FHA domain boundaries of the Dun1p and Rad53p cell cycle checkpoint kinases

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Abstract Dun1p and Rad53p of the budding yeast *Saccharomyces cerevisiae* are members of a conserved family of cell cycle checkpoint protein kinases that contain forkhead-associated (FHA) domains. Here, we demonstrate that these FHA domains contain 130–140 residues, and are thus considerably larger than previously predicted by sequence comparisons (55–75 residues). In vivo, expression of the proteolytically defined Dun1p FHA domain, but not a fragment containing only the predicted domain boundaries, inhibited the transcriptional induction of repair genes following replication blocks. This indicates that the non-catalytic FHA domain plays an important role in the transcriptional function of the Dun1p protein kinase.

Key words: Forkhead-associated domain; Dun1p; Rad53p; Chk2; Protein kinase; Cell cycle

1. Introduction

Cell cycle checkpoints play an essential role in maintaining the integrity of the genome by delaying the progression through the cell cycle in response to DNA damage, replication blocks or mitotic spindle assembly defects [1]. The importance of such surveillance mechanisms is underscored by the existence of human genetic disorders with impaired checkpoint functions, such as ataxia–telangiectasia (AT), Nijmegen breakage syndrome and Li–Fraumeni syndrome, resulting in a predisposition to cancer [2,3]. As might be expected for a cellular event of such fundamental importance, checkpoint signalling pathways are highly conserved at the molecular level from budding yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*) through nematodes and flies to humans [1,4,5].

The *S. cerevisiae* checkpoint proteins Dun1p [6] and Rad53p [7] are members of a conserved family of forkhead-associated (FHA) domain-containing protein kinases that includes Cds1 in *S. pombe*, and Chk2/Cds1 in mammals [8–10]. These kinases are activated by phosphorylation in response to checkpoint signals, and the activation depends on the ATM protein kinase [9,10], that is mutated in AT patients, or its homologues Mec1p (*S. cerevisiae*) [11,12] and Rad3 (*S. pombe*) [13], respectively. Mutations in these kinases cause a failure of cell cycle checkpoints, resulting in reduced viability following DNA damage or replication blocks in yeast, and, importantly, are the cause of a subset of human patients with the Li–Fraumeni multicancer syndrome [3].

FHA domain-containing kinases have also been identified in *Drosophila* and *Caenorhabditis elegans*, and *S. cerevisiae* contains a third such kinase, Mek1p, that is involved in the regulation of meiosis [14]. All these kinases contain a single FHA domain in their N-terminus, except for Rad53p which has another FHA domain in the C-terminus. However, modular FHA domains are not restricted to protein kinases, and are found in more than 120 diverse proteins (see http://smart.embl-heidelberg.de). Based on sequence comparisons, the FHA domain was predicted to contain 55–75 amino acid residues [15]. To date, little is known about the function of FHA domains, but the available data indicate that FHA domains act as protein–protein interaction modules, possibly in a phosphorylation-dependent manner. For example, the C-terminal FHA domain (FHA2) of Rad53p can directly interact with Rad9p, but only when the latter is phosphorylated [16]; likewise, the FHA domain of a plant kinase-associated protein phosphatase (KAPP) binds only to the phosphorylated form of a receptor-like protein kinase [17]; and finally, it has recently been shown that FHA domains can directly bind to phosphothreonine-containing peptides in vitro [18], reminiscent of SH2 domain interactions with phosphotyrosine residues.

As little is known about the structure and functional specificity of FHA domains in general, we characterized the N-terminal FHA domains of Rad53p and Dun1p. The data presented here indicate that FHA domains are considerably larger than apparent from sequence comparisons, and that the Dun1-FHA domain plays a role in the transcriptional response to replication blocks.

2. Materials and methods

2.1. Generation of FHA domain constructs

FHA domain constructs were generated by polymerase chain reaction (PCR) using synthetic oligonucleotides (Dun1(1–184)forward, 5′-ATG AAT TCA TGA GTT TGT CCA CGA AAA G-3′; Dun1(1–184)reverse, 5′-GCT CTA GAT TAG TGA TGG TGA TGG TGA TGT GCG GCA CTC GTT GTA GC-3′; Dun1(19–159)forward, 5′-GAT CGA ATT CAA AAG ACA GCA ACG AAG-3′; Dun1(19–159)reverse, 5′-GAT CGT CGA CTT AAT ATG AAC GAC TTT CTG-3′; Dun1(56–112)forward, 5′-GAT CGA ATT CAC TAC AAT CGG TAG AAG-3′; Dun1(56–112)reverse, 5′-GAT CGT CGA CTT ACA AAC GAT TAC CAT TAA TAA AAG-3′; Rad53(1–199)forward, 5′-CAA CTA ACC ATG GAA AAT ATT ACA CAA C-3′; Rad53(1–199)reverse, 5′-CTT GTT GGA TCC CAT TGA TGA TGC AGT AGA TG-3′; cloning sites are underlined) and the Expand

