Characterization of DNA Damage-stimulated Self-interaction of Saccharomyces cerevisiae Checkpoint Protein Rad17p*

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Saccharomyces cerevisiae Rad17p is necessary for cell cycle checkpoint arrests in response to DNA damage. Its known interactions with the checkpoint proteins Mec3p and Ddc1p in a PCNA-like complex indicate a sensor role in damage recognition. In a novel application of the yeast two-hybrid system and by immunoprecipitation, we show here that Rad17p is capable of increased self-interaction following DNA damage introduced by 4-nitroquinoline-N-oxide, camptothecin or partial inactivation of DNA ligase I. Despite overlap of regions required for Rad17p interactions with Rad17p or Mec3p, single amino acid substitutions revealed that Rad17p-Rad17p complex formation is independent of Mec3p. E128K (rad17-1) was found to inhibit Rad17p interaction with Mec3p but not with Rad17p. On the other hand, Phe-121 is essential for Rad17p self-interaction, and its function in checkpoint arrest but not for Mec3p interaction. These differential effects indicate that Rad17p-Rad17p interaction plays a role that is independent of the Rad17p-Mec3p complex, although our results are also compatible with Rad17p-mediated supercomplex formation of the Rad17p-Mec3p-Ddc1p heterotrimer in response to DNA damage.

Eukaryotic cells are endowed with checkpoint controls that ensure the correct succession of cell cycle events (1, 2). In certain stress situations provoked by DNA damage, inhibition of replication or incorrect formation of the mitotic spindle, reversible arrest at checkpoints can provide a critical time window in which to remedy a condition that would otherwise result in irreversible cellular damage. Unless these cells are inviable or actively eliminated (e.g. by apoptosis), such damage may manifest itself as genetic change. Therefore, checkpoint mechanisms are critically important for preserving genetic stability. Inactivation of checkpoint controls can facilitate the multistep process of malignant transformation, and consequently, mutations in checkpoint genes have frequently been found to be associated with human cancer-prone syndromes and sporadic cancer (3, 4).

Analysis of lower eukaryotes such as Saccharomyces cerevisiae, Schizosaccharomyces pombe, or Aspergillus nidulans has shaped our current understanding of the signal transduction processes resulting in cell cycle arrest in response to DNA damage (5–10). DNA damage triggers arrest responses in G1, G2, or M and at several levels in S-phase. It became clear that the identity of the targeted components of the cell cycle machinery may depend on the species-specific features of cell cycle organization. For instance, DNA damage induced by ionizing radiation elicits a G2 arrest in S. pombe and mammalian cells, primarily by preventing the dephosphorylation of a tyrosine residue of the cyclin-dependent kinase Cdk1p (Cdc2p) (7). In the budding yeast S. cerevisiae, however, where budding and formation of the mitotic spindle is already completed in S-phase, and no G2-phase can be discerned, a similar checkpoint pathway triggers arrest at the metaphase/anaphase transition by targeting Pds1p, an inhibitor of sister chromatid separation (11–13).

However, the available data strongly indicate that the damage-sensing mechanism(s) that initiate the arrest signal are evolutionarily well conserved. The molecular nature of the recognized lesion(s) is still not entirely clear. Studies in yeast, human cells, and Xenopus extracts have demonstrated convincingly that double strand breaks or stretches of single-stranded DNA can be efficient triggers (14–18). Whereas such structures can be the consequence of exposure to ionizing radiation and certain chemicals, the checkpoint signal following UV irradiation, with dipyrimidine lesions as the most prominent damage, remains to be explained. For UV-induced G1 arrest prior to replication initiation, a critical role for nucleotide excision repair (NER)1 has been demonstrated (18–20). This could be interpreted as a dependence of the arrest signal on the presence of intact NER proteins themselves or on a certain NER-mediated DNA repair intermediate.

Among the checkpoint proteins, several sensor candidates have been identified based on structure predictions, protein interactions, and their role in damage-induced phosphorylation of further downstream acting “transducer” proteins. The same sensors not only trigger cell cycle arrest but also, through largely overlapping pathways, other regulatory responses connected with DNA repair. In S. cerevisiae, this includes the transcriptional induction of repair-associated genes (21–24) as well as the relocation and modification of certain strand break repair proteins (25–28). The following discussion will focus on candidate sensor proteins functioning outside of S-phase where damage-sensing appears to occur by structure-recognition mechanisms that function largely independent of particular cell cycle events. In budding yeast, it is clear that in S-phase, many of these sensors are substituted by components of the replicative machinery, such as polymerase α (21, 29).

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1 The abbreviations used are: NER, nucleotide excision repair; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; 4-NQO, 4-nitroquinoline oxide; AD, activation domain; Me3SO, dimethyl sulfoxide; BD, DNA-binding domain; GST, glutathione S-transferase; HA, hemagglutinin; PCNA, proliferating cell nuclear antigen.
An example of a conserved damage-recognition protein is represented by S. cerevisiae Rad17p and its various homologues in lower and higher eukaryotes, e.g. Rec1p in Ustilago maydis, Rad1p in S. pombe, hRad1p in humans (16, 30–38). (No uniform nomenclature has been adapted. In the following, we will refer to the S. cerevisiae proteins unless a species designation is given.) The phenotype of Caenorhabditis elegans mutants defective in the homologous protein revealed other areas besides checkpoint arrest that may be affected by such a protein in multicellular eukaryotes: ionizing-radiation-induced apoptosis and expression of telomerase in germ cells (37, 39). Rad17p shares structural similarities with proliferating cell nuclear antigen (PCNA), forming homotrimeric ring structures around DNA and commonly referred to as a “sliding clamp” that tethers replicative polymerases onto their template (40).

