Location-Specific Functions of the Two Forkhead-Associated Domains in Rad53 Checkpoint Kinase Signaling[†]

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ABSTRACT: Signaling proteins often contain multiple modular protein–protein interaction domains of the same type. The *Saccharomyces cerevisiae* checkpoint kinase Rad53 contains two phosphothreonine-binding forkhead-associated (FHA) domains. To investigate if the precise position of these domains relative to each other is important, we created three *rad53* alleles in which FHA1 and FHA2 domains were individually or simultaneously transposed to the opposite location. All three mutants were approximately 100-fold hypersensitive to DNA lesions whose survival requires intact Rad53 FHA domain functions, but they were not hypersensitive to DNA damage that is addressed in an FHA domain-independent manner. FHA domain-transposed Rad53 could still be recruited for activation by upstream kinases but then failed to autophosphorylate and activate FHA domain-dependent downstream functions. The results indicate that precise FHA domain positions are important for their roles in Rad53, possibly via regulation of the topology of oligomeric Rad53 signaling complexes.

Various types of modular protein–protein interaction domains play critical roles in the assembly of macromolecular signaling complexes in eukaryotic cells (*1*). The concept of modularity is underscored by findings that signaling pathways can be experimentally rewired by molecular transplantation of specific domains; for example, fusion of the Grb2 SH2 domain to the FADD death-effector domain leads to apoptosis in response to normally mitogenic signals (*2*). Signaling proteins frequently contain more than one modular domain, presumably to enable simultaneous interaction with multiple binding partners. Proteins often contain multiple copies of the same type of modular domain; this can be in tandem (for example, the adaptor protein Nck contains three consecutive SH3 domains) or interrupted by a member of another domain family (for example, two SH3 domains flanking an SH2 domain in Grb2). In principle, such domain repetition could simply serve to bind multiple ligands at the same time. However, if distinct ligands are recruited to the repeated modules, the precise spatial arrangement of specific domains would also predetermine the overall topology of how recruited ligands are positioned relative to each other in ternary complexes. Surprisingly, although there are several examples in which modular domains have been experimentally exchanged between different proteins, to our knowledge it has not yet been investigated if repetitive domain family members can be "swapped" within the same protein.

We have chosen the yeast Rad53 checkpoint kinase as a model for determining if the positions of repeated domain modules are functionally interchangeable. Rad53 contains two phosphothreonine-binding FHA[1] domains that have partially redundant as well as specific functions in Rad53 activation and recruitment of downstream effectors (*3–14*). Although the two Rad53 FHA domains differ considerably in their primary sequence and the length of some loops, both form three-dimensionally remarkably similar 11-stranded β-sandwiches (*15*). While the Rad53 FHA1 and FHA2 domains were originally believed to differ fundamentally in their binding specificity for the "plus three" position C-terminal of the phosphothreonine on the basis of in vitro peptide binding studies (*15, 16*), subsequent studies have indicated that the rules for preferred binding specificities do not seem to be as strict in vivo (*9, 17–19*). In addition to the FHA domains, Rad53 contains two SQ/TQ cluster domains (SCDs) that also play a role in the phosphorylation-dependent activation of Rad53 by the ATM/ATR-like upstream kinases Mec1/Tel1 and the recruitment of the downstream kinase Dun1 to propagate the DNA damage signal (*10, 20–24*) (Figure 1A). As the exact boundaries of both FHA domains are known from three-dimensional structural analyses (*16, 25, 26*), this system allows for the precise transposition of the FHA domains with a reduced risk of nonspecific protein destabilization and without disruption of the nearby SCDs. Furthermore, powerful genetic tools are available for *Saccharomyces cerevisiae* for altering domain-coding regions in the

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[1] Abbreviations: FHA, forkhead-associated; MMS, methylmethanesulfonate; SCD, SQ/TQ cluster domain.

