Diverse but Overlapping Functions of the Two Forkhead-associated (FHA) Domains in Rad53 Checkpoint Kinase Activation*§

Forkhead-associated (FHA) domains are phosphothreonine-binding modules prevalent in proteins with important cell cycle and DNA damage response functions. The yeast checkpoint kinase Rad53 is unique in containing two FHA domains. We have generated novel recessive rad53 alleles with abolished FHA domain functions resulting from Ala substitution of the critical phosphothreonine-binding residues Arg70 and Arg605. In asynchronous cells, inactivation of the N-terminal FHA1 domain did not impair Rad53 activation and downstream functions, whereas inactivation of the C-terminal FHA2 domain led to reduced Rad53 activation and significantly increased DNA damage sensitivity. Simultaneous inactivation of both FHA domains abolished Rad53 activation and all downstream functions and dramatically increased the sensitivity to DNA damage and replication blocks similar to kinase-defective and rad53 null alleles, but did not compromise the essential viability function of Rad53. Interestingly, in G2/M synchronized cells, mutation of either FHA domain prevented Rad53 activation and impaired the cell cycle arrest checkpoint. Our data demonstrate that both FHA domains are required for normal Rad53 functions and indicate that the two FHA domains have differential but partially overlapping roles in Rad53 activation and downstream signaling.

FHA§ domains contain ~100–180 amino acid residues forming an 11-stranded β-sandwich and act as protein-protein interaction modules by binding to phosphothreonine (Thr(P)) residues in target ligands (1). FHA domains are present in a large number of proteins in all phyla from bacteria to mammals and seem to be prevalent among proteins with cell cycle and DNA damage response functions. Important human FHA domain-containing checkpoint proteins include NBS1 that is mutated in the Nijmegen breakage syndrome (2), CHK2 that is mutated in a subset of cases of the Li-Fraumeni multicancer syndrome (3), the spindle checkpoint protein CHFR (4), and the recently identified NFBP1/MDC1 (5).

The yeast homolog of the CHK2 kinase, Rad53, is the only known protein to contain two FHA domains (6). Rad53 plays central roles in yeast DNA damage and replication block checkpoints (7). The finding that the C-terminal FHA2 domain plays an important role in the DNA damage-dependent activation of Rad53 by binding to the phosphorylated upstream protein Rad9 was instrumental in defining FHA domains as Thr(P)-binding modules (8). Phosphorylated Rad9 can also bind to the N-terminal FHA1 domain, but the in vivo relevance of this interaction is unknown (9–11). Other proposed Rad53 FHA domain ligands include Dbf4 that can interact in yeast two-hybrid and in vitro assays with both the FHA1 and FHA2 domain (12) and the protein phosphatases Ptc2 and Ptc3 that can interact with the FHA1 domain and then down-regulate Rad53 activity in the recovery from DNA damage (13). An important question considering the presence of two FHA domains in Rad53 compared with a single N-terminal FHA domain in other related kinases is: do the two FHA domains have independent specialized functions, or do they act in a redundant manner?

To address this question we have now used chromosomal site-directed mutagenesis to generate novel rad53 alleles leading to amino acid substitutions that specifically inactivate the Thr(P)-binding site in the two Rad53 FHA domains, and we compare the functions of single FHA1 and FHA2 mutants, FHA1/2 double mutant, kinase-defective, and rad53Δ alleles.

MATERIALS AND METHODS

Yeast Strains and Cultures—All haploid yeast strains were derived from the W303-1A strain U952–3B (MATa ade2–1 can1–100 leu2–3,112 trp1–1 ura3–1 sml1::HIS3 RAD5) (14). rad53R70A, rad53S227A, and rad53Δ alleles were described previously (15). The rad53R605A and rad53R70AR605A alleles were generated by similar PCR-based allele replacement (16). To obtain diploids, these strains were mated to a W303-1B strain containing wild type SML1 and RAD53. After sporulation, a MATa rad53R70A rad53Δ SML1 haploid was mated to the original rad53Sml1Δ strain. All incubations were at 30 °C in 2% yeast extract, 1% peptone, 2% glucose (YPD), except for 0.05% MMS. For liquid assays, 100 m M HU or 0.1% MMS was added to log-phase cultures for the indicated times. For cell cycle arrest studies, asynchronous cells were spotted in serial 10-fold dilutions onto plates containing 5–20 mM hydroxyurea (HU) or 0.02–0.025% mithramycin sulfate (MMS). For liquid assays, 100 mM HU or 0.1% MMS was added to log-phase cultures for the indicated times. For cell cycle arrest studies, log-phase cultures were treated with 15 μg/ml nocodazole for 2 h and synchronized (>95%) assessed by phase contrast microscopy. Cultures were kept in nocodazole for another 30 min in the presence or absence of 0.05% MMS and washed and released into YPD ± 0.05% MMS. Aliquots were fixed in 70% ethanol, washed, and stained using 0.5 μg/ml 4',6'-diamidino-2-phenylindole hydrochloride in phosphate-buff ered saline. 100 cells per sample were scored at ×400 magnification using a Zeiss Axiosvert 25 phase contrast/fluorescence microscope.