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Abbreviations: AT, ataxia–telangiectasia; FHA, forkhead-associated; HU, hydroxyurea; KAPP, kinase-associated protein phosphatase

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High Fidelity DNA polymerase mixture (Roche). Dun1 constructs were generated using the yeast *DUN1* cDNA [6] as template, Rad53 constructs were generated by PCR using random hexamer primed cDNA synthesized from poly(A)$^+$-RNA of the yeast strain AB1380 [*MAT**a**, ura3-52, lys2-1, ade2-1, his5, trp1, can1-100*, Ile$^-$, Thr$^-$]. For bacterial expression, cDNAs were cloned into pQE60 (Qiagen) between the *Nco*I and the *Bam*HI or *Hin*dIII sites, respectively. For yeast expression, constructs were ligated to the Gal4p DNA binding domain for nuclear targetting by initial cloning into pGBT9 (Clontech) followed by ligation of the resulting *Hin*dIII–*Sal*I restriction fragment into the inducible vector p416Gal1 [19]. Plasmids were transformed into the *Escherichia coli* strain XL1-Blue (Stratagene) and sequences of all PCR-generated fragments were confirmed by automated DNA sequence analysis using dye terminator kits (Perkin-Elmer).

2.2. Bacterial expression, protein purification and limited proteolysis experiments

For bacterial expression, plasmids were transformed into the *E. coli* strain BL21(DE3) that was pretransformed with the pREP4 plasmid (Qiagen) to achieve tighter control of induction. Cultures were grown to mid-log phase in rich medium and expression was induced for 3 h at 30°C using 0.5 mM IPTG. Cells were lysed using Triton X-100 and lysozyme, and soluble His_6-tagged protein was purified to near homogeneity using Ni^{2+} affinity chromatography followed by SP-Sepharose (Pharmacia) cation-exchange chromatography essentially as described [20]. Purified proteins were dialyzed against 20 mM HEPES, pH 7, 1 mM DTT and concentrated to 10–20 mg/ml using Centricon membranes (Amicon), snap-frozen and stored at −20°C until use.

Proteolysis experiments were performed at room temperature using 1 mg/ml Dun1(1–184), Rad53(1–199) and Rad53(547–729), respectively, and 10 μg/ml of the endopeptidase (Roche) indicated in the figure legends and Table 1. 10 μl aliquots were removed at the times indicated and the reaction was terminated by immediately boiling the samples in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer. Reaction products were separated in 15% SDS–polyacrylamide gels and visualized by Coomassie brilliant blue R250 (Bio-Rad) staining.

2.3. Protein sequencing and mass spectrometry

For N-terminal sequencing, aliquots of the proteolysis experiments were transferred onto polyvinyldifluoride membranes after SDS–PAGE and stained with Ponceau-S. Bands corresponding to the protease-resistant fragments were excised and sequenced with a Hewlett Packard G1000A Protein Sequencer as described [21]. Protease-resistant fragments were purified by chromatography on a Brownlee Aquapore RP300 column (300 Å, 7 μm, C_8, 250×2.1 mm) using an acetonitrile gradient in trifluoroacetic acid, identified by SDS–PAGE and subjected to mass analysis using a PerSeptive Biosystems Voyager DE mass spectrometer (MALDI-TOF) or a Perkin-Elmer Sciex API *III* triple quadrupole mass spectrometer (ES-MS) as described [21].

