blue). No other positive clone was obtained by the screen with the other baits.

An initial investigation for searching Cut5-interacting proteins by the two-hybrid screening method (Fields and Song 1989), five constructs (Fig. 1C) were made as bait, each of which contained a region of Cut5 fused to the GAL4B. GAL4DB-R1R2 thus contained the R1 and R2 region, and so on. More than 1 × 10^6 S. pombe cut5 cDNA clones were screened for each plasmid. Two identical clones (designated crb2+, cut5-repeat binding) were obtained by the bait of R1R2, whereas eight identical clones (crb3+) were isolated by the bait of R3. Combinations of R1R2 and Crb2, and of R3 and Crb3, produced β-galactosidase activities (Fig. 2A), respectively, as strong as the control interaction (p53 and T-antigen). Other control combinations showed low activities. The interaction of Cut5 with Crb2 or Crb3 was specific as seen by pairwise combinations (Fig. 2B; β-galactosidase positives are blue). No other positive clone was obtained by the screen with the other baits.

**Results**

Mutations of cut5/rad4 in the conserved amino-terminus

To locate an essential region in Cut5, we determined mutation sites of cut5. The mutant gene was amplified from three cut5/rad4 alleles [rad4-116; Duck et al. (1976); cut5-580, Hirano et al. (1986); cut5-T401, Samejima et al. (1993)] by PCR and sequenced. Surprisingly, all three mutations contained the same substitution at the 45th codon (from ACG to ATG), leading to the amino acid change T45M (Fig. 1A). An additional alteration (resulting in K62O) was present in cut5-T401. The T45M mutation should create a new EcoT22I site (ATGCAT, Fig. 1B). This was verified by Southern hybridization (Fig. 1B, right), which produced expected sizes of the EcoT22I fragments for the mutant genomic DNAs. The T45M mutation resided in the middle of the first amino-terminal repeat R1 (consensus is indicated in the bottom of panel A; Saka et al. 1994b). T45 is present within the conserved stretch (consensus, VTHLIA), hereafter designated as the TH domain.

Isolation of Crb2

For the two-hybrid screening method (Fields and Song 1989), five constructs (Fig. 1C) were made as bait, each of which contained a region of Cut5 fused to the GAL4B. GAL4DB-R1R2 thus contained the R1 and R2 region, and so on. More than 1 × 10^6 S. pombe cut5 cDNA clones were screened for each plasmid. Two identical clones (designated crb2+, cut5-repeat binding) were obtained by the bait of R1R2, whereas eight identical clones (crb3+) were isolated by the bait of R3. Combinations of R1R2 and Crb2, and of R3 and Crb3, produced β-galactosidase activities (Fig. 2A), respectively, as strong as the control interaction (p53 and T-antigen). Other control combinations showed low activities. The interaction of Cut5 with Crb2 or Crb3 was specific as seen by pairwise combinations (Fig. 2B; β-galactosidase positives are blue). No other positive clone was obtained by the screen with the other baits.

Interaction of Crb2 with Cut5

An in vitro-binding assay described below supported a direct interaction between Crb2 and Cut5. The cDNAs of Crb2 and Crb3 were expressed under the T7 promoter by the in vitro reticulocyte lysate system by use of (35S)metanione for labeling Crb2 and Crb3 (Fig. 2C, left; luciferase was also labeled as control). To examine binding, GST–R1R2 and GST–R3 fusion proteins produced in Escherichia coli and purified by glutathione-agarose beads were incubated with 35S-labeled proteins. The beads were washed, and bound labeled proteins were analyzed by SDS-PAGE and autoradiography (Fig. 2C, right). Radiolabeled Crb2 and Crb3 were detected to be bound to GST–R1R2 and R3, respectively, but luciferase was not. A Crb2 fragment cleaved during incubation was also efficiently bound to GST–R1R2. Another control
with the beads containing only GST showed that neither bound Crb2 nor Crb3.

To examine whether mutant Cut5 T45M retains the ability to interact with Crb2, the mutant R1R2 was isolated and fused to the GAL4DB. Two-hybrid interaction between Crb2 and mutant R1R2 was completely abolished at both 26°C and 36°C (Fig. 2D). Interaction of Cut5 with Crb2, at least at the level of the two-hybrid system, thus requires the TH domain.

