Structure and Function of a New Phosphopeptide-binding Domain Containing the FHA2 of Rad53

Hua Liao, In-Ja L. Byeon and Ming-Daw Tsai*

Departments of Chemistry and Biochemistry, The Ohio State Biochemistry Program, and Campus Chemical Instrument Center, The Ohio State University, Columbus, OH 43210, USA

The forkhead-associated (FHA) domain is a 55-75 amino acid residue module found in >20 proteins from yeast to human. It has been suggested to participate in signal transduction pathways, perhaps via protein-protein interactions involving recognition of phosphopeptides. Neither the structure nor the ligand of FHA is known. Yeast Rad53, a checkpoint protein involved in DNA damage response, contains two FHA domains, FHA1 (residues 66-116) and FHA2 (residues 601-664), the second of which recognizes phosphorylated Rad9. We herein report the solution structure of an “FHA2-containing domain” of Rad53 (residues 573-730). The structure consists of a α-b-sandwich containing two antiparallel β-sheets and a short, C-terminal α-helix. Binding experiments suggested that the FHA2-containing domain specifically recognizes pTyr and a pTyr-containing peptide from Rad9, and that the binding site involves residues highly conserved across FHA domains. The results, along with other recent reports, suggest that FHA domains could have pTyr and pSer/Thr dual specificity.

Introduction

Interactions between two proteins in signaling events are often mediated by small modular domains, as reviewed by Pawson & Scott (1997). Forkhead-associated (FHA) domains were first identified within a subset of forkhead transcriptional factors, located outside of the conserved DNA-binding forkhead domain (Hofmann & Bucher, 1995). They were subsequently found in a number of other proteins by sequence homology analyses. As shown in Figure 1(a), the FHA domains consist of ca 55-75 amino acid residues with three highly conserved regions. Between the regions are more divergent spacers of varying length. The family of proteins containing FHA domains, which includes proteins of diverse functional roles, is growing rapidly. Examples of the FHA family include: Dun1 and Rad53 (or Spk1), which are involved in DNA damage repair and the S phase checkpoint in Saccharomyces cerevisiae (Allen et al., 1994; Navas et al., 1995; Sun et al., 1998; Zheng et al., 1993); Cds1, the Schizosaccharomyces pombe homolog of Rad53 (Murakami & Okayama, 1995); Mek1, which is related to meiotic recombination of yeast (Leem & Ogawa, 1992); KAPP, kinase-associated protein phosphatase from Arabidopsis thaliana, which binds to the receptor-like protein kinase RLK5 (Stone et al., 1994); and Ki-67, a human nuclear protein related to cell proliferation (Schluter et al., 1993). The two most recent members are HuCds1 (or Chk2) in humans (Brown et al., 1999; Matsuoka et al., 1998), and Dmnk in Drosophila melanogaster (Oishi et al., 1998). Both proteins participate in the cellular response to DNA damage. The focus of this study is on the yeast protein Rad53.

Results and Discussion

Structural determination of an FHA2-containing fragment of Rad53

Previous two-hybrid analyses identified a minimal fragment, residues 549-730 encompassing the FHA2 domain (residues 601-664), which is responsible for the interaction with phosphorylated Rad9 (Sun et al., 1998). Our structural study began with the expression of the fragment 549-730 in Escherichia coli. In the course of NMR analyses, it was found that residues 549-575 were unstructured in the construct, which resulted in undesirably strong
resonances at random coil chemical shifts. After deletion of the first 24 residues, the resonances of the remaining residues were unchanged and the structure was subsequently completed for this shorter version (residues 573-730; Figure 1(b)). Resonance assignments and structure calculations were performed as described in Materials and Methods. The final ensemble of 15 low-energy structures (Figure 2(a)) was generated in X-PLOR from 2571 inter-proton distance restraints (871 intra-residue, 547 sequential, 230 medium-range, 923 long-range) including 80 hydrogen bond restraints and 142 $^{13}$C$_a$ and 129 $^{13}$C$_b$ chemical shift restraints. As shown in Table 1, the resulting structures satisfy the experimental restraints very well. The stereochemical quality of the ensemble is good, with >92% of the backbone phi/psi angles occupying the most favored or additionally favored regions. There are two disordered loops, 632-643 and 707-713, which display few nuclear Overhauser enhancement (NOE) constraints (particularly the first one). The rest of the structure is very well defined. Excluding the disordered regions, the r.m.s.d. values are 0.51(±0.09) Å for backbone heavy-atoms and 0.97(±0.08) Å for all heavy-atoms. The disorder of the loop 632-643 should arise from its flexible nature rather than lack of NMR data, based on the observation that the $^{15}$N HSQC cross-peaks of the loop residues are much more intense than the rest.