2.4. Yeast cultures, RNA preparation and Northern blot experiments

All experiments were performed in the K699 strain [*MAT**a**, ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, can1-100*]. Yeasts were transformed with p416Gal1 constructs using lithium acetate/polyethyleneglycol 3350, and selected on synthetic medium lacking uracil. Before experiments, overnight cultures in selective medium containing 2% sucrose were diluted to $OD_{600} = 0.1$ and grown for 6 h at 30°C in selective medium containing 2% sucrose and 4% galactose to induce expression of Gal4–FHA fusion proteins, followed by addition of hydroxyurea (HU). Total RNA was prepared by resuspending yeast pellets from 10 ml cultures in 300 μl 0.5 M NaCl, 200 mM Tris–HCl, pH 7.4, 10 mM EDTA and vigorous vortexing after addition of 300 μl phenol and 300 μl glass beads, extraction of the supernatant with $CHCl_3$/isoamylalcohol (24:1) and precipitation with three volumes ethanol. For Northern blot analysis, 20 μg total RNA was loaded per lane after denaturation with formamide/formaldehyde and electrophoresed in 1.2% agarose gels containing formaldehyde, followed by capillary transfer onto Hybond-N membranes (Amersham) using standard procedures. A probe specific for the *RNR2* mRNA was generated by PCR from random-primed yeast cDNA using synthetic oligonucleotides (RNR2forward, 5′-TAC CAT GAA ATC TGG CAG C-3′; RNR2reverse, 5′-TTG AAG AGA CTG CGT AAA AAG-3′). This probe was cloned into pGEM-T (Promega) and confirmed by automated sequencing as above. As a control, membranes were rehybridized using an *18S* RNA antisense oligonucleotide (5′-GCT TAT ACT TAG ACA TGC AT-3′). After high-stringency washes, RNA blots were quantified by phosphorimaging using ImageQuant software (Molecular Dynamics).

3. Results

3.1. Determination of FHA domain boundaries using limited proteolysis

The currently known FHA domains share overall less than 25% sequence identity in a region of 55–75 amino acid residues with only four residues comprising an over 80% consensus, and three further residues comprising the over 65% consensus sequence (http://smart.embl-heidelberg.de). As the prediction of the boundaries of such highly diverse domains by sequence comparisons may miss residues flanking the domain core, we chose to experimentally determine FHA domain boundaries by using a limited proteolysis approach. This approach is based on the assumption that under native conditions, proteases preferentially cleave freely accessible peptide bonds, but do not cut near residues that are inaccessible as part of compact domain structures. To identify the boundaries of the FHA domain of the yeast Dun1p protein kinase, a recombinant fragment containing the entire N-terminal region of Dun1p preceding its catalytic domain (Dun1(1–184)) was incubated with chymotrypsin, a protease that cleaves C-terminal of bulky hydrophobic residues. Analysis of reaction products by SDS–PAGE revealed that, within 2 min, this fragment was quantitatively converted to a ∼16 kDa fragment that was then remarkably stable over the course of the 1 h experiment (Fig. 1A). Similar results were also obtained from the digest of the Dun1(1–184) fragment using endopeptidase Glu-C, that cleaves C-terminal of glutamic acid residues (Fig. 1B). The subsequent analysis of these protease-resistant fragments by N-terminal sequence analysis and mass spectrometry (Table 1) revealed them to contain residues 19–159 (chymotrypsin) and 9–155 (endopeptidase Glu-C). This experiment indicated that the Dun1p FHA domain contains approximately 137 residues from Lys-19 to Glu-155, and that it is thus considerably larger than the 57 residue motif first identified by sequence alignments (Val-56–Leu-112).

To independently confirm that FHA domains are indeed twice as large as previously thought, similar experiments were performed for the Rad53p FHA1 domain. A recombinant fragment (Rad53(1–199)) containing the entire N-terminal region preceding the protein kinase catalytic domain was purified and incubated with chymotrypsin (Fig. 2A) and endopeptidase Glu-C (Fig. 2B), respectively. In both experiments, the Rad53(1–199) fragment was converted to protease-resistant fragments similar to those in the corresponding experiments with the Dun1p N-terminus (Fig. 1). However, in both cases, the predominant protected bands were generated on a slower time-scale than for Dun1(1–184). Particularly in the case of the endopeptidase Glu-C digest, two reaction intermediates persisted to some extent over the 3 h experiment, indicating that the flanking sequences may interact with the Rad53-FHA1 domain. Nevertheless, the band that accumulated most noticeably during the experiment (indicated by the arrow in Fig. 2B) had an electrophoretic mobility similar to the protected bands in the other three experiments (Figs. 1A,B