Rad17p forms a stable three-way complex with two additional checkpoint proteins resembling PCNA: Ddc1p and Mec3p (41, 42). Complexes of similar structure have been identified in S. pombe (43, 44) and human cells (45–47). Molecular modeling suggests indeed the formation of a PCNA-like ring complex by Rad17p, Ddc1p, and Mec3p (36, 40) but direct evidence for such a structure is missing. Interestingly, 3’→5’exonuclease activity seems to be associated with this complex. Purified Rec1p (the U. maydis Rad17p homologue) has such activity (38) whereas contradicting results have been reported for the human protein (32, 34). However, such activity was also demonstrated for the human Ddc1p homologue (48). The significance of these findings remains unclear. An exonuclease may be required to convert DNA damage such as double strand breaks, single strand breaks, and gaps into a recognized arrest-triggering substrate or to facilitate a yet to be defined repair function of the Rad17p complex.

S. cerevisiae Rad24p is another potential sensor protein required for damage-induced cell cycle arrest that may interact with the Rad17p-Ddc1p-Mec3p complex. Rad24p forms a stable complex with four of five subunits of replication factor C (Rfc2p-Rfc5p) (49–51). Because replication factor C recognizes primer-template structures and functions as a “clamp-loader” to recruit PCNA, it is tempting to speculate about a similar relationship between the Rad24p-Rfc complex and the PCNA-like Rad17p-Ddc1p-Mec3p heterotrimer (9, 36). There are indeed indications for physical interaction of S. pombe and human Rad17p and Rad24p protein homologues (44, 52). The Rad24p-containing complex might function as the initial damage sensor by binding to a double-stranded/single-stranded DNA junction, recruit the Rad17p sliding clamp complex and somehow create a platform that enables cross-talk with and activation of downstream-acting kinases such as Rad53p. With respect to Rad53p kinase activation and subsequent transcriptional regulation following UV irradiation, Rad17p and Rad24p have indeed been placed in the same epistasis group, separate from Rad9p (24), yet another potential sensor protein of unknown function interacting with Rad53p (53, 54).

The last candidate for a damage sensor among the budding yeast checkpoint proteins is Mec1p, a protein kinase with similar function interacting with Rad53p (53, 54). Because of the involvement of Mec1p in arrest and transcriptional signaling in all cell cycle stages in response to various types of DNA damage and to inhibition of replication (58, 59), a function as a universal signal transducer was initially assumed. However, more recent data hint at a role more proximal to the damage recognition step. First, phosphorylation of assumed sensor proteins such as Ddc1p or Rad9p, which occurs rapidly after DNA damage is largely dependent on Mec1p (42, 53). Second, phosphorylation of S. pombe checkpoint protein Rad26p in response to DNA damage has been found to be only dependent on the S. pombe Mec1p homologue Rad3p but not on any other checkpoint protein, thus placing Rad3p at the very beginning of the signal transduction pathway (60). In support of its potential role as a damage sensor, the Xenopus homologue was found to partition to the DNA fraction following external addition of broken DNA to extracts (61).

In summary, the basic mechanisms of DNA damage recognition during the course of checkpoint arrest and the molecular organization of the sensor proteins involved are far from clear and many scenarios can be envisioned. For instance, several separate damage-sensing complexes may exist that detect different types or structural aspects of DNA damage with different affinity. To explain the high sensitivity and specificity of the system, a re-inforcing cross-talk between sensors as the precondition for the origination of a transducible signal may be assumed.

In the present study, we attempted to identify novel protein-protein interactions of S. cerevisiae Rad17p that may be enabled or increased in response to DNA damage. Using a novel application of the yeast two-hybrid system, we found an elevated level of self-interaction of Rad17p following checkpoint activation by inhibition of DNA ligase or upon treatment with DNA-damaging agents such as 4-NQO or camptothecin. Although a similar region of Rad17p is critical for both Rad17p-Rad17p and Rad17p-Mec3p interactions, homomeric complex formation was independent of the Rad17p-Mec3p-Ddc1p interaction and was differentially influenced by amino acid substitutions. In addition, several novel point mutations that abolish Rad17p function were identified.

EXPERIMENTAL PROCEDURES

Yeast Strains—For yeast two-hybrid experiments we used an ADE^ derivative of Y190, originally from S. Elledge (62), and CG1945 (63) as reporter yeast strains. A thermoconditional ligase-deficient cdc9 reporter strain (WS3019/89) was created by crossing Y190 with a cdc9− strain. WS3019/89 was also reverted to wild-type by transformation with a CDC9^ PCR fragment. Strain BY4741 trp1Δ (from the Euroscarf yeast stock center) was used for co-immunoprecipitation studies. For complementation experiments, we used strain SX46A (20) deleted for RAD17. Complete chromosomal deletions of RAD17, MEC3, or TOP1 were created by PCR amplification of existing transplacements with kanMX4 into BY4741-derived strains (Euroscarf), followed by transformation of the target strain by the lithium acetate method (64). Media recipes can be found elsewhere (65).

Plasmid Constructs—For two-hybrid experiments, an in-frame fusion of full-length Rad17p (minus the N-terminal three amino acids) with the Gal4p DNA-binding domain was constructed from vector pGBT9 (CLONTECH) and a subcloned library plasmid insert (30). C-terminal truncations of Rad17p (Δ136, Δ100) were made by digesting the resulting plasmid pGBT9-Rad17 with Pac1 and blunt-ending before re-ligation (Δ136) or by subcloning of an EcoRI fragment of pGBT9-Rad17 into pGBT9 (Δ100). The amino acid substitutions L119A, I120A, F121L, F121D, and F121A in Rad17p were introduced by PCR using primers that included at their 5’ ends sequence homology to the pGBT9 vector. pGBT9-Rad17 was gapped with Smal and Pac1 and co-transformed with each PCR product into yeast strain Y190, resulting in reconstitution of plasmids with mutated RAD17 coding sequence by recombination (66). These plasmids were recovered and verified following electroporation of Escherichia coli with yeast DNA preparations (67). C-terminal fusions of Rad17p or E128KRad17p with the transcriptional activation region of Gal4p were made in pCAD1, a vector containing codos 768–881 of Gal4p downstream of the ADH1 promoter (Ref. 68, kindly provided by Megan Keniry, University of Oregon). We also constructed an N-terminal fusion of full-length Mec3p with the Gal4p transcriptional activation domain in vector pGAD424 using the described PCR/recombination strategy.
For immunoprecipitation experiments, we epitope-tagged Rad17p on inducible overexpression plasmids containing TRP1 or LEU2 as selectable markers. First, a GST-Rad17 fusion was constructed in vector pYEXt1 (CLONTECH). We transferred the GST-Rad17 fusion to pEGL6h (provided by T. Zhang, University of Kentucky) and created a vaccinia-inducible His-tag-GST-Rad17 fusion (plasmid pEGL6h-GST-Rad17). Similarly, RAD17 was cloned into pEGTIIa (69), resulting in an HA-Rad17p fusion under the control of the galactoside inducible GAL1/GAL10 promoter. A centromeric plasmid containing full-length untagged RAD17 under control of its chromosomal promoter was constructed from pWSU174 (30) and vector YCp50. Further construction details and primer sequences can be provided upon request.