Crb2 resembles Rad9 and p53-binding protein

An S. pombe cosmid that contained the genomic crb2+ gene was isolated with cDNA as the probe. Hybridization to an ordered cosmid bank indicated that crb2+ was located near nda2+ in the left arm of chromosome II (Mizukami et al. 1993). The 9-kb PstI fragment containing crb2+ was subcloned from the cosmid (the map shown in Fig. 3A). Plasmid carrying crb2+ (pFE15) fully suppressed the UV sensitivity of cut5-T401 at 26°C, and this suppression was employed for subcloning of crb2+ (+ indicates suppression). The same plasmid, however, did not suppress the temperature sensitive phenotype of cut5-T401 at 36°C (data not shown). Sequencing showed that crb2+ encodes a 778 amino acid protein (predicted molecular mass of 87.5 kD; database accession no. D86478). The near amino-terminal region is required for interacting with Cut5 (data not shown). Database search revealed the highest scores for Rad9 of S. cerevisiae (Weinert and Hartwell 1988) and human 53BP1, which interacts with p53 (Iwabuchi et al. 1994). The carboxy-terminal region of Crb2 is 30% and 25% identical to that of Rad9 and 53BP1, respectively. Crb2 is also similar to the carboxyl terminus of BRCA1, a mammary cancer gene, and other repair and cancer-related genes (Koonin et al. 1996; Callebaut and Mornon 1997). A common domain termed BRCT was proposed from detailed sequence analyses (Bork et al. 1997); a number of damage-responsive proteins including Cut5 also contained the BRCT motif (Fig. 3D). The BRCT-containing domain in BRCA1 is implicated in transcriptional activation.

Crb3 is a WD repeat containing protein

The nucleotide sequence of Crb3 cDNA has been determined (database accession no. D45883): crb3+ encodes a protein with the WD repeats (Neer et al. 1994) partly resembling β-transducin (Fig. 4). The predicted sequence contains 446 amino acids (calculated molecular mass of 49.5 kD) with 6 WD repeats. It is an essential protein for viability and may be implicated in the G1/S progression (T. Matsusaka and M. Yanagida, unpubl.). Structure and functional analyses of the crb3+ gene will be described elsewhere.

Identification and localization of Crb2

Rabbit antibodies against Crb2 were affinity-purified for detection of Crb2 in S. pombe extracts. Diffused immunoblot bands were obtained at the position of 100-110 kD (Fig. 5A, lane 2), the intensity of which increased in cells carrying plasmid pCRB2 (lane 3), but which were absent in acrb2 strain (lane 1). These diffuse bands may be caused by post-translational modification (see below).

Jellyfish green fluorescence protein (GFP) was tagged to the amino-terminus of Crb2 under a moderate promoter REP41 and used to determine intracellular localization of Crb2 (Figure 5B). The nuclear chromatin region (verified by double stain with DAPI; data not shown). To determine whether mutant Cut5 T45M retains the ability to interact with Crb2, the mutant R1R2 was isolated and fused to the GAL4DB. Two-hybrid interaction between Crb2 and mutant R1R2 was completely abolished at both 26°C and 36°C (Fig. 2D). Interaction of Cut5 with Crb2, at least at the level of the two-hybrid system, thus requires the TH domain.

Figure 1. cut5/rad4 mutations reside in the highly conserved T45. (A) Mutation sites for rad4-116, cut5-580, and cut5-T401 were determined to be the identical T45M, residing in the R1 repeat. The amino-terminal regions R1 and R2 are similar to oncoprotein Ect2 (Miki et al. 1993), DNA repair proteins XRCC1 (Thompson et al. 1990), and Rev1 (Larimer et al. 1989). The consensus is indicated below. (B) The cut5/rad4 mutation created a new site for EcoT221 (left). This was verified by hybridization probed with EcoRI fragment containing the cut5+ gene (right). Genomic DNAs from wild-type and mutant DNAs digested with EcoT221 and EcoRI gave rise to the fragments with the expected sizes. (C) Construction of bait for two-hybrid screening. Each of five DNA fragments was placed under GAL4DB. The S. pombe cDNA library associated with the GAL4 activation domain was employed for screening. Crb2 and Crb3 cDNAs were obtained when the amino-terminal R1R2 and R3 were used as bait, respectively.
shown) showed green fluorescence when the promoter was repressed in the presence of thiamine (+Thi). When Crb2 was mildly overproduced in the absence of thiamine (−Thi), the same nuclear region was intensely fluorescent. Hence, Crb2 appears to be a nuclear chromatin protein like Cut5 (Saka et al. 1994). We have been unsuccessful in detecting the stable complex formation between Cut5 and Crb2 in S. pombe extracts, however.

Immunoprecipitation by anti-cut5 antibodies was performed under different buffer conditions. Cut5 was clearly detected in the precipitates, whereas Crb2 was not (data not shown). Conversely, Crb2 was immunoprecipitated by anti-crb2 antibodies, but Cut5 was not detected in the precipitates.