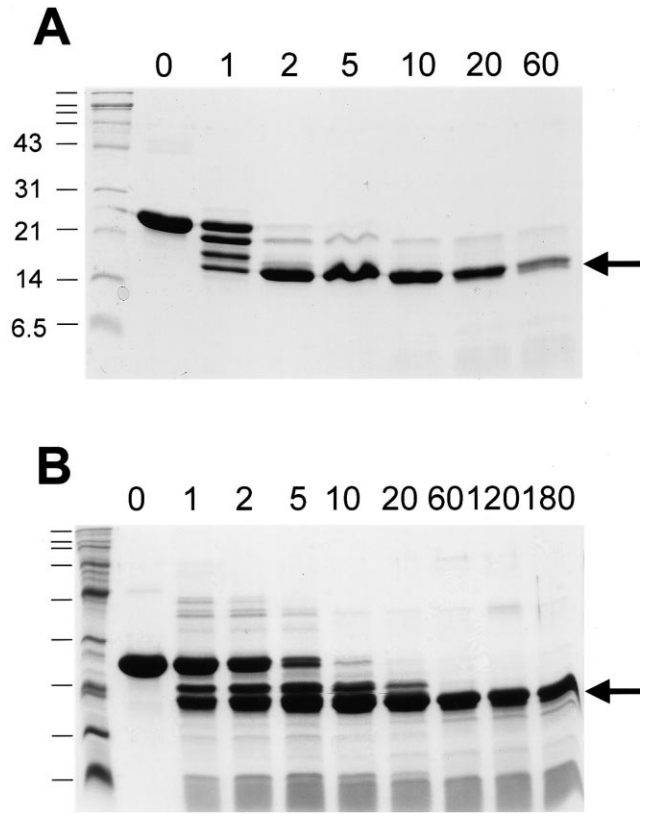

Fig. 1. Identification of protease-resistant Dun1-FHA domain fragments. Dun1(1–184) was incubated with chymotrypsin (A) or endopeptidase Glu-C (B) for the times (min) indicated on top. Mass standards are indicated on the left (from top: 200, 116, 97.4, 69, 43, 31, 21, 14 and 6.5 kDa). Arrows point to the major protected fragment in each experiment.

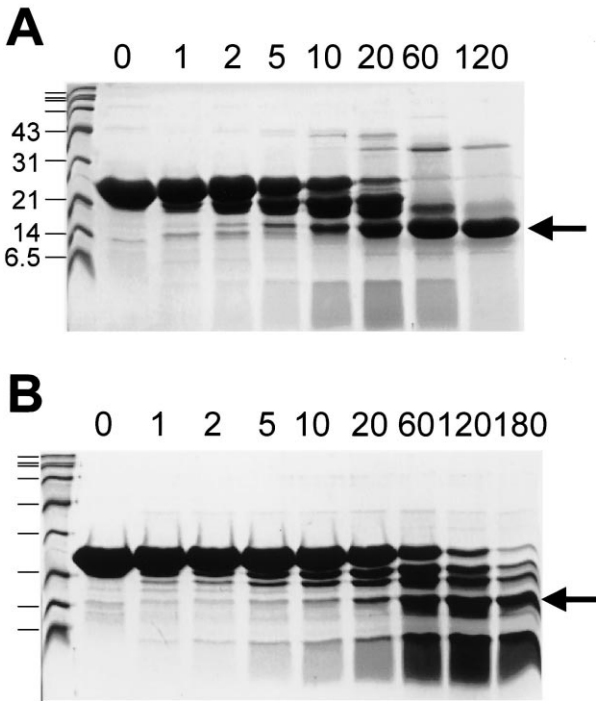

Fig. 2. Identification of protease-resistant Rad53-FHA1 domain fragments. Chymotrypsin (A) or endopeptidase Glu-C (B) were added for the times (min) indicated on top. Mass standards are indicated on the left (from top: 200, 116, 97.4, 69, 43, 31, 21, 14 and 6.5 kDa). Arrows point to the major protected fragment in each experiment. Proteolysis products that migrate as a smear at the bottom of the gels are smaller than the predicted FHA domain core and were not further characterized.

and 2A). N-terminal sequencing and mass spectrometry demonstrated that the protease-resistant fragments encompassing the Rad53-FHA1 domain corresponded to residues 24–167 (chymotrypsin) and 22–156 (endopeptidase Glu-C), respectively (Table 1), indicating that this domain contains approximately 133 residues located between Ser-24 and Glu-156.