**Yeast Two-hybrid Interaction Assays**—Following co-transformation of yeast strains Y190, CG1945, or WS3019/89, individual colonies were streaked on synthetic medium plates without tryptophan and leucine and grown for 2 days. To test for HIS3 reporter gene activation in the presence of DNA damage, 3 × 10⁶ (strains Y190, WS3019/89) or 1.2 × 10⁶ (strain CG1945) cells were spread per plate of medium lacking tryptophan, leucine, and histidine, containing 25 μM (Y190, WS3019/89) or 1.25 μM (CG1945) aminotriazole. Filter paper discs containing 10 μl of 4-nitroquinoline-N-oxide (Sigma, 100 μg/ml in Me2SO) or camptothecin (Sigma, 10 μg/ml in Me2SO) were placed in the center of each plate. Growth was evaluated after 6 days of incubation at 30 °C or at 33 °C for cdc9–1 strains. The two-hybrid yeast cDNA library used here was a yeast cDNA library constructed from Y190 (Baylor College of Medicine, Houston, TX). We constructed a new plasmid containing a C-terminal fusion of Gal4p-AD with the full-length open reading frame of RAD17 (pCAD1-Rad17). Using this plasmid and the original Rad17p bait, we confirmed the initial selection result, as shown in Fig. 1. When the cdc9–1 strain was co-transformed with pGBT9-Rad17 and pCAD1-Rad17, significant growth was observed in histidine-free plates incubated at 33 °C (or 30 °C), although the same was not true for control combinations with vector plasmids (Fig. 1A and data not shown). A dependence of this effect on the chromosomal ligase defect was demonstrated in a CDC9 revertant where the frequency of colony growth was significantly reduced as compared with the original ligase-defective strain (Fig. 1B). Similarly, when a conventional, non-thermoconditional two-hybrid reporter strain such as Y190 was co-transformed with both Rad17p fusions, growth on histidine-free medium was very much reduced albeit still above the background seen with vector combination controls (Fig. 1C and data not shown). On the basis of this observation, we explored if such reporter gene activation can be increased by exposure to external DNA-damaging agents.

**Rad17p Self-interaction in the Two-hybrid System Is Increased by DNA-damaging Agents**—By controlling for the plated number of co-transformant cells, DNA-damaging agent, 3-aminotriazole concentration in the selection plates and for the duration of incubation, we detected a colony growth pattern that can be interpreted as increased Rad17p self-interaction in response to DNA damage (Fig. 2). We used 4-NQO as a genotoxic agent with a long half-life in plates. Because the effective concentration could not easily be predicted, the agent was applied in form of a concentration gradient using a centrally placed filter disc containing 4-NQO solution or the solvent Me2SO as a control. In a certain distance from the filter paper, a zone of HIS3+ colony growth clearly developed. Such a pattern was absent in the solvent control; however, a background of occasional colony growth remained evident. Essentially no growth is detectable with vector controls (Fig. 2). Similar results were obtained in another reporter strain (CG1945, Fig. 2) and the specificity of the effect was further supported by the fact that we were able to characterize point mutants in Rad17p that abolished the observed growth pattern (see below). As a particularly effective agent we identified the topoisomerase I inhibitor camptothecin, which induces a broad zone of increased colony growth without causing significant lethality (data not shown). Increased colony growth was clearly dependent on the presence of the known camptothecin target topoisomerase I (Top1p) because a Y190 reporter strain derivative deleted for TOP1 did not show a similar growth pattern (data not shown). Other genotoxic agents such as methyl nitrotreso-guanidine or cis-platinum also induced colony growth in the same assay but less so than 4-NQO or camptothecin (data not shown).
To confirm the two-hybrid results by a different, biochemical method, we overexpressed differently epitope-tagged Rad17p versions on plasmids pEGTHa-Rad17 and pEGLh6-His-GST-Rad17 in the same yeast strain and immunoprecipitated HA-Rad17p using an agarose bead-bound anti-HA antibody.

**Fig. 1.** A DNA ligase I defect enhances HIS3 reporter gene activation by Rad17p two-hybrid fusions. The growth assays shown were performed on plates lacking histidine with streaks or cell suspensions of several independent co-transformants. Strain WS3019/89 (cdc9–1 or CDC9+) (A, B) was co-transformed with pGBT9-Rad17 and pCAD1-Rad17 or the vector control pCAD1, as indicated in the figure. The conventional reporter strain Y190 (CDC9+) was used in C. Plates were incubated at 33 °C (A, B) or 30 °C (C).

**Fig. 2.** 4-NQO exposure enhances HIS3 reporter gene activation by Rad17p two-hybrid fusions. Strains Y190 (upper panel) or CG1945 (lower panel) were transformed with pGBT9-Rad17 and pCAD1-Rad17 or vector controls as indicated. Cells of individual co-transformant clones were plated and incubated for 6 days at 30 °C on histidine-free 4-NQO gradient plates or plates without 4-NQO, containing the solvent Me2SO (DMSO).