Hypermodification of Crb2 upon irradiation

We examined whether Crb2 was further modified after cells were UV irradiated (100 J/m²) at 26°C. Additional upper bands (apparent molecular mass of 120–150 kD) became visible, greatly intensified after 1–2 hr, and diminished after 4–5 hr (Fig. 5C, top). This transient band-shift clearly indicated the occurrence of strong post-translational modification in Crb2 after irradiation. The intensity of 74-kD Cut5 increased (approximately two-fold) after irradiation (Fig. 5C; second panel). Chk1 kinase has been reported to be transiently phosphorylated in UV irradiated cells (Walworth and Bernards 1996). We compared the timing of Crb2 band-shift with that of Chk1 phosphorylation by immunoblot with anti-PSTAIRE antibodies and a strain integrated with the HA-tagged chk1+ gene (a kind gift of Dr. N. Walworth, Robert Wood Johnson Medical School, Piscataway, NJ; this strain was also used to obtain data for Crb2 modification described above). The upper phosphorylated band of Chk1 was seen at approximately the same timing (Fig. 5C, third panel) as Crb2 modification. Cdc2 detected by anti-PSTAIRE antibodies was shown as control (Fig. 5C, bottom panel). UV irradiation thus induced a change in Crb2 during the arrest of cell cycle (see below).

We wanted to know whether the band shift of Crb2 was produced by phosphorylation, and performed phosphatase treatment of UV irradiated cell extracts as described in Walworth and Bernards (1996) at 30°C and 0°C. The intensity of the unmodified sharp 100-kD band clearly increased after the phosphatase treatment at 30°C (Fig. 5E, second lane). The control Chk1 was also dephosphorylated after phosphatase treatment. The upper 110-kD and 120- to 150-kD bands of Crb2 thus formed at least partly because of phosphorylation. Crb2 in nonirradiating cells thus appeared to be already phosphorylated and this phosphorylation was greatly enhanced further following UV irradiation. This hypermodification of Crb2 is not caused by the cell-cycle arrest, because it did not occur in G2-arrested cdc25 mutant cells (data not shown). UV-induced DNA damage is probably the direct cause of this hypermodification.

To know whether Crb2 and Cut5 were present in mo-
nomeric or oligomeric complexes, sucrose gradient centrifugation of *S. pombe* extracts was run to determine the sedimentation profiles of Crb2 and Cut5 (Fig. 5F).

Cell extracts were first centrifuged at 14,000 rpm for 20 min, and the supernatants were overlaid on the top of a 15%-40% linear gradient, followed by centrifugation at 40,000 rpm for 12 hr. A broad peak ranging from 20S to the near bottom of the centrifugal tube (>50S) was obtained by immunoblot with anti-crb2 antibodies (Fig. 5F, top panel, α-Crb2), suggesting that Crb2 was associated with relatively large heterogeneously sized particles in extracts. For Cut5, the native 74-kD band was also present in the heavy fractions, whereas the cleaved 61-kD band (Saka et al. 1974) sedimented much more slowly (α-Cut5). The cleavage of 74-kD Cut5 occurs during extract preparations and centrifugations, and is difficult to control; the cleaved 61-kD Cut5 band is enriched in slowly sedimenting fractions. Chk1 kinase sedimented very broadly from the top to the bottom of sucrose gradient (α-HA). Fractions of Crb2, Cut5, and Chk1 thus sedimented as large structures though they did not form any cosedimenting peak.

Sedimentation profiles in cell extracts prepared 1 hr after UV irradiation (100 J/m²) differed strikingly from those in nonirradiated cells. The modified forms of Crb2 sedimented very slowly at ∼3–5S (Fig. 5F, bottom panel, α-Crb2), suggesting that Crb2 was associated with relatively large heterogeneously sized particles in extracts. For Cut5, the native 74-kD band was also present in the heavy fractions, whereas the cleaved 61-kD band (Saka et al. 1974) sedimented much more slowly (α-Cut5). The cleavage of 74-kD Cut5 occurs during extract preparations and centrifugations, and is difficult to control; the cleaved 61-kD Cut5 band is enriched in slowly sedimenting fractions. Chk1 kinase sedimented very broadly from the top to the bottom of sucrose gradient (α-HA). Fractions of Crb2, Cut5, and Chk1 thus sedimented as large structures though they did not form any cosedimenting peak.

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We examined whether UV-induced damage checkpoint is abolished in Δcrb2. When wild-type cells were irradiated with UV (100 J/m² at 26°C), their mitotic entry was greatly delayed, and cell number increase was arrested (Fig. 7A, WT). The septation index (SI) decreased for ~2 hr because of the activation of damage checkpoint control (Al-Khodairy and Carr 1992; Rowley et al. 1992). The damage checkpoint mutant rad1-1, however, did not show such delay nor a decrease in SI. We found that Δcrb2 also lost the delay, basically showing the identical damage phenotype to that of rad1-1 (Fig. 7A).

UV-irradiated wild-type cells are known to be elongated with the single nucleus (Fig. 6B, 2 hr after irradiation). In sharp contrast, after irradiation Δcrb2 and rad1-1 cells were divided and the septated cells, with occasional cut phenotype, were frequently seen. The deletion of crb2+ thus led to loss of the cell-cycle arrest in response to UV irradiation. The UV sensitivity of the double mutant cut5 Δcrb2 was identical to Δcrb2, and its UV-induced checkpoint control was also abolished (data not shown).