The domain boundaries determined here for the Dun1-FHA and Rad53-FHA1 domains have a size similar to the part of Rad53p (residues 547–729) encompassing the FHA2 domain that was found to interact with Rad9p in yeast two-hybrid assays [16]. Therefore, we also produced a Rad53(547–729) fragment for proteolytic analysis. Digestion of this protein with a variety of proteases produced protected fragments with just slightly faster mobility than the undigested protein (data not shown). Sequencing of the chymotrypsin-generated fragment revealed His-559 as its N-terminal residue, but as the products of three different enzymes did not yield results in the mass spectrometric analysis (Table 1), the C-terminus of the Rad53-FHA2 domain could not be assigned.

The alignment of the sequences in Fig. 3 shows that the proteolytic cleavage sites determined here are located in close proximity to each other, indicating similar domain boundaries for all three FHA domains. The fact that endopeptidase Glu-C cleaves the Rad53-FHA1 domain at Glu-21 but not at the nearby Glu-26 and Glu-30 residues, and its cleavage of the Dun1-FHA domain at Glu-155 but not at Glu-150 or Glu-146, respectively, underscores the precision of the assignment of the FHA domain boundaries. Furthermore, Dun1p and Rad53p each contain three protease-resistant glutamic acid residues located between the N-terminal domain boundaries determined here and the predicted FHA domain core, and two (Dun1p) or three (Rad53p) protected glutamic acid resi-

Table 1
Identification of proteolytically stable FHA domains

Substrate	Protease	N-terminus	Mass (observed)	Mass (theory)	Protected fragment
Dun1(1–184)	Chymotrypsin	KRQQRSNKPS	16 089[a]	16 096	19–159
	Endo-Glu-C	HSGDV	16 630[a]	16 635	9–155
Rad53(1–199)	Chymotrypsin	SQEQI	16 153[b]	16 152	24–167
	Endo-Glu-C	KFSQE	15 104[b]	15 103	22–156
Rad53(549–729)	Chymotrypsin	HSNNTE	n.d.[c]		559–?

[a]MALDI-TOF.
[b]ES-MS.
[c]Proteolytically stable fragments containing the RAD53-FHA2 domain using chymotrypsin, thermolysin and trypsin did not give results in mass spectrometric analyses.