Detection of Rad17p Self-interaction by Co-immunoprecipitation—To confirm the two-hybrid results by a different, biochemical method, we overexpressed differently epitope-tagged Rad17p versions on plasmids pEGTHa-Rad17 and pEGLh6-His-GST-Rad17 in the same yeast strain and immunoprecipitated HA-Rad17p using an agarose bead-bound anti-HA anti-
body. We probed for both HA-Rad17p and GST-Rad17p among the eluted proteins (Fig. 3). The initial protein levels of HA-Rad17p and GST-Rad17p in the crude extracts were approximately identical (lanes 5–8), and the amounts of immunoprecipitated HA-Rad17p in all samples expressing HA-Rad17p were also comparable (lanes 1–4, upper panel). However, a higher fraction of GST-Rad17p was co-immunoprecipitated in the 4-NQO treated sample compared with the untreated sample where only a faint band was visible (lanes 1 and 2, lower panel). No signal was detected in control samples from cells transformed with vector plasmids and a single tagged Rad17p fusion (lanes 3 and 4, lower panel). This indicates increased HA-Rad17p-GST-Rad17p complex formation in response to 4-NQO. Similar results were found after treatment with camptothecin (data not shown).

Rad17p Self-interaction Is Independent of Rad17p Mec3p Complex Formation—Next, we characterized protein regions and individual residues that are necessary for homomeric interaction of Rad17p. First, two C-terminal deletions of Rad17p in the Gal4p-DB fusion plasmid (pGBT9-Rad17) were created that were tested for interaction with the full-length Rad17p-Gal4p-AD fusion (pCAD1-Rad17). As before, interaction was measured in reporter strain Y190 by HIS3 gene activation using 4-NQO gradient plates lacking histidine. Reducing the protein to the N-terminal 136 residues (Δ136) decreased the interaction significantly (Table I). Further shortening to 100 residues completely abolished reporter gene activation (Table I). In parallel tests, we determined the effect of the same deletions on interaction with Mec3p. Here, the N-terminal Δ136 fragment was capable of wild-type-like interaction whereas the shorter fragment (Δ100) did not interact at all (Table I). This suggests that overlapping but not identical regions of Rad17p are essential for both Rad17p/Rad17p and Rad17p/Mec3p complex formations. Conceivably, regions downstream of Leu-136 are additionally required for efficient Rad17p-Rad17p interaction but not for Rad17p-Mec3p interaction.

The region between residues 100 and 136 seem to be critical for both types of interactions. The rad17-1 mutation (ΔE128K/Rad17p) (30) known to eliminate Rad17p-Mec3p complex formation (41) maps to this region, which is conserved in various Rad17p and PCNA protein homologues (Fig. 4). When this mutation was introduced into the Rad17p-Gal4p-AD fusion protein, elimination of Rad17p-Mec3p interaction was indeed confirmed (Table I). However, the same substitution did not reduce Rad17p-Rad17p interaction but reproducibly increased constitutive interaction up to a level where a stimulating effect of 4-NQO was difficult to ascertain (Table I). This differential effect of the E128K mutation implies that Rad17p-Rad17p interaction can occur independently of Rad17p-Mec3p interaction. Indeed, Rad17p-Rad17p two-hybrid interaction remains unchanged when the reporter strain is deleted for MEC3 (data not shown).

Phe-121 is Required for Rad17p Self-interaction and Complementation Activity—We analyzed the influence of other conserved residues in the vicinity of the rad17–1 mutation and within the region defined by deletions Δ136 and Δ100 on Rad17p protein interactions. We introduced targeted base pair substitutions in the plasmid carrying the Rad17p-Gal4p-BD fusion and thus converted the conserved Phe-121 residue to Leu, Asp, or Ala (Fig. 4). This position is invariably Phe in PCNA from various organisms but Val or Leu in all other known Rad17 versions. The conservative F121L substitution did not notably affect interaction with Rad17p or Mec3p (Table I). In contrast, an F121D exchange resulted in markedly reduced homomeric Rad17p-Rad17p complex formation, but some stimulation by 4-NQO was still evident (Table I). An F121A substitution resulted in a more severely reduced Rad17p-Rad17p interaction with complete elimination of any 4-NQO effect (Table I). (In contrast to the C-terminal deletion Δ100, occasional colony formation independent of DNA-damaging agents is still possible and may reflect a less severely reduced constitutive level of interaction.) None of these mutations had any effect on Rad17p-Mec3p interaction that could be detected in two-hybrid assays (Table I).

To correlate phenotypic consequences with defective Rad17p protein interactions, we tested for functional activity of these altered proteins by measuring complementation of damage sensitivity of a strain deleted for RAD17. First, it was established that an unaltered Gal4p-DB-Rad17p fusion carried on a multicopy plasmid (pGBT9-Rad17) was fully functional and could indeed fully complement rad17 mutant phenotypes (Fig. 5). Identical results were found with a single-copy plasmid carrying full-length RAD17 under control of its chromosomal promoter (YCP50-Rad17) (Fig. 5A). It was therefore justified to perform complementation analysis with the Gal4p fusion plasmids and thus with the identical system used to construct and characterize Rad17p mutations.