Northern Blots, Western Blots, and Kinase Assays—Northern and Western blots (17), Rad53 autophosphorylation (18), and Dun1 immunoprecipitation kinase assays (19) were performed essentially as de-
Rad53 FHA Domain Functions in DNA Damage Survival—

RESULTS

Rad53 FHA Domain Functions in DNA Damage Survival—

Based on NMR spectroscopic and x-ray crystallographic analyses, a conserved Arg residue (Arg⁷⁰ in the FHA1 and Arg⁶⁰⁵ in the FHA2) is pivotal for direct FHA domain interactions with the phosphate group of Thr(P)-containing peptide ligands (9, 11, 20). Substitution of the critical Arg by Ala specifically abolishes the ability of FHA domains to bind to Thr(P)-containing ligands without affecting other biophysical properties of the FHA domains such as protein folding and stability in vitro (1). We have previously shown that an FHA1 mutated rad53R70A allele is expressed at wild type protein levels but confers a moderately increased DNA damage sensitivity in vivo (1). To expand these studies and to systematically address the contribution of the two FHA domains to Rad53 functions under the most physiological conditions possible, we generated novel rad53 alleles with Thr(P)-binding site inactivating alanine substitutions of the FHA2 domain (rad53R605A) and both FHA domains (rad53R70A/605A) for comparison with the FHA1-defective rad53R70A, kinase-defective rad53K227A, and rad53Δ alleles (Fig. 1A).

In DNA damage sensitivity assays, the FHA1 or FHA2 single mutant strains had no noticeable survival defect on plates containing the replication blocking agent HU or up to 0.02% of the DNA damaging agent MMS (Fig. 1B). However, in the presence of higher MMS doses the FHA2 mutant rad53R605A allele resulted in ~100-fold reduced colony formation compared with the wild type and rad53R70A (Fig. 1B, 0.025% MMS). Importantly, on 20 mM HU or 0.02% MMS, where the single mutants had no survival defect, the FHA1/2 double mutant strain was dramatically damage hypersensitive similar to the kinase-defective rad53K227A and the rad53Δ strains (Fig. 1B). Moreover, on low dose HU plates the rad53R70AR605A strain had a more severe growth defect than the kinase-defective strain (Fig. 1B).

FHA Domain Functions in Rad53 Activation—To explore these DNA damage hypersensitivity phenotypes further, we investigated how FHA domain mutations affect Rad53 activation. DNA damage-dependent Rad53 activation results in slower electrophoretic mobility forms detectable on Western blots as a result of Rad53 autophosphorylation and phosphorylation by the upstream kinase Mec1 (18). In asynchronous cultures, the FHA1 mutation did not reduce Rad53 mobility shifts in response to MMS, while the FHA2 mutation significantly reduced Rad53 activation (Fig. 2A). Importantly, the double FHA1/2 mutation completely abolished Rad53 mobility shifts, in contrast to kinase-defective Rad53 that was efficiently modified to an intermediate mobility form. Consistent results were obtained in Rad53 autophosphorylation assays, where the kinase activity of the Rad53-R70A protein was similar to the wild type, but activity of Rad53-R605A was significantly reduced, and activity of the FHA1/2 double mutant was essentially abolished with levels similar to the kinase-defective...
Rad53 FHA Domain Functions

Rad53 FHA Domain Functions in \( G_{2}/M \) Checkpoint Activation of Rad53—A major function of Rad53 is to delay mitosis in the presence of DNA damage in the \( G_{2}/M \) checkpoint (22). To assess \( G_{2}/M \) checkpoint function, nocodazole synchronized cells were released into normal medium or treated with 0.05% MMS for the final 30 min under nocodazole arrest and released into MMS-containing medium and then scored for binucleated late anaphase and telophase cells. In wild type cells, DNA damage clearly delayed the completion of mitosis (−60% reduced binucleation index 60 min after release; see Fig. 3C and Supplemental Fig. S1). This delay was impaired in all Rad53 FHA domain mutants to an extent similar to the kinase-defective strain (−40% reduced binucleation), but not as much as in rad53Δ (−20% reduction; Fig. 3C and Supplemental Fig. S1), which may be partly due to much slower release kinetics of this strain in the absence of DNA damage.