Structural characteristics

The secondary structures of the FHA2-containing domain derived from NMR data are defined in Figure 1(b) (primary sequence) and Figure 2(b) (tertiary structure). The structure consists of a β-sandwich containing two twisted antiparallel β-sheets and a short α-helix. One of the β-sheets consists of β1, β2, β6, β7, β9 and β10, and the other sheet consists of β3, β4, β5, β8 and β11. These two β-sheets intimately interact with each other through hydrophobic side-chains including those of Leu578, Leu580, Ile594, Phe601, Ile603, Ile625, Ile647, Tyr649, Leu659, Met664, Phe670, Ile678, Phe692, Val694 and Val716. The short C-terminal helix packs against the latter sheet by hydrophobic interactions with β3 and β4. Since this is not the actual C terminus in the native Rad53 protein, the structure could be distorted by truncation and should not be over emphasized.

The tertiary structural fold of FHA2 appears to be unique on the basis of VAST search. The most similar structural fold was found from the Smad4 tumor suppressor C-terminal domain, a functionally unrelated protein (Shi et al., 1997). Both structures show a similar β-sandwich, but the other domain contains several prominent helices and also shows different topology of the loops.

Two important structural features are worth pointing out. One is that, while the β-sheet structure is likely to be the common feature of all FHA domains, the five absolutely conserved residues in FHA domains are located outside of the β-sheets: Gly604, Arg605, Ser619 and His622 are located in a loop between β3 and β4, and Asn655 is located in a turn between β5 and β6 (Figure 2(b)). It is particularly notable that Ser619, His622 and Asn655 are closely interacting in space forming a triangle, with a hydrogen bond formed between the imidazole ring of His622 and the side-chain of Asn655. These three residues have been shown by mutagenesis to be essential for binding to Rad9 (Sun et al., 1998).

The other important structural feature is that the “FHA2 domain” (residues 601-664), as defined by sequence analysis, does not constitute a separate structural domain. While the FHA domain barely
forms one β-sheet, the flanking sequences are required to form the stable β-sandwich structure. It is possible that the fragment 573-730 (or a major part of it) represents the minimal structural domain of FHA. This is supported by a previous finding that deletion of 52 amino acid residues from either end of the functional fragment 549-730 abolished the interaction with Rad9 (Sun et al., 1998), even though in both cases the FHA domain itself is untruncated in the resulting fragments. Another recent paper reported that about 30 aa flanking sequences are required on each side for the function of another FHA domain (Li et al., 1999).
Phospho amino acid specificity of FHA2 and FHA1 domains

Since FHA domains have been suggested to recognize phosphoproteins, we first examined the specificity of the FHA2-containing domain toward single amino acid ligands. Binding of pTyr, pThr, pSer, or P_i was monitored by {^1H, ^15N}HSQC spectra. As shown by the titration curves of 1H chemical shifts (Figure 3(a)), pTyr induces a set of shifts with \( K_d \approx 20 \text{ mM} \). On the other hand, pSer induces much smaller shifts with \( K_d \) values >150 mM (Figure 3(b)). Furthermore, the residue shifted most by pSer, L730, is also shifted similarly by pTyr with a large \( K_d \), as shown by the broken line in both panels. Similar results were shown by 15N chemical shifts, where K728 is shifted the most by non-specific effects. The effects of pSer and pTyr binding (at 70 mM ligands) are further illustrated by bar diagrams in Figure 4(a) and (b). These data

![Figure 3](image_url)