We explored if the severity of the defect in Rad17p self-interaction of the various mutant versions correlated with their complementation activity. Whereas the F121L mutant protein was indistinguishable from the wild-type protein in restoring UV resistance, the F121A mutant protein showed no complementation at all and was comparable with empty vector controls or Rad17-1p (Fig. 5, Table I). A chromosomal rad17–1 mutation had indeed been previously characterized as equivalent to a complete deletion of the gene (30). However, despite reduced homomeric complex formation, [F121D]Rad17p was not significantly different from the wild-type protein in terms of restoration of macrocolony formation following treatment

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**Fig. 3.** 4-NQO exposure enhances co-immunoprecipitation of differently epitope-tagged Rad17p versions. Cells of BY4741 that had been co-transformed with both expression plasmids pEGTHa-Rad17 and pEGLh6-GST-Rad17 (lanes 1, 2, 5, 6) or vector controls (lanes 3, 4, 7, 8) were treated with 4-NQO (lanes 2, 6) or mock-treated (lanes 1, 3, 4, 5, 7, 8) as indicated in the figure. HA-Rad17p was immunoprecipitated with monoclonal anti-HA agarose beads. Proteins eluted from beads were first detected with anti-GST (lower panels), then with anti-HA antibodies following stripping (upper panels). The Western blot signals obtained from the crude extracts used for the immunoprecipitation are also shown (right).
Self-interaction of Rad17p in Response to DNA Damage

Table I

Activity of mutated Rad17 protein versions in interaction with wild-type Rad17p, Mec3p, and in phenotypic complementation of a rad17 deletion mutant

| Rad17 protein (Gal4p-BD fusion) | Interaction with Rad17p (Gal4p-AD fusion) | Interaction with Mec3p (Gal4p-AD fusion) | Complementation of Rad17 radiation sensitivity | Complementation of Rad17 checkpoint defects |
|--------------------------------|------------------------------------------|-----------------------------------------|----------------------------------|---------------------------------|
| WT                            | ++                                      | +++                                    | +                                | +                              |
| Δ136                          | +                                       | +                                      | +                                | +                              |
| Δ100                          | +                                       | +                                      | +                                | +                              |
| E128K                         | +                                       | +++                                    | +                                | +                              |
| F121L                         | +                                       | +++                                    | +                                | +                              |
| F121D                         | +                                       | ++                                     | +                                | +                              |
| F121A                         | (+)                                     | (+)                                    | (+)                              | (+)                            |
| H120A                         | (+)                                     | (+)                                    | (+)                              | (+)                            |
| L119A                         | +                                       | +                                      | +                                | +                              |

Fig. 4. Sequence alignment of the Saccharomyces cerevisiae Rad17p region from amino acid residues 101–136 with similar regions of its eukaryotic homologs. The represented species are Schizosaccharomyces pombe (SP), Ustilago maydis (UM), Caenorhabditis elegans (CE), Drosophila melanogaster (DM), and Homo sapiens (HS). The consensus residues derived from PCNA proteins of various eukaryotic sources are depicted underneath. Identical and similar amino acids are shown by different shading. The investigated single amino acid exchanges in the S. cerevisiae protein are indicated above the sequence. Modified after Ref. 36.

with UV or γ irradiation where at best a marginal sensitization was found (Fig. 5A, Table I, and data not shown).

Colony survival of irradiated cells may not always correlate well with the extent of checkpoint arrest in mutants with a partial checkpoint defects (see e.g. Ref. 71). Consequently, we directly analyzed the effect of mutated versions of Rad17p on checkpoint arrest following irradiation of synchronized cells. We placed cells that had been synchronized as large budded cells with hydroxyurea on solid medium and determined the fraction of microcolonies with more than 2 cell bodies as a function of time after γ or UV irradiation (Fig. 5B and data not shown). This method measures the extent of arrest at checkpoints downstream of S before entry into the next S-phase and provides a reliable estimate of the prevailing M-phase arrest (13, 71). As predicted, [F121L]Rad17p was equally active as the wild-type protein whereas [F121A]Rad17p was inactive and not different from Rad17p–1p or empty vector controls. However, we reproducibly detected abbreviated arrest for [F121D]Rad17p and thus demonstrated a partial defect in activity (Fig. 5B, Table I).

We also identified additional conserved residues that are required for both Rad17p-Rad17p and Rad17p-Mec3p interactions. L119A and I120A mutations (Fig. 4) abolished Rad17p/Mec3p complex formation while also inhibiting efficient homeric interaction (Table I). Confirming the importance of Rad17p-Mec3p interactions, the degree of radiation sensitivity and defect in checkpoint arrest conferred by these mutations approached that of a null mutant (Table I).

A considerable amount of information is now available on the cell cycle effectors that are targeted during DNA damage-induced checkpoint arrest. However, the molecular details of the organization of the damage sensor proteins and of their interactions with DNA structures have remained largely unknown. Based on structure predictions and protein interaction studies, it has been hypothesized that one initial step of damage recognition can be attributed to an alternative version of replication factor C, consisting of Rad24p and Rfc2–5p subunits, which binds to DNA damage and recruits the PCNA-like sliding clamp complex of Rad17p-Mec3p-Ddc1p (9, 36, 52). The increased nuclear retention of the human Rad17p and Ddc1p homologues following DNA damage may indeed result from the formation of such a higher-order complex (52, 72).

In the present study, we screened for Rad17 protein interactions that may only be enabled in the presence of DNA damage by use of a novel variation of the two-hybrid screen. Inactivation of DNA ligase creates DNA damage structures that trigger G2/M checkpoint arrest in S. cerevisiae (73, 74). Following the introduction of the DNA-ligase I mutation into the reporter strain, we performed a library screen, with a fusion of Rad17p to the Gal4p-DNA-binding domain as bait, at semi-restrictive temperature. Using positive selection based on a Gal4p responsive HIS3 reporter gene, we were able to identify Rad17p itself as an interacting partner. We interpreted increased colony formation on histidine omission plates following partial ligase inactivation or during exposure to DNA-damag-
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It remains to be demonstrated if such two-hybrid-system based identification of conditional interactions has broader applications. Obvious candidates include other checkpoint, repair, and recombination proteins. However, the agents or conditions that can be used successfully must meet several criteria. Because a continued damage signal is almost certainly necessary for long-term reporter gene activation, a long half-life of the agent in solid medium will be required. Furthermore, a damage level must be achievable that triggers interaction while not being incompatible with growth. As described here, a range of concentrations can easily be probed in a gradient assay. Such an assay may even be applied in a high-throughput characterization of chemical agents that may influence protein interactions of biological importance.