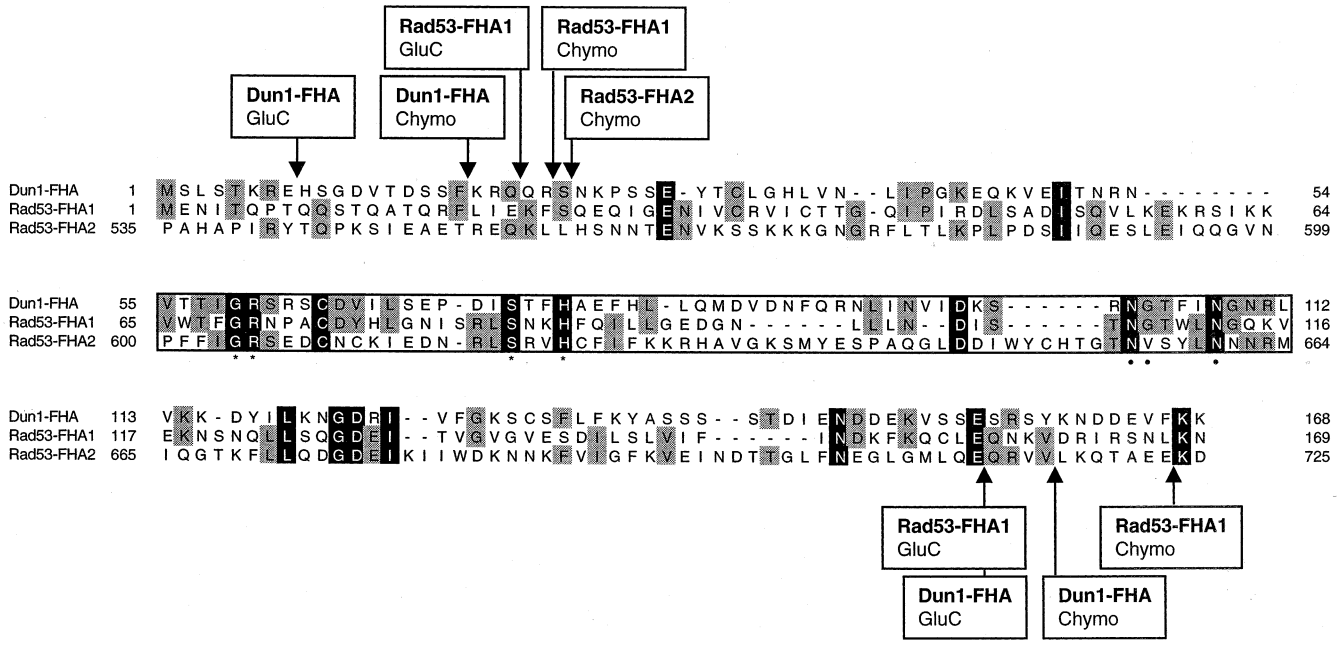

Fig. 3. Sequence alignment of the Dun1-FHA, Rad53-FHA1 and Rad53-FHA2 domains. The positions of cleavage sites in limited proteolysis experiments are indicated. The FHA core domain predicted by sequence comparisons is boxed. Asterisks indicate residues that are conserved in 80% of all FHA domains, circles indicate additional residues conserved in 65% of FHA domains. Identical residues in all three sequences are indicated by black boxes, those conserved in two sequences by gray boxes. The alignment was generated using SMART [28] and CLUSTAL-W [29] and manually modified to improve similarity.

dues between the predicted core and the assigned C-terminal domain boundary. Likewise, there are several bulky hydrophobic amino acids located between these domain boundaries and the predicted core that are not cleaved by chymotrypsin. The fact that all these residues are protease-resistant strongly indicates that the sequences flanking the predicted FHA domain core are part of a compact domain structure and not just loosely associated with it.

3.2. *Role of the Dun1-FHA domain in the transcriptional response pathway*

Dun1p is involved in the inactivation of the Crt1p transcriptional repressor protein by phosphorylation in response to checkpoint signals [22]. This results in the induction of repair genes including *RNR* subunits [6]. *DUN1* has additional functions in the G_2/M arrest in response to DNA damage [23,24]. However, no direct kinase substrates nor the role of the FHA domain in these pathways are currently known. To analyze the role of the FHA domain in the regulation of Dun1p, we overexpressed Dun1-FHA constructs in yeast in vivo and tested if this could interfere with the function of the endogenous Dun1p protein kinases by competing for either upstream activators or downstream effectors. If the FHA domain is involved in the transcriptional function of Dun1p, a fragment corresponding to the proteolytically defined FHA domain boundaries should result in a reduced transcriptional induction of repair proteins in response to replication blocks, but a truncated construct containing only the predicted FHA domain core should have little or no effect.

Yeast strains were transformed with inducible FHA domain constructs. Upon induction, DNA replication of these yeast strains was blocked in mid-log phase by addition of 150 mM HU. Aliquots of the cultures were removed for RNA preparation at time points indicated in Fig. 4A. RNA blot analysis of the samples revealed that the Dun1(19–159) construct, but not the Dun1(56–112) construct, caused an approximately 60% reduction in the transcriptional induction of the *RNR2* mRNA in response to the HU-dependent replication block, when compared to the vector only control (Fig. 4A). This indicates that the FHA domain plays a crucial role in the function of the Dun1p protein kinase and confirms the results of the limited proteolysis studies. The Dun1(19–159) expressing yeast cells remained fully viable during the 3 h replication block (Fig. 4B) and had normal growth properties on plates containing methylmethanesulfonate to induce DNA damage (data not shown). This indicates that the FHA domain did not cause a general checkpoint defect resulting in diminished cell survival.

4. Discussion

DUN1 has previously been shown to be involved in the transcriptional induction of repair genes and the G_2/M arrest in response to cell cycle checkpoint signals, but the molecular mechanisms of these functions are currently unclear. Our data show that the FHA domain plays a crucial role in the transcriptional function of the Dun1p kinase, as overexpression of the Dun1-FHA domain reduced the transcriptional response by 60%. The residual levels of *RNR2* induction in the Dun1(19–159) strain are similar to those elicited by an uncharacterized alternative pathway in *dun1* null strains [6,22,25]. This indicates that overexpression of the Dun1(19–159) fragment can almost completely abolish the *DUN1*-dependent component of the transcriptional response. However, yeast overexpressing this FHA domain remained fully viable under replication block (Fig. 4B) and DNA damage promoting conditions (data not shown), in contrast to the checkpoint defect observed in *dun1* null strains [23,24]. Therefore, the