Polymerase mutant-induced arrest abolished in Δcrb2

We wanted to know whether the cell-cycle arrest phenotypes of polymerase mutants at 36°C were maintained in the absence of crb2+. Double-mutants were constructed by crossing of Δcrb2 with cdc6-121, swi7-H4, or cdc20-M10 (Singh and Klar 1993; Francesconi et al. 1995; Murakami and Okayama 1995). These single polymerase mutants (α, δ, and ε) were arrested at 36°C and the SI decreased (Fig. 7C). The double-mutants, however, were not arrested at 36°C, and the level of SI remained the same after the temperature-shift. The double-mutant cells frequently displayed the cut phenotype at 36°C for 4 hr (Fig. 7D, right), whereas single mutant cells were elongated with the single nucleus (left). Checkpoint control was thus lost if the crb2+ gene was disrupted in the polymerase mutants at 36°C. The phenotype of the other double-mutant with cdc17-K42 (defective in DNA ligase) was also examined. The arrest phenotype was abolished in the double-mutant (data not shown). We concluded from these results that Crb2 was required for the cell-cycle arrest induced by defects in the replication machinery enzymes.

Nucleotide pool-dependent replication checkpoint retained in Δcrb2

We then determined whether the HU-induced arrest was maintained in Δcrb2. In sharp contrast to the results with polymerase mutations, Δcrb2 cells were arrested in the presence of HU (4HU) at 36°C (Fig. 8A). Δcrb2 cells in the presence of HU were elongated with the single nucleus as wild-type cells, and did not show the cut phenotype (right panel). The SI index was strikingly lowered (data not shown). Similar results were obtained at 26°C (data not shown). The HU-induced cell-cycle arrest was thus completely retained in Δcrb2.

Basically the same result was obtained for the double mutant Δcrb2 temperature sensitive cdc22. The cdc22+ gene encodes the large subunit of ribonucleotide reductase (Gordon and Fantes 1986; Fernandez-Sarabia et al. 1993), and is known to be directly inhibited by HU. The double mutant Δcrb2 cdc22 grew normally at 26°C, and was arrested at 36°C, displaying the identical arrest phenotypes of HU-added Δcrb2 or single cdc22 mutant cells (Fig. 8B). These results established that the cell-cycle ar-
rest induced by HU or cdc22 mutation did not require Crb2.

Interaction of crb2+ with other checkpoint genes

We examined whether the UV sensitivity of Δcrb2 can be suppressed by overexpression of other checkpoint genes. For this purpose, plasmids carrying crb2+, cut5+, rad1+, rad3+, rad9+, rad17+, rad26+, or chk1+/rad27+ (Al-Khodairy and Carr 1992; Rowley et al. 1992; Sheldrick and Carr 1993; Al-Khodairy et al. 1994) placed at the downstream of the moderate promoter REP41 (a generous gift of Dr. A.M. Carr, Medical Research Council Cell Mutation Unit, University of Sussex, Brighton, UK, except for the first two strains constructed in the present study) were introduced into Δcrb2, cut5-T401 and Δchk1 strains, and the UV-sensitive phenotype of transformants was investigated. The results are summarized in Table 1. None of the damage-sensing checkpoint genes (rad1+, rad3+, rad9+, rad17+, rad26+) could suppress the UV sensitivity of Δcrb2, cut5-T401, and Δchk1. In contrast, moderate overexpression of Chk1 kinase, a checkpoint signal transmitter, suppressed the UV phenotype of Δcrb2 (see Fig. 6C) and cut5-T401. Moreover, pCRB2...
suppressed the UV sensitivity of cut5, but pCUT5 suppressed neither D\(\text{crb2}\) nor D\(\text{chk1}\). These results suggested that Crb2 played an upstream role for Chk1 and acted at the downstream of, or parallel to, other checkpoint gene products in the UV damage-signaling pathway (see Discussion).

Interactions of Chk1 with Crb2 and Cut5

More evidence for genetic interactions among Crb2, Cut5, and Chk1 was obtained in following experiments. HU, as well as UV, sensitivities were examined for D\(\text{crb2}\) or D\(\text{chk1}\) carrying multicopy vector plasmid (1, 2 in Fig. 9A), pCHK1 (3, 4), pCRB2 (5, 6), or pCUT5 (7, 8). These plasmid-borne cells were spotted after dilution, and cultured at 26°C with the addition of HU to the medium (right, HU) or the pretreatment by UV irradiation (100 J/m\(^2\), UV, middle, +UV) as control. pCHK1 suppressed the HU-phenotype of D\(\text{crb2}\), whereas pCRB2 did not suppress that of D\(\text{chk1}\).