Apart from providing a convenient screening system for mutations of the bait protein that ablate constitutive or induced interaction, the effect of selected yeast mutant backgrounds can be determined easily before a more cumbersome characterization (e.g. by immunoprecipitation) is attempted. In our case, we found no dependence of induced interaction on intact checkpoint arrest in general and no critical role of Mec3p, Ddc1p, Rad9p, or Rad24p in this process (data not shown). However, absence of Rad9p or Rad24p seemed to delay or weaken the Rad17p self-interaction to some extent (data not shown).

Analysis of truncated versions of Rad17p defined the N-terminal 136 amino acid residues as a region sufficient for Rad17p-Mec3p interaction, thus refining a previous estimate of 179 N-terminal residues (41). This fits well with an analysis of the human orthologue (hRad1) where the N-terminal 140 residues were found to be sufficient for physical interaction with human Mec3p (hHus1; Ref. 46). The same region was also sufficient for some Rad17p-Rad17p complex formation, but wild-type levels of interaction seem to require additional regions of the protein. Shortening the N-terminal fragment to 100 residues completely abolished Rad17p-Rad17p and Rad17p-Mec3p interactions.

Overlap of regions of Rad17p required for both types of interaction could indicate that the homomeric interaction of Rad17p is dependent on complex formation with Mec3p and indeed, we did not detect enhanced interaction or its increase in response to DNA damage (data not shown). Additionally, the interaction phenotype conferred by single amino acid substitutions within the same region of Rad17p argues for separable contacts. The rad17-1 mutation has been identified as the chromosomal mutation in the first isolated rad17 mutant strain and represents a phenotypic null allele (Fig. 5, see also Ref. 30). Confirming an independent study (41), we found that this mutation, a E128K substitution, is associated with a severe disruption of Mec3p interaction; however, Rad17p self-interaction remained unaffected.

The opposite effect was found for substitutions of Phe-121 that is highly conserved among Rad17p and PCNA versions from various species (Fig. 4). In Rad17p, Phe at this position is associated with a severe disruption of Mec3p interaction; however, Rad17p self-interaction remained unaffected.

or to affect only a subset of Rad17 protein molecules, even in cells that had suffered DNA damage.

It remains to be demonstrated if such two-hybrid-system based identification of conditional interactions has broader applications. Obvious candidates include other checkpoint, repair, and recombination proteins. However, the agents or conditions that can be used successfully must meet several criteria. Because a continued damage signal is almost certainly necessary for long-term reporter gene activation, a long half-life of the agent in solid medium will be required. Furthermore, a damage level must be achievable that triggers interaction while not being incompatible with growth. As described here, a range of concentrations can easily be probed in a gradient assay. Such an assay may even be applied in a high-throughput characterization of chemical agents that may influence protein interactions of biological importance.

Apart from providing a convenient screening system for mutations of the bait protein that ablate constitutive or induced interaction, the effect of selected yeast mutant backgrounds can be determined easily before a more cumbersome characterization (e.g. by immunoprecipitation) is attempted. In our case, we found no dependence of induced interaction on intact checkpoint arrest in general and no critical role of Mec3p, Ddc1p, Rad9p, or Rad24p in this process (data not shown). However, absence of Rad9p or Rad24p seemed to delay or weaken the Rad17p self-interaction to some extent (data not shown).

Analysis of truncated versions of Rad17p defined the N-terminal 136 amino acid residues as a region sufficient for Rad17p-Mec3p interaction, thus refining a previous estimate of 179 N-terminal residues (41). This fits well with an analysis of the human orthologue (hRad1) where the N-terminal 140 residues were found to be sufficient for physical interaction with human Mec3p (hHus1; Ref. 46). The same region was also sufficient for some Rad17p-Rad17p complex formation, but wild-type levels of interaction seem to require additional regions of the protein. Shortening the N-terminal fragment to 100 residues completely abolished Rad17p-Rad17p and Rad17p-Mec3p interactions.

Overlap of regions of Rad17p required for both types of interaction could indicate that the homomeric interaction of Rad17p is dependent on complex formation with Mec3p and indeed, we did not detect enhanced interaction or its increase in response to DNA damage (data not shown). Additionally, the interaction phenotype conferred by single amino acid substitutions within the same region of Rad17p argues for separable contacts. The rad17-1 mutation has been identified as the chromosomal mutation in the first isolated rad17 mutant strain and represents a phenotypic null allele (Fig. 5, see also Ref. 30). Confirming an independent study (41), we found that this mutation, a E128K substitution, is associated with a severe disruption of Mec3p interaction; however, Rad17p self-interaction remained unaffected.

The opposite effect was found for substitutions of Phe-121 that is highly conserved among Rad17p and PCNA versions from various species (Fig. 4). In Rad17p, Phe at this position is associated with a severe disruption of Mec3p interaction; however, Rad17p self-interaction remained unaffected.
This observation emphasizes the significance of the structural similarity of Rad17p with PCNA. However, F212D and F212A substitutions compromised Rad17p homomeric interaction to different degrees (Table I). Interestingly, whereas [F212A]Rad17p fails to complement, the milder F212D mutation confers a partial checkpoint arrest defect but has no effect on radiation resistance. Apparently, the residual capacity for checkpoint arrest is sufficient to fully complement the DNA damage sensitivity of macrocolony formation. Importantly, none of these mutations affects Rad17p-Mec3p interaction.

Consequently, we conclude that Rad17p-Rad17p interaction can occur independently of the Rad17p-Mec3pDdc1p complex and participation of Rad17p in two separate, possibly competing complexes can be assumed. One wonders if Rad17p might be able to form PCNA-like homotrimERIC structures as originally suggested (40). However, MEc3 expressed from a multicopy plasmid does neither enhance nor reduce Rad17p-Rad17p interaction and thus no indication for a competition between different Rad17p-containing complexes was found (data not shown). Alternatively, the data are also consistent with a simultaneous complex formation of Rad17p with itself and Mec3p. One could envision multimeric aggregation of a PCNA-ring-like Rad17p-Mec3pDdc1p complex on DNA, mediated by Rad17p homomeric interactions. We currently favor this hypothesis because Rad17p but also Mec3p self-interactions have been indicated for the human homologues (hRad1, hHus1, Ref. 46), thus possibly hinting at an evolutionary conserved capability of the heterotrimer to form higher order complexes. On the other hand, protein elution profiles in S. pombe did not indicate any shift in Hus1 protein association following DNA damage by ionizing radiation (44). However, all of the complexes detected in this study were larger in size than the predicted single heterotrimERIC complex.