It was surprising that the single FHA1 mutation impaired the \( G_{2}/M \) checkpoint to the same extent as the single FHA2 and FHA1/2 double mutations, in contrast to its modest effects on Rad53 activation and signaling in asynchronous cells. We therefore monitored Rad53 activation in response to MMS treatment of nocodazole-arrested cells. Interestingly, mutation of either FHA domain, completely abolished Rad53 activation under these conditions (Fig. 2D). These data demonstrate that both FHA domains are essential for Rad53 activation and cell cycle arrest signaling in the \( G_{2}/M \) checkpoint.

FHA Domain Mutant Phenotypes Are Recessive and Unrelated to the Essential Function of Rad53—To test whether DNA damage hypersensitivities in FHA mutants (Fig. 1B) are loss-of-function phenotypes or a dominant-negative consequence of aberrant signaling caused by stray FHA ligands no longer targeted to the correct cellular sites, heterozygous diploid strains were generated that contained the respective rad53 mutant alleles in combination with the wild type \( RAD53 \) allele. All of these strains had normal viability in the presence of HU or MMS (Fig. 1C). Furthermore, the mutant Rad53 forms did not interfere with activation of the wild type form in these strains (Fig. 2E). The FHA1/2 double mutant in combination with \( RAD53 \) also resulted in extreme HU and MMS hypersensitivity (Fig. 1C) and failed to be activated in diploids (Fig. 2E). Altogether, these results demonstrate that the FHA domain mutations behave in a recessive manner.

In the absence of exogenous DNA damaging agents, \( rad53Δ \) is lethal unless balanced by extragenic suppressors such as \( sml1Δ \) (14). Surprisingly, \( rad53\text{-K227A} \) is viable without \( sml1Δ \), a phenomenon attributed to residual but barely detectable kinase activity in this mutant (23). As the \( rad53\text{-R70AR605A} \) strain was more sensitive to low level replication blocks than the kinase-defective strain (Fig. 1B), we sporulated the \( rad53\text{-R70AR605A}/RAD53\text{-sml1Δ}/SML1 \) diploid and analyzed resulting haploids for the segregation of \( RAD53 \) and \( SML1 \) alleles. As expected, all viable \( rad53Δ \) colonies from a similar sporulation experiment contained \( sml1Δ \) and failed to be activated in diploids (Fig. 2E). Altogether, these results demonstrate that the FHA domain mutations are specific for the DNA damage response function but not the essential function of Rad53.

Discussion

The results presented here demonstrate that the two FHA domains play important roles in activation of the Rad53 kinase in response to DNA damage. Overall, effects of FHA domain mutations on Rad53 activation correlate well with their defects in checkpoint signaling downstream of Rad53 (cell cycle arrest, transcriptional response, Dun1 activation), indicating that re-
Rad53 activation in G2/M-synchronized cells (24). The simplest Rad17 pathways leads to only partially reduced Rad53 activation (7) and implies that Rad53 activation in vivo depends critically on its direct phosphorylation by Mec1. This in vivo situation is in contrast to the in vitro model in which Rad53 can be activated by autophosphorylation when bound to Mec1-phosphorylated Rad9 without requiring direct phosphorylation of Rad53 by Mec1 (25).

Interestingly, under conditions where the FHA1 mutation alone did not impair Rad53 activation, it actually enhanced some Rad53 functions (Rad53 gel shifts in HU, RNR3 induction, and Dun1 activation; Figs. 2 and 3). This is fully consistent with the proposed role of the FHA1 domain in down-regulation of Rad53 activity by recruiting Ptc2/3 (13). The results for the FHA1 domain therefore indicate that a single FHA domain can have multiple functions in vivo (Fig. 4B).

Finally, our data indicate that the conserved Arg should be the residue of choice for mutational analyses of FHA domain functions as its substitution results in very specific phenotypes without affecting protein levels.

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Fig. 4. FHA domains are not required for the essential viability function of Rad53. A, genotyping of haploid strains obtained from sporulation of heterozygous diploids with the Rad53 allele combination shown above and SML1×sml1Δ. Impaired growth on HU indicates rad53 mutations; growth on −His indicates sml1Δ. B, summary diagram of proposed Rad53 FHA domain functions. Both FHA domains are required for Rad53 activation, but the FHA2 contributes more to activation in asynchronous cells. Both FHA domains may interact with Dbf4/Cdc7 as a Rad53 target, but only the FHA1 interacts with Ptc2/3 in Rad53 dephosphorylation.