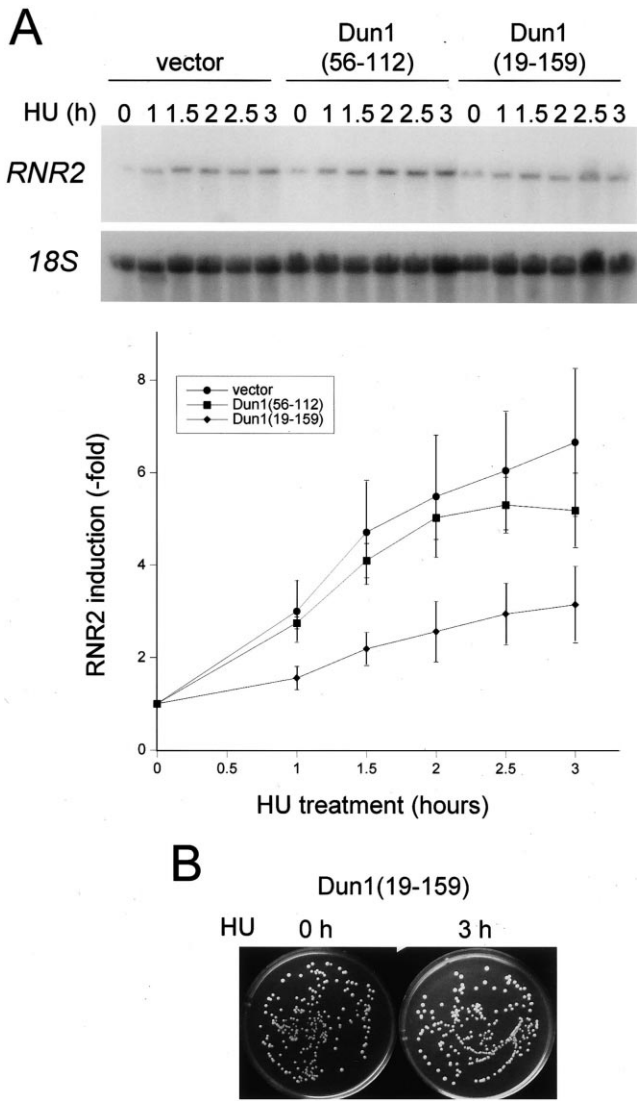

Fig. 4. Inducible expression of the Dun1-FHA domain in yeast. (A) Northern blot analysis of *RNR2* induction by replication blocks in transformed yeast strains. The graph shows the mean (±S.E.M.) of three independent experiments of yeast strains transformed with vector only (circles), the Dun1(56–112) construct (squares) or the Dun1(19–159) construct (diamond). Results of one experiment are shown on top. Numbers indicate time after addition of 150 mM HU. The *RNR2* signals were corrected for loading differences revealed by probing for the *18S* RNA. The relative induction is the normalized *RNR2* signal divided by the *RNR2* signal at $t=0$. All cultures were grown in 4% galactose for 6 h before addition of HU. (B) Equal aliquots of the Dun1(19–159) expressing culture were removed immediately (0 h) and 3 h after the addition of HU and immediately diluted on YPD plates to assess cell viability.

Dun1-FHA domain seems to function specifically in the transcriptional response but not in cell cycle arrest pathways.

The data presented here indicate that FHA domains in general are much larger than predicted by sequence comparisons. It was recently reported that the interaction of the plant KAPP with receptor-like protein kinases also required some 30 additional residues on either side of its predicted FHA domain [26]. This was interpreted as an indication that these additional residues enhance the binding of the predicted FHA domain by directly interacting with the target [26]. However, our proteolysis experiments strongly suggest that additional residues flanking the predicted FHA domain core are actually an integral part of a compact domain structure. As the Dun1-FHA and Rad53-FHA1 domain boundaries correspond well with the flanking sequences required for protein–protein interactions by the KAPP-FHA and Rad53-FHA2 domains [16,26], the only other FHA domains characterized in some molecular detail, we propose that FHA domains are considerably larger (up to 140 residues) than the 55–75 residues predicted by sequence comparisons. This proposal is supported by the nuclear magnetic resonance structure of the Rad53-FHA2 domain [27], that was published while this manuscript was in preparation, showing that all residues of a recombinant Rad53(573–730) fragment form a compact β-sandwich consisting of 11 β-strands arranged in two antiparallel sheets and a short C-terminal α-helix.