In vitro and in vivo data suggest that homomeric interactions are quite common among checkpoint proteins, especially among the signal-transducing kinases (71, 76, 77). Facilitation of autophosphorylation may be a convenient mechanism to amplify a DNA damage signal on each level of the signal transduction cascade and contribute to the extraneous sensitivity and specificity of checkpoint controls. A damage dependence of homomeric interactions among yeast checkpoint proteins is also not without precedence. The potential sensor protein Rad9p exhibits enhanced self-interaction following treatment with UV irradiation that is mediated by the BRCA1 (BRCA1 carboxyl terminus) domain and associated with phosphorylation (78). Another damage-dependent heteromeric interaction of Rad9p involves the Rad53p kinase as a partner. Here, the contact is made through a FHA (forkhead-associated) domain following Mec1p-dependent phosphorylation of Rad9p (53, 54).

The mechanism by which homomeric Rad17p interaction in S. cerevisiae is increased in response to DNA damage remains to be established. An obvious explanation involves some kind of covalent modification of Rad17p. However, using a chromosomal Rad17-Myc fusion we were not able to identify a Rad17p species of altered mobility in one-dimensional SDS-PAGE gels, even after extended treatment with the agents used in this study (data not shown). Nevertheless, given the recent demonstration of such a phosphorylated nuclear form of the human homologue following DNA damage (72), an analysis by more sensitive methods may be warranted. Although Rad17p-Rad17p interaction does not depend on interaction with Mec3p or Ddc1p, the latter known to exhibit damage-dependent phosphorylation (79), another yet unidentified protein may mediate Rad17p self-interaction and may be a target of damage-dependent modification. Interestingly, self-interaction of the human Rad17p orthologue could be demonstrated by co-immunoprecipitation but not by yeast two-hybrid assays and therefore, the existence of another protein(s) mediating such interaction (that cannot be substituted by a yeast protein) has been suggested (46). Alternatively, a release of Rad17p from sequestration that increases the pool available for homomeric complex formation could be envisioned. Altered cellular localization in response to DNA damage might be indicative of such a process. However, we found (overexpressed) Rad17p to be predominantly nuclear, irrespective of DNA damage (data not shown) but a more refined subnuclear localization has not yet been performed. Nuclear sequestration has been identified as a regulatory mechanism for several eukaryotic proteins involved in cell cycle progression (80). Interestingly, this seems to include the human Rad24p homologue but no evidence for a similar DNA damage-dependent subnuclear relocalization was detected for the human orthologue of Rad17p (81). Lastly, self-interaction may simply increase by bringing Rad17p monomers closer to each other in the context of damaged DNA.

The present study advances our knowledge of the cross-talk between DNA damage sensors and provides novel information on residues that are critical for Rad17p activities. Ultimately, the significance of Rad17p self-interaction for checkpoint arrest or damage processing will have to be addressed. Although not providing any proof, the fact that a mutant version of Rad17p with compromised self-interaction (F121A) but normal Mec3p complex formation does not complement a RAD17 deletion mutant is consistent with a critical role of this effect in checkpoint arrest.