We wanted to know whether the loss of UV-induced checkpoint arrest in D\(\text{crb2}\) could be restored by moderate overproduction of Chk1 (Fig. 9B). REP41, a moderate inducible promoter, was derepressed in the absence of thiamine (−Thi). The ability to arrest cells following UV damage was restored in D\(\text{crb2}\) cells that overexpressed Chk1 (−Thi; shown by a temporal decrease of the SI) but not in cells without overproduction (+Thi), showing that moderate overexpression of Chk1 also suppressed the loss of damage checkpoint. Hence, the UV-induced checkpoint is partly restored in D\(\text{crb2}\) mutant by overproducing Chk1, suggesting that the gene product other than Crb2 might be able to activate Chk1, but in a way much less efficiently.

We found that phosphorylation of Chk1 after UV irradiation required functional Crb2. The upper band of Chk1 formed 1 hr after UV irradiation at 26°C (+UV) in wild type and cut5 mutant cells but did not in D\(\text{crb2}\) (Fig. 9C, +UV); the intensity of Chk1 upper band in cut5 mutant was significantly reduced, however. However, after UV irradiation, Crb2 may become essential for cells to survive by sending a signal for Chk1 phosphorylation, that leads to the accumulation of activated Chk1 (Walworth and Bernards 1996).

Moreover, strong two-hybrid interactions existed between Chk1 and Crb2 and between Chk1 and the central region (Acidic R3R4) of Cut5 (Fig. 9D). The degree of interactions were the highest for Chk1 and Crb2, whereas the interaction between Chk1 and the region of Cut5 spanning from the acidic to R3R4 region (acidicR3R4) was high. No two-hybrid interaction was found between Chk1 and R1R2 of Cut5, however. The interaction between Chk1 and R3R4 of Cut5 was relatively weak. Immunoprecipitation experiments, however, did not show the presence of a detectable stable complex. Neither Chk1 and Crb2, nor Chk1 and Cut5, existed as the stable complex in cell extracts.

Discussion

We report in this study (1) identification and characterization of crb2\(^+\), an S. pombe checkpoint gene, which is required for both DNA damage and replication machinery checkpoint; and (2) interactions among three checkpoint gene products Cut5, Crb2, and Chk1 for proper maintenance of the genome. Cut5 and Crb2 contain BRCT-motif present in a number of damage-responsive proteins, whereas Chk1 is a protein kinase. Although the stable complex has not been detected in S. pombe extracts, evidence for their putative direct interaction is presented. Functions of Crb2 and Cut5 are distinct, but overlapping. Crb2 is responsive to the DNA damage, whereas Cut5 responds to the impaired precursor nucleotide supply for replication. Both Crb2 and Cut5,
Cut5 and Crb2 may act as sensors that sense damage and/or replication defect, and generate signals to activate Chk1 for regulating Cdc2. Thus, Cut5, Crb2, and Chk1 may form a checkpoint sensor-transmitter pathway to arrest the cell cycle.

The present paper also shows that replication checkpoints present in *S. pombe* may be categorized into two classes: one caused by the defect in replication machinery (mutations in polymerases and ligase), and the other caused by the inhibition of normal nucleotide supply (HU and cdc22 mutation). Crb2 is required only for the former, but Cut5 is necessary for both (Saka et al. 1994a,b). A possible explanation for this difference is that the mutant polymerase and ligase enzymes may

Table 1. UV sensitivity of Δcrb2 suppressed by plasmid pCHK1 carrying the chk1+rad27+ gene

| Plasmids | pCRB2 | pCUT5 | pCHK1 |
|----------|-------|-------|-------|
| pRAD1    | +     | −     | +     |
| pRAD3    | +     | +     | +     |
| pRAD9    | +     | +     | +     |
| pRAD17   | −     | −     | −     |
| pRAD26   | −     | −     | −     |

(+ Normal sensitivity; (−) hypersensitivity; Δcrb2, Δchk1) deletion mutants of crb2+ and chk1+, respectively.
Figure 9. Chk1 interacts with Crb2 and Cut5. (A) Elevated gene dosage of chk1* suppresses the UV- and HU-sensitive phenotypes of Δcrb2. Each of the four transformant strains of Δcrb2 (top) and Δchk1 (bottom) strains carrying plasmids (1 and 2, vector; 3 and 4, pCHK1; 5 and 6, pCRB2; 7 and 8, pCUT5) were spotted after dilution, and incubated at 26°C (left). Identical cells were also incubated after UV irradiation (right). The plates did not contain thiamine. (B) Δcrb2 null cells carrying plasmid with the chk1* gene under a moderate inducible promoter REP41 were precultured at 26°C for 18 hr in the presence of thiamine (+Thi; repressed condition) or the absence (−Thi; derepressed condition), and then irradiated by UV and cultured for 4 hr at 26°C. The cell number and the septa- tion index were measured. (C) Phosphorylation of Chk1 does not take place in Δcrb2 on UV irradiation. Three strains (wild type, cut5-T401 and Δcrb2) integrated with chk1* tagged with the HA antigen (Walworth and Bernards 1996) were irradiated with UV (+UV) and cultured at 26°C for 1 hr. The level of Cdc2 (anti-PSTAIRE) is shown as control. The intense upper band of Chk1 appeared after UV irradiation.