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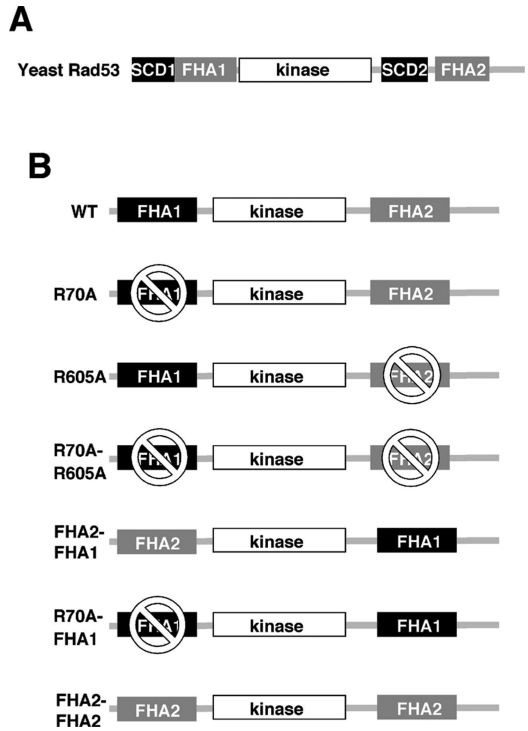

FIGURE 1: Schematic diagram of the Rad53 domain organization and alleles used in this study. (A) Diagram of the Rad53 domain organization. FHA, forkhead-associated domain; SCD, SQ/TQ cluster domain; kinase, kinase domain. (B) Diagram of FHA domain constructs expressed from the endogenous *RAD53* locus. R70A and R605A specifically disable the pThr binding function of FHA1 and FHA2 domains, respectively.

endogenous chromosomal gene locus so that expression of altered proteins occurs in a physiological context (*27*). Here, we have utilized these experimental advantages to alter the positions of the Rad53 FHA domains relative to each other, and we show that either domain can fulfill its function only when in its natural position.

MATERIALS AND METHODS

Yeast Strains. All experiments were performed in the W303-1A background containing *sml1::HIS3* and corrected *RAD5* (kindly provided by R. Rothstein) (*28*). Our strain numbers for the FHA domain-transposed alleles were Y427 (*rad53-FHA2-FHA2*), Y428 (*rad53-R70A-FHA1*), and Y492 (*rad53-FHA2-FHA1*). Control strains Y53 (*RAD53*), Y57 (*rad53-R70A*), Y59 (*rad53-K227A*), Y60 (*rad53::URA3*), Y205 (*rad53-R605A*), and Y206 (*rad53-R70A-R605A*) were described previously (*5*).

Shuffling of FHA Domains. Fragments for pop-in transformation were generated in three consecutive PCRs. In the first step, the FHA1 and FHA2 domains were amplified by PCR using primers Rad53-1−Rad53-4. At the 3′-end, each primer contains 20 nucleotides matching the FHA domain boundaries (FHA1 domain, amino acid residues 27–158, nucleotide positions 81–474; FHA2 domain, amino acid residues 573–730, nucleotide positions 1719–2190); at the 5′-end, each primer contains 20 nucleotides flanking the respective other FHA domain position (N, K1, K2, or C). In parallel, short (~100 bp) targeting extensions adjacent to the FHA domains were amplified using adaptamer A and B compatible primers. In the second step, these fragments were mixed and fused by PCR using only the outside primers. In the third step, the resulting fragments were fused to partially truncated yet overlapping *Kluyveromyces lactis URA3* fragments for standard PCR-based pop-in/pop-out mutagenesis (*27*) similar to methods used for generating *rad53-R605A* and *rad53-R70A-R605A* (*5*).

Primer combinations for moving the FHA1 domain were as follows. Round 1: FHA1, Rad53-1 + Rad53-2; K2 ext, Rad53-5 + Rad53-11; C ext, Rad53-12 + Rad53-6. Round 2: Rad53-5 + Rad53-6. Round 3: Rad53-5 + URA 3′ and Rad53-6 + URA 5′. Primer combinations for moving the FHA2 domain were as follows. Round 1: FHA2, Rad53-3 + Rad53-4; N ext, Rad53-7 + Rad53-9; K1 ext, Rad53-10 + Rad53-8. Round 2: Rad53-7 + Rad53-8. Round 3: Rad53-7 + URA 3′ and Rad53-8 + URA 5′.