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REFERENCES

1. Elledge, S. J. (1996) Science 274, 1664–1672
2. Murray, A. W. (1992) Nature 359, 599–604
3. Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998) Nature 396, 643–649
4. Hartwell, L. H., and Kastan, M. B. (1994) Science 266, 1821–1828
5. Osmani, S. A., and Ye, X. S. (1997) Trends Cell Biol. 7, 283–288
6. Weinert, T. (1999) Curr. Opin. Genet. Dev. 9, 145–193
7. O’Connell, M. J., Walworth, N. C., and Carr, A. M. (2000) Trends Cell Biol. 10, 296–303
8. Caspary, T., and Carr, A. M. (1999) Biochimie (Paris) 81, 173–181
9. Lowndes, N. F., and Murguia, J. R. (2000) Curr. Opin. Genet. Dev. 10, 17–25
10. Longhese, M. P., Foiani, M., Muzi-Falconi, M., Luechini, G., and Plevani, P. (1998) EMBO J. 17, 5525–5528
11. Sanchez, J., Bachant, J., Guacci, V., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S. J. (1999) Science 286, 1166–1171
12. Cohen-Fix, O., and Koshland, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14361–14366
13. Yenamando, I., Guacci, V., and Koshland, D. (1996) J. Cell Biol. 133, 99–110
14. Huang, L.-C., Clarkin, K. C., and Wahl, G. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4827–4832
15. Lee, S. E., Moore, J. K., Holmes, A., Umezu, K., Kolodner, R. D., and Haber, J. E. (1998) Cell 94, 399–409
16. Lydall, D., and Weinert, T. (1995) Science 270, 1488–1491
17. Gao, Z., and Dnuphy, W. G. (2000) Mol. Cell. Biol. 11, 1535–1546
18. Nelson, W. G., and Kastan, M. B. (1994) Mol. Cell. Biol. 14, 1815–1823
19. Neecke, H., Luchini, G., and Longhese, M. P. (1999) EMBO J. 18, 4485–4497
20. Siede, W., Friedberg, A. S., Dianoova, I., and Friedberg, E. C. (1994) Genetics 138, 271–281
21. Navas, T. A., Sanchez, Y., and Elledge, S. J. (1996) Genes Dev. 10, 2623–2643
22. Aboussous, H., Vialard, J. E., Morrison, D. E., de la Torre-Ruiz, M. A., Cerna ´kova ´, L., Fabre, F., and Lowndes, N. F. (1996) EMBO J. 15, 3912–3922
23. Kiser, G. L., and Weinert, T. A. (1996) Mol. Biol. Cell 7, 703–718
24. de la Torre-Ruiz, M.-A., Green, C. M., and Lowndes, N. F. (1998) EMBO J. 17, 2667–2676
25. Mills, K. D., Sinclair, D. A., and Guarente, L. (1997) Cell 97, 609–620
26. Martin, S. G., Larche, T., Suka, N., Grunstein, M., and Gasser, S. M. (1999) Cell 97, 621–634
27. Bashkirov, V. I., King, J. S., Bashkirova, E. V., Schmuckli-Maurer, J., and Heyer, W.-D. (2000) EMBO J. 19, 1166–1171
28. Bashkirov, V. I., King, J. S., Bashkirova, E. V., Schmuckli-Maurer, J., and Heyer, W.-D. (2000) Mol. Biol. Cell 11, 4383–4404
29. Downs, J. A., Lowndes, N. F., and Jackson, S. P. (2000) Nature 408, 1001–1004
30. Navas, T. A., Zhou, Z., and Elledge, S. J. (1995) Cell 80, 29–39
31. Siede, W., Nusspaumer, G., Portillo, V., Rodriguez, R., and Friedberg, E. C. (1996) Nature Acids Res. 24, 1669–1675
32. Bluysens, H. A. R., van Os, R. I., Nuss, N. C., Jaspers, I., Hoeijmakers, J. H. J., and de Klein, A. (1996) Genomics 45, 331–337
33. Parker, A. E., Van de Weyer, I., Laus, M. C., Oostoven, I., Yon, J., Verhasselt, P., and Luyten, W. H. M. J. (1998) J. Biol. Chem. 273, 18332–18339
33. Marathi, U. K., Dahlen, M., Sunnerhagen, P., Romero, A. V., Ramagli, L. S., Siciliano, M. J., Li, L., and Legerksi, R. J. (1996) Genomics 34, 344–347
34. Freire, R., Murugia, J. R., Tarsounas, M., Lowndes, N. F., Moens, P. B., and Jackson, S. P. (1996) Nature 382, 2550–2573
35. Udell, C. M., Lee, S. K., and Davey, S. (1998) Nucleic Acids Res. 26, 3971–3976
36. Venclovas, C., and Thelen, M. P. (2000) Nucleic Acids Res. 28, 2481–2493
37. Ahmed, S., and Hodgkin, J. (2000) Nature 403, 159–164
38. Thelen, M. P., Oneil, K., and Hengartner, M. O. (1996) J. Biol. Chem. 271, 747–754
39. Gartner, A., Milstein, S., Ahmed, S., Hodgkin, J., and Hengartner, M. O. (2000) Mol. Cell 5, 435–443
40. Thelen, M. P., Venclovas, C., and Fidelis, K. (1999) Cell 97, 769–770
41. Kondo, T., Matsumoto, K., and Sugimoto, K. (1999) Mol. Cell. Biol. 19, 1136–1143
42. Piacenti, V., Luchini, G., Plevani, P., and Longhese, M. P. (1998) EMBO J. 17, 4199–4209
43. Kostrub, C. P., Kudrun, K., Subramani, S., and Enach, T. (1998) EMBO J. 17, 2055–2066
44. Caspari, T., Dahlen, M., Kanter-Smolker, G., Lindsay, H. D., Hofmann, K., Papadimitriou, K., Sunnerhagen, P., and Carr, A. M. (2000) Mol. Cell Biol. 74, 1254–1262
45. St. Onge, R. P., Udell, C. M., Casselman, R., and Davey, S. (1999) Mol. Biol. Cell 10, 1985–1995
46. Hang, H., and Lieberman, H. B. (2000) Genomics 65, 24–33
47. Volkmer, E., and Karnitz, L. M. (1999) J. Biol. Chem. 274, 567–570
48. Besheo, T., and Sanacir, A. (2000) J. Biol. Chem. 275, 7451–7454
49. Nakai, T., Shimomura, T., Kondo, T., Matsumoto, K., and Sugimoto, K. (2000) Mol. Cell. Biol. 20, 5888–5896
50. Shimomura, T., Ando, S., Matsumoto, K., and Sugimoto, K. (1998) Mol. Cell. Biol. 18, 5499–5501
51. Green, C. M., Erdjument-Bromage, H., Tempst, P., and Lowndes, N. F. (2000) Curr. Biol. 10, 39–42
52. Rauen, M., Burtelow, M. A., Dufaute, V. M., and Karnitz, L. M. (2000) J. Biol. Chem. 275, 28434–28438
53. Schiestl, R. H., Reynolds, P., Prakash, S., and Prakash, L. (1989) Mol. Cell. Biol. 9, 1882–1896
54. Weinert, T. A., and Hartwell, L. H. (1993) Genetics 134, 63–80
55. Chan, M. T., Capasso, H., and Walworth, N. (1999) Yeast 15, 821–828
56. Bentley, N. J., Holtzman, D. A., Flagg, G., Koonin, P., and Lieberman, H. B. (1999) EMBO J. 18, 6561–6572
57. Soulier, J., and Lowndes, N. F. (1999) Curr. Biol. 9, 551–554
58. Piacenti, V., Clerici, M., Luchini, G., and Longhese, M. P. (2000) Genes Dev. 14, 2046–2056
59. Visintin, R., and Amon, A. (2000) Curr. Opin. Cell Biol. 12, 372–377
60. Chang, M.-S., Sasaki, H., Campbell, M. S., Kraeft, S. K., Sutherland, R., Yang, C.-Y., Liu, Y., Autzler, D., Hase, L., Sonoda, H., Ferland, L. H., and Chen, L. B. (1999) J. Biol. Chem. 274, 36544–36549
Characterization of DNA Damage-stimulated Self-interaction of *Saccharomyces cerevisiae* Checkpoint Protein Rad17p

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