Saka et al.

synthesize bad or damaged DNAs that are sensed by Crb2, whereas such bad DNAs are not made in HU-arrested cells. This implies that Crb2 may monitor the damaged DNAs accumulated during the S phase, consistent with the concept that DNA damage causes replication to be blocked (Paulovich and Hartwell 1995).

Chk1 appeared to act at the downstream of Crb2 and Cut5 as a checkpoint signal transmitter. This hypothesis explains a number of results presented in this paper (Table 1; Figs. 5, 6, and 9). Consistent with this hypothesis, Chk1 is required not only for the damage checkpoint (Walworth et al. 1993; Al-Khodairy et al. 1994) but also for the replication checkpoint (Francesconi et al. 1997). Cdc2 kinase is affected by Chk1, which was recently shown to regulate Tyr15 phosphorylation of Cdc2 through Cdc25 and/or Wee1 (O’Connell et al. 1997; Rhind et al. 1997).

We showed that a fraction of Crb2 was transiently modified, probably phosphorylated, after UV irradiation. In sucrose gradient centrifugation, the modified Crb2 was slowly sedimenting, possibly released from the large complex structures. Similarly, the phosphorylated form of Chk1 kinase, which was reported to be activated by autophosphorylation when damaged (Walworth and Bernards 1996), was also present in the slowly sedimenting fractions. We speculate that damage leads to Crb2 modification, and the modified Crb2 can produce a signal to activate Chk1, possibly by direct interaction. This hypothesis is consistent with two-hybrid interaction, the suppression of Δcrb2 by the elevated gene dosage of chk1* and the absence of Chk1 phosphorylation in Δcrb2 cells. The occurrence of Crb2 modification induced by damage in Δchk1 cells supports the upstream nature of Crb2 to Chk1.

Following UV irradiation, the products of the damage-sensing checkpoint genes (rad1+, rad3+, rad9+, rad17+, and rad26+; Al-Khodairy et al. 1994) may act in conjunction with Crb2 to generate the signal for the cell-cycle block. Functional relationship between Crb2 and these five checkpoint genes is unclear. The fact that Crb2 is not modified upon UV irradiation in these rad mutants and that plasmids carrying any one of these rad* genes fail to suppress the UV phenotype of Δcrb2 cells suggests that Crb2 functions in the upstream of these Rad gene products. Crb2 may be activated by the Rad gene products. It is also possible, however, that Crb2 independently senses damaged DNAs and sends an essential signal for promoting cell-cycle arrest, separately from the Rad genes. A more complex relationship is likely, as pCRB2 can suppress the UV sensitivity of rad1-1, but not that of rad3, rad9, or rad17 mutants (F. Esashi and M. Yanagida, unpubl.). Crb2 may thus have a close functional link to Rad1. The S. cerevisiae Rad17, an S. pombe Rad1 homolog, has an exonuclease activity to produce a gap in DNA and plays an important role in the damage checkpoint (Lyddall and Weinert 1995; Nugent et al. 1996).

The predicted amino acid sequence of Crb2 is signifi-
Crb2, a linker between damage and replication checkpoints

cantly similar to the budding yeast Rad9 (Weinert and Hartwell 1988; Hartwell and Weinert 1989) that acts in the processing of DNA damage for repair (Lydall and Weinert 1995), as a component in the sensor/transducer pathway of UV damage outside of S phase (Navas et al. 1996). However, the similarity between budding yeast Rad9 and fission yeast Crb2 is restricted to the carboxyl termini. Hence, RAD9 and crb2+ are unlikely to be true homologs. Elevated dosage of the budding yeast RAD9 or the fission yeast crb2+ gene introduced into the fission yeast Jcrb2 or the budding yeast rad9 mutant, respectively, did not suppress the UV sensitivity (S. Mochida and M. Yanagida, unpubl.).

Mammalian proteins involved in oncogenesis, 53BP1 (Iwabuchi et al. 1994) and BRCA1 (Koonin et al. 1996), contain regions similar to the carboxyl termini of Crb2. 53BP1 binds to a tumor suppressor protein p53 by the two-hybrid method, and the carboxyl terminus of 53BP1 is the site for binding to p53 (Iwabuchi et al. 1994). Mutations in BRCA1 lead to familial breast and ovarian cancers. The common carboxy terminal motifs present in Crb2, Rad9, 53BP1, and BRCA1 (and other related proteins, Koonin et al. 1996) may be the sites for interaction with proteins implicated in the maintenance of the genome in response to damages. It is of considerable interest to determine whether any protein bound to the carboxyl terminus of Crb2 exists in fission yeast, and whether such protein(s) is a transcription factor sharing a property with p53.