The precise molecular function of the FHA domains in cell cycle checkpoint kinases such as Dun1p and Rad53p will ultimately only be elucidated by the identification of specific binding proteins, and our definition of the domain boundaries should be helpful in this process.

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References

[1] Elledge, S.J. (1996) Science 274, 1664–1672.
[2] Shiloh, Y. (1997) Annu. Rev. Genet. 31, 635–662.
[3] Bell, D.W., Varley, J.M., Szydlo, T.E., Kang, D.H., Wahrer, D.C., Shannon, K.E., Lubratovich, M., Verselis, S.J., Isselbacher, K.J., Fraumeni, J.F., Birch, J.M., Li, F.P., Garber, J.E. and Haber, D.A. (1999) Science 286, 2528–2531.
[4] Rhind, N. and Russell, P. (1998) Curr. Opin. Cell Biol. 10, 749–758.
[5] Longhese, M.P., Foiani, M., Muzi-Falconi, M., Lucchini, G. and Plevani, P. (1998) EMBO J. 17, 5525–5528.
[6] Zhou, Z. and Elledge, S.J. (1993) Cell 75, 1119–1127.
[7] Allen, J.B., Zhou, Z., Siede, W., Friedberg, E.C. and Elledge, S.J. (1994) Genes Dev. 8, 2401–2415.
[8] Zeng, Y., Forbes, K.C., Wu, Z., Moreno, S., Piwnica-Worms, H. and Enoch, T. (1998) Nature 395, 507–510.
[9] Matsuoka, S., Huang, M. and Elledge, S.J. (1998) Science 282, 1893–1897.
[10] Brown, A.L., Lee, C.H., Schwarz, J.K., Mitiku, N., Piwnica-Worms, H. and Chung, J.H. (1999) Proc. Natl. Acad. Sci. USA 96, 3745–3750.
[11] Sanchez, Y., Desany, B.A., Jones, W.J., Liu, Q., Wang, B. and Elledge, S.J. (1996) Science 271, 357–360.
[12] Sun, Z., Fay, D.S., Marini, F., Foiani, M. and Stern, D.F. (1996) Genes Dev. 10, 395–406.
[13] Lindsay, H.D., Griffiths, D.J., Edwards, R.J., Christensen, P.U., Murray, J.M., Osman, F., Walworth, N. and Carr, A.M. (1998) Genes Dev. 12, 382–395.
[14] de los Santos, T. and Hollingsworth, N.M. (1999) J. Biol. Chem. 274, 1783–1790.
[15] Hofmann, K. and Bucher, P. (1995) Trends Biochem. Sci. 20, 347–349.
[16] Sun, Z., Hsiao, J., Fay, D.S. and Stern, D.F. (1998) Science 281, 272–274.
[17] Stone, J.M., Collinge, M.A., Smith, R.D., Horn, M.A. and Walker, J.C. (1994) Science 266, 793–795.

[18] Durocher, D., Henckel, J., Ferscht, A.R. and Jackson, S.P. (1999) Mol. Cell 4, 387–394.
[19] Mumberg, D., Müller, R. and Funk, M. (1994) Nucleic Acids Res. 22, 5767–5768.
[20] Heierhorst, J., Kobe, B., Feil, S.C., Parker, M.W., Benian, G.M., Weiss, K.R. and Kemp, B.E. (1996) Nature 380, 636–639.
[21] Mitchelhill, K.I., Michell, B.J., House, C.M., Stapleton, D., Dyck, J., Gamble, J., Ullrich, C., Witters, L.A. and Kemp, B.E. (1997) J. Biol. Chem. 272, 24475–24479.
[22] Huang, M., Zhou, Z. and Elledge, S.J. (1998) Cell 94, 595–605.
[23] Pati, D., Keller, C., Groudine, M. and Plon, S.E. (1997) Mol. Cell Biol. 17, 3037–3046.
[24] Gardner, R., Putnam, C.W. and Weinert, T. (1999) EMBO J. 18, 3173–3185.
[25] Navas, T.A., Zhou, Z. and Elledge, S.J. (1995) Cell 80, 29–39.
[26] Li, J., Smith, G.P. and Walker, J.C. (1999) Proc. Natl. Acad. Sci. USA 96, 7821–7826.
[27] Liao, H., Byeon, I.J. and Tsai, M.D. (1999) J. Mol. Biol. 294, 1041–1049.
[28] Ponting, C.P., Schultz, J., Milpetz, F. and Bork, P. (1999) Nucleic Acids Res. 27, 229.
[29] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.