Primer sequences were as follows: Rad53-1, 5′-TCA AGA GCT CAA AGA AAA AGC AGA TCG GCG AAA ACA TTG TG-3′; Rad53-2, 5′-TGA GCT GCC ATC ATC TGG GTG TTC TGC TCG AGG CAC TGC T-3′; Rad53-3, 5′-TTG AGA AGT TTT CTC AAG AAG GTA ATG GTA GGT TTT TAA C-3′; Rad53-4, 5′-GAT CTT ATG CGA TCA ACT TTT TAC TTT TTC ACC AAA TCT T-3′; Rad53-5, 5′-AAT TCC AGC TGA CCA CCA TGC AAG ATG GAA AAA TTC AAG G-3′; Rad53-6, 5′-GAT CCC CGG GAA TTG CCA TGG ACT CTA CCA AGT CGT TTA G-3′; Rad53-7, 5′-AAT TCC AGC TGA CCA CCA TGG CTT TAA AAG AGA GAA TAG TG-3′; Rad53-8, 5′-GAT CCC CGG GAA TTG CCA TGC ACC ACT TCG TCA ATA ATC G-3′; Rad53-9, 5′-TTC TTG AGA AAA CTT CTC AA-3′; Rad53-10, 5′-AAA GTT GAT CGC ATA AGA TC-3′; Rad53-11, 5′-CTT TTT CTT TGA GCT CTT GA-3′; Rad53-12, 5′-ACC CAG ATG ATG GCA GCT CA-3′.

DNA Damage Response Assays. MMS and bleomycin sensitivity assays, Northern blots, Western blots, and in-gel autokinase assays were performed as described previously (*5, 8, 29*).

RESULTS AND DISCUSSION

Rad53 FHA Domain Transposition Results in Increased MMS Sensitivity. To test if the precise location of the FHA domains is important for Rad53 function, we shuffled their positions by moving the FHA1 domain (amino acid residues 27–158) to the position of the FHA2 domain (amino acid residues 573–730) and vice versa (Figure 1B). Interestingly, despite intact FHA1 and FHA2 domains, this *rad53-FHA2-FHA1* allele resulted in severe hypersensitivity to the DNA methylating agent MMS (Figure 2A). This MMS hypersensitivity was comparable to that of the *rad53-R70A-R605A* allele (Figure 2A,B), where both FHA domains are specifically disabled by alanine substitution of the critical arginine residues R70 and R605 (*5*) in the phosphothreonine binding site. This result indicates that simultaneous transposition of the two intact FHA domains impairs Rad53 functions in the MMS response. To distinguish if either the FHA1 domain, the FHA2 domain, or both have to be in their natural position for normal Rad53 function, we generated two strains in which individual FHA domains were transposed. First, to specifically investigate the importance of the location of the FHA1 domain, the *rad53-R70A-FHA1* strain was generated, where the natural FHA1 domain is disabled by R70A substitution and a single intact FHA1 domain is located in the FHA2 domain position (Figure 1B). This strain was as MMS-

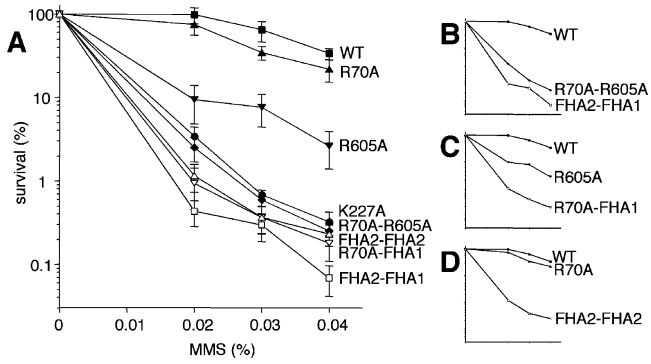

FIGURE 2: MMS sensitivity assays of FHA domain-transposed rad53 alleles. (A) Cell survival in response to 0.02–0.04% MMS treatment for 3 h (mean ± standard error of three or four independent experiments). (B−D) Simplified plots of the same data.