During the preparation of this paper, Wilson et al. (1997) reported that one of the fission yeast methylmethane sulfonate (MMS)-sensitive alleles, rhp9+, was required for the DNA damage checkpoint but not the replication checkpoint. Comparison of the sequences indicated that Rhp9 was identical to Crb2. Though their conclusion apparently differed from ours, the experimental results on rhp9 null were not inconsistent with ours, because only the replication checkpoint induced by HU was investigated for rhp9 null. Crb2/Rhp9 may respond to a variety of DNA toxins.

XRCC1, a human repair protein, the amino acid sequence of which partly resembles Cut5, plays a scaffold role in interactions with polymerase β and ligase III (Kubota et al. 1996). Cut5 might also have a scaffold structure, which makes complex regulations possible through protein–protein interactions. The amino-terminal T45, which appears to be necessary for interacting with Crb2, is the temperature sensitive mutation site. Because the mutants were also defective in replication, the amino terminus might also interact with an unidentified protein(s) essential for replication. The central-to-carboxy-terminal region is probably the site for interaction with Chk1 and Crb3. The amino-terminal and the central-to-carboxy-terminal regions contain multiple BRCT motifs (Saka et al. 1994; Bork et al. 1997). As both Cut5 and Crb2 contain BRCT motif, pursuing their protein functions will shed light on the actual role of BRCT motif in maintaining the genome.

It is not a simple matter to explain the reason cut5 mutants are sensitive to UV because the UV- and 4N-QO-induced checkpoint is maintained in cut5 mutant at both 26°C and 36°C (Saka et al. 1994a,b; Saka et al., unpubl.). We speculate that the interaction between Cut5 and Crb2 is needed for promoting efficient repair of the damaged DNA. In cut5/rad4 mutants, where the interaction of Cut5 with Crb2 might be lost even at 26°C, repair replication may become inefficient, leading to UV hypersensitivity. However, Crb2, which is responsible for generating a damage signal for cell-cycle arrest is present. The reason for the HU sensitivity in ∆crb2 is also unclear because the HU-induced checkpoint is retained in ∆crb2. We propose that only Cut5 is required for HU-induced checkpoint and that the Cut5-Crb2 interaction is needed for an unidentified function to facilitate replication under an inhibited nucleotide supply. Under the HU-induced checkpoint arrest, Cut5 may send an arrest signal through a protein other than Crb2 or directly to Chk1. Two-hybrid interaction favors the latter case. To explain the result that both Crb2 and Cut5 are necessary for the cell-cycle arrest by defects in DNA polymerases or ligase, we propose that neither Crb2 nor Cut5 alone can detect the replication defect caused by defects of polymerases or ligase.

It remains to be determined whether the stable complex between Crb2 and Cut5 is actually present in the nucleus in vivo but difficult to detect in S. pombe extracts, or if the stable complex never exists in vivo and the interaction is only transient such as is true of a substrate–enzyme relationship. In sucrose gradient centrifugation, fractions of Crb2 and Cut5 were sedimented as large heterogeneous materials that might contain both Crb2 and Cut5. A small fraction of Chk1 was also in the large complex form. The properties of these large complexes are unknown, and whether Cut5, Crb2, and Chk1 exist in the same large structure remains to be determined. Alternatively, slowly sedimenting forms of Crb2, Cut5, and Chk1 were obtained after UV irradiation, and these smaller forms possibly phosphorylated may interact each other in a transient style.

Materials and Methods
Strains and media
Haploid wild-type S. pombe h∞972 and h∞975 (Gutz et al. 1974) and their derivative mutant strains were used. cut5 mutants (cut5-T401, cut5-580, rad4-116; Duck et al. 1976; Saka and Yanagida 1993; Samejima et al. 1993), rad mutants (rad1-1, rad3::ura4+, rad9::ura4+, rad17::ura4+, rad26::ura4+, and rad27::ura4+ mutants; Al-Khodairy et al. 1994; gifts of Dr. A. M. Carr), cdc mutants (cdc6-121, cdc17-K42, cdc20-M10, cdc22-C1, Nourse et al. 1976; Gordon and Fantes 1986) and swi7-H4 (Singh and Klar 1993) mutants were described previously. Rich YPD (2% glucose, 2% polypeptone, and 1% yeast extract) and minimal EMM2 (Mitchison 1970) were employed.