**Figure 3.** Titration curves for (a) pTyr and (b) pSer. Only the chemical shift changes in ^1H dimension are presented for some of the affected residues. The starting protein concentration is 0.4 mM. The pH of the samples was maintained at 6.5 throughout the titration. The changes for N655 and S619 are too small to be presented for pSer. R617 shows similar changes in proton chemical shifts for both pTyr and pSer, but significant change in ^15N dimension only for pTyr.
suggest that both ligands induce a set of nonspecific shifts, but only pTyr induces specific shifts. The conclusion that FHA2 binds pTyr specifically is further supported by the following experiments and arguments: (a) The non-specific shifts induced by pSer were also observed with pThr, P_i, and NaCl (data not shown); (b) Unphosphorylated tyrosine did not induce notable changes at 2 mM (its limiting conc.); (c) The residues shifted by pTyr, R605, S619, R620, H622, T654, N655, and V656, include most of the highly conserved residues. On the other hand, the residues most shifted by pSer, K728 and L730, are not conserved residues and are right at the C terminus. (d) The shifts induced by pTyr cannot be caused primarily by the ring current effect of the Tyr ring since some residues are separated far apart and the K_d of pTyr is distinct from that of pSer.

To provide further support that pTyr specificity is indeed a property of FHA domains, we expressed and purified a fragment containing the FHA1 domain of Rad53 (residues 2-175). The assignments of this fragment is yet to be completed. However, assignments are not required to obtain K_d values, and we have shown that this fragment binds pTyr with an even higher affinity (K_d = 4 mM) but does not bind pSer specifically.

**Phosphopeptide specificity of FHA2**

Since the biological binding target of FHA2 domain is believed to be phosphorylated Rad9 in yeast (that of FHA1 is not known), we proceeded to identify peptide fragment(s) from Rad9 that binds tightly to the FHA2-containing domain of Rad53. We focused on the fragment 553-1056 of Rad9, which has been shown to interact with Rad53 (Sun et al., 1998). By using the same NMR method, we studied the interactions between FHA2 and the 13 synthetic heptapeptides (general sequence: XXX(pY)XXX) corresponding to the 13 tyrosine residues within the fragment 553-1056 of Rad9. One of the heptapeptides from this region, 826EDI(pY)YLD32, induced chemical shift changes that were significantly greater than other peptides did. The dissociation constant is estimated to be ~ 0.1 mM from the titration curves in Figure 5. Unphosphorylated peptide showed little binding affinity. Similar residues are perturbed by the peptide and by pTyr, but there are clear differences in the magnitude of chemical shift changes for some of the residues, as illustrated in Figure 4(c). While the backbone NH resonances of R617, W682, V689, and I690 were shifted only slightly by pTyr, they were shifted significantly by the peptide. Residues R605, R620, H622, T654, N655, and V656 showed similar perturbations upon binding with pTyr and the peptide. The observed differences possibly reflect the fact that the peptide is a more complete ligand than pTyr alone. The structural locations or the residues significantly affected by this peptide are mainly at the loop regions where the conserved residues are located. Some of the key residues are shown in Figure 6.

To provide further support that the FHA2 residues whose chemical shifts are perturbed by the phosphopeptide are indeed involved in binding, we constructed a single mutant H622A and a triple mutant NVS655-657AAA. Both mutants have been shown to prevent the two-hybrid interaction between Rad53 and Rad9 (Sun et al., 1998). In our results, H622A is insoluble and could not be further characterized, but the triple mutant displayed a reduced affinity by a factor of 10 (1 mM K_d) for the peptide 826EDI(pY)YLD32.
Of the 13 possible pTyr-peptides tested, the second best peptide is the one with the neighboring tyrosine phosphorylated, i.e. 827DIY(pY)L833. The $K_d$ value of this peptide is estimated to be five to tenfold higher than that of the best binding peptide. It appears that sequences at both sides of pTyr are recognized by FHA2, since peptides with residues at only one side showed weaker binding affinity.

**Structural comparison with other phosphoprotein-binding domains**

The global structural fold and binding site of the FHA2-containing domain are clearly different from other phosphoprotein-binding modules, such as SH2 and PTB domains that recognize pTyr, and 14-3-3 proteins that bind pSer. The SH2 family has over 100 members with relatively well-conserved sequences (Kuriyan & Cowburn, 1997). Structural studies have revealed striking similarities in the overall structure and the pTyr-binding mechanism within the family. PTB domains