hypersensitive as the entirely FHA domain-disabled *rad53-R70A-R605A* strain (Figure 2A). Interestingly, this dramatic MMS hypersensitivity was a remarkable contrast to the intermediate MMS hypersensitivity of the *rad53-R605A* allele (Figure 2A,C) that also contains only the FHA1 domain as the sole intact FHA domain, in this case because the endogenous FHA2 domain is disabled by R605A substitution (Figure 1B). The increased DNA damage hypersensitivity of "FHA1-only" Rad53 with the transposed domain (*rad53-R70A-FHA1*) compared to that of the FHA1-only *rad53-R605A* allele therefore indicates that the FHA1 domain is fully functional only when in its natural position. For unknown reasons, repeated attempts to engineer a similar *rad53-FHA2-R605A* allele to study the role of a single intact FHA2 domain in the natural FHA1 domain position were unsuccessful. However, we were able to generate a *rad53-FHA2-FHA2* strain in which Rad53 contains an extra FHA2 domain at the FHA1 domain position in addition to the FHA2 domain in its natural location (Figure 1B). Again, this strain was profoundly MMS-hypersensitive (Figure 2A). This result was surprising considering the very modest MMS hypersensitivity of the *rad53-R70A* strain (Figure 2A,D). Both *rad53-R70A* and *rad53-FHA2-FHA2* strains encode functional FHA2 domains in the natural position, and neither encodes a functional FHA1 domain. The fact that substitution of a defective FHA1 domain with an intact FHA2 domain results in much greater sensitivity to methylating DNA damage therefore indicates that misplacement of the FHA2 domain does not simply result in a loss-of-function phenotype but that it misguides Rad53 in a dominant-negative manner.

FHA Domain Transposition Does Not Impair FHA Domain-Independent Rad53 DNA Damage Response Functions. The main reason for MMS hypersensitivity of *rad53* mutants is believed to be their inability to stabilize stalled replication forks and to prevent their irreversible collapse during the S phase (*30, 31*). To determine if FHA domain transposition interferes with DNA damage response functions in general, we analyzed the response to a mechanistically distinct agent, bleomycin, that gives rise to 3′-phosphoglycolate-blocked DNA double-strand breaks as the major cytotoxic lesion (*32, 33*). Interestingly, in response to chronic bleomycin exposure, the entirely FHA domain-defective *rad53-R70A-R605A* allele was able to sustain survival rates very similar to that of the wild type under conditions where a rad53Δ null allele was lethal (Figure 3). This differential requirement for FHA domains indicates that there may be an alternative or

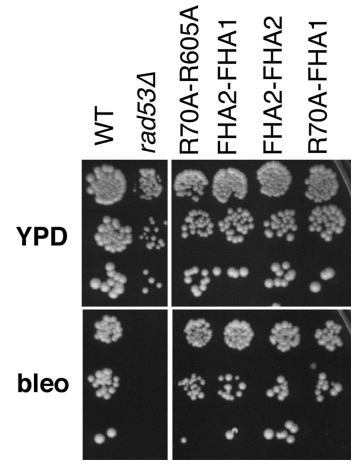

FIGURE 3: Bleomycin sensitivity assays of FHA domain-transposed rad53 alleles. Cell growth of 10-fold serial dilutions of cells (from top to bottom) on control plates (top) and in the continuous presence of 75 ng/mL bleomycin for 3 days.

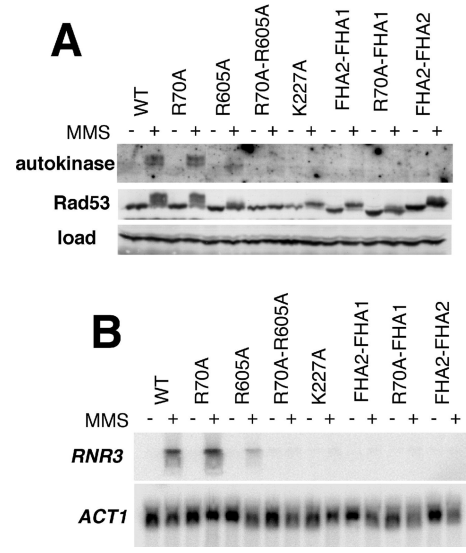

FIGURE 4: Rad53 activation and downstream signaling. (A) Rad53 in situ autokinase activity and Western blot analysis of identically loaded lysates from MMS-treated or untreated cells as indicated. (B) Northern blot analysis of MMS-induced *RNR3* expression and *ACT1* loading control of the indicated strains.

redundant Rad53 activation mechanism for the response to bleomycin that is not available for the response to MMS damage. Importantly, all three FHA domain-transposed strains (*rad53-FHA2-FHA1*, *rad53-R70A-FHA1*, and *rad53-FHA2-FHA2*) also had bleomycin survival rates similar to that of the wild type (Figure 3). These results therefore indicate that FHA domain transposition affects Rad53 functions only in response to lesions that are handled in an FHA domain-dependent manner and, thereby, demonstrate that FHA domain transposition does not generally impede *RAD53* functions as a nonspecific quasi-null allele.

FHA Domain-Transposed Rad53 Can Be Recruited by Upstream Kinases but Is Impaired in Autoactivation and Downstream Signaling. All three FHA domain-transposed Rad53 variants were expressed at essentially wild-type levels, further supporting the notion that they were correctly folded (Figure 4A, middle panel). Consistent with the presence of an extra 27 amino acid residues in the FHA2 domain compared to the FHA1 domain, Rad53-R70A-FHA1 had

slightly accelerated electrophoretic mobility and Rad53 with the FHA2 domain duplication had slightly slower mobility compared to those of the wild-type protein (Figure 4A). Rad53 activation in response to DNA damage involves its initial phosphorylation by the upstream kinases Mec1 and Tel1 and subsequent autophosphorylation that can be followed by electrophoretic mobility shifts on Western blots (*10, 34*). Interestingly, in contrast to the entirely FHA domain-defective R70A/R605A variant, all three FHA domain-transposed Rad53 proteins were still shifted to slower electrophoretic mobility forms in response to MMS-induced DNA damage (Figure 4A, middle panel). This indicates that FHA domain transposition does not impair the recruitment of Rad53 by checkpoint adapters (including, for example, Rad9) into the pathway as a prerequisite for its phosphorylation by the upstream kinases in vivo. However, all three FHA domain-transposed Rad53 variants lacked in vitro autokinase activity (Figure 4A, top panel) and largely lacked supershifted bands after MMS treatment as a marker for autokinase activity in vivo, very similar to the case for the kinase-defective *rad53-K227A* allele (Figure 4A, middle panel). Likewise, transcriptional induction of *RNR3* after MMS treatment as a marker for Rad53 downstream signaling in vivo was also severely impaired in all three FHA domain-transposed alleles, in a manner similar to that of the FHA domain-defective *rad53-R70A-R605A* and kinase-defective *rad53-K227A* alleles (Figure 4B). Taken together with the MMS sensitivity assays, these results indicate that Rad53 with intact FHA domains in the transposed location can in principle still be primed by the upstream signaling pathway but then fails to further autophosphorylate and autoactivate as a prerequisite for the recruitment of downstream effectors.

Location-Specific Constraints on Repetitive Domain Functions in Multimodular Signaling Proteins. Our results indicate that, despite largely redundant roles in the DNA damage response, each of the two Rad53 FHA domains can only completely fulfill its functions when in its native position. The position of the FHA domains relative to each other seems to be less important for DNA damage-dependent Rad53 phosphorylation, which tends to be proportional to Rad53 activation (e.g., compare intermediate MMS sensitivity, mobility shift, autokinase, and *RNR3* induction of *rad53-R605A* relative to other strains in Figures 2 and 4). However, "activated" Rad53 with transposed FHA domains fails to properly engage downstream signaling components, but only in response to DNA lesions that are normally handled in an FHA domain-dependent manner. As each FHA module is structurally intact in our system (and should therefore be able to interact with its natural ligands even when in the wrong position), these results indicate that location is pivotal for Rad53 FHA domain functions by regulating not only which proteins are bound but also where they are bound. A possible explanation for these findings could be that the FHA domains contribute to a scaffold function of Rad53, in a manner where the location of recruited ligands also determines their orientation toward each other. Our results should be relevant for signaling proteins beyond the DNA damage response and suggest that multiple repeats of the same modular domain in a given protein not only allow for simultaneous recruitment of multiple ligands by a similar interaction mode (e.g., pThr binding in the case of FHA domains) but also play crucial roles in predetermining the precise topology of recruited ligands in ternary signaling complexes.

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