Determination of UV sensitivity
The procedures for examining UV sensitivity were described previously (Saka and Yanagida 1993; Saka et al. 1994). Briefly, S. pombe cells diluted were plated and incubated for 30 min, followed by UV irradiation (0–300 J/m²; Stratalinker, Stratagene)
and culturing for several days at 26°C (the permissive temperature for temperature sensitive mutants). The percent septation index was obtained by Calicoflour staining after glutaraldehyde fixation. To study the checkpoint response, exponentially growing S. pombe cells were irradiated by 100 J/m².

FACSscan analysis, Southern transfer, and sequencing
The procedures for FACSscan were described previously (Costello et al. 1986; Saka and Yanagida 1993). Southern hybridization was performed by the procedures described (Saka et al. 1994a). The dye-terminator method was employed for nucleotide sequencing by use of the ABI 373A Sequencing System.

Cell extracts and immunoblotting
Cells were collected and suspended in ice chilled phosphate buffer-saline at pH 7.5 containing 10 mM Na3Vo4 and 50 mM NaF, rapidly frozen by liquid nitrogen and kept at −20°C. Cells were then thawed and suspended at a concentration of 1 × 10⁶/ml in HB buffer (25 mM Tris-HCl at pH 7.5 containing 15 mM MgCl2, 15 mM EGTA, 0.1% NP-40, 1 mM DTT, 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 0.5 mM Na3Vo4, 0.1 mM NaF, 1 mM PMSF). Cells were disrupted by glass beads and centrifuged at 4°C at 14,000 rpm for 20 min. Supernatants were used for immunoblotting. As secondary antibodies, HRP-labeled protein A (200-fold dilution, Bio-Rad) or HRP-labeled sheep anti-mouse antibody (Amersham, 500-fold dilution) was employed.

Two-hybrid screening
The S. pombe cDNA library adapted to the two-hybrid system in yeast was purchased from Clontech, Inc. (XL4000AA), and the instruction procedures for screening were followed by use of the HF7c strain. Yeast cells were grown in the SD synthetic medium supplemented with the dropout solution at 30°C. DNAs of different domains of the cut5 gene were obtained by amplification by the PCR method (the R1R2 region) or by isolating the restriction fragments (the central acidic domain, R3, R4, and the carboxy-terminal region). These DNAs were ligated in frame with pGBT9 containing the GAL4 DNA-binding domain. All of these plasmids were verified by nucleotide sequencing. The filter assay for interaction was also done following the company’s instructions by use of the SFY526 strain.

Preparation of GST fusion proteins and in vitro binding assay
NdeI–BamHI fragment of cut5 gene containing the R1R2 region was blunt-end ligated at the Xhol site of plasmid pGEX-KG (Guan and Dixon 1991), whereas BamHI–SalI fragment containing the R3 region was inserted at the same site of pGEX-KG. Resulting plasmids pY5583 and pR308, respectively, were used for producing fusion proteins that were dissolved in the presence of 8 M urea, followed by successive dialysis in PBS containing reduced concentrations of urea and 10 mM DTT. Resulting GST proteins were in vitro translated in the absence of urea were incubated with glutathione beads. In vitro translation of Crb2 and Crb3 proteins was performed with the Promega TNT Coupled reticulocyte lysate system and [³⁵S]methionine (ICN). Luciferase, Crb2, and Crb3 proteins were in vitro translated and mixed with the beads bound to GST–R1R2 or GST–R3, and incubated at 4°C for 1 hr. Beads were washed three times by 10 volumes of 20 mM Tris-HCl at pH 7.5 containing 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM PMFS, 1 μg/ml of leupeptin, 0.5 μg/ml of aprotinin, followed by SDS-PAGE and autoradiography by use of the Amer sham AmphiLyte system.

Sucrose gradient centrifugation and phosphatase treatment
The procedure of sucrose gradient centrifugation was followed to that reported previously (Yamashita et al. 1996; Yamada et al. 1997). Extracts prepared from each 4 × 10⁷ cells nonirradiated or irradiated with UV (100 J/m²) and cultured 1 hr at 26°C were run at 40,000 rpm for 12 hr at 4°C with a SW50.1 rotor. BSA (4.5S) and thyroglobulin (16.5–19S) were used as the markers for the S values. The procedure of Walworth and Bernards (1996) was followed for treatment of Crb2 and Chk1 in cell extracts by β protein phosphatase (New England Biolab).

GFP tagging and immunofluorescence microscopy
The jellyfish GFP gene was introduced to the amino-terminal site of the crb2 gene by creating a new BamHI site at the amino-terminus. A mild inducible promoter REPA1 (Maudrell 1990) was placed upstream of the fused GFP–Crb2 gene by use of pGFT41 (Heilm et al. 1995). Resulting plasmid was introduced into acr2 strain and fluorescence was observed in the presence (repressed) or the absence (induced) of thiamine (Nabesima et al. 1995). Cells cultured in the presence or the absence of thiamine carrying pGFT–Crb2 were observed by blue light without fixation.

Gene disruption
The procedure of gene disruption was as reported previously (Rothstein 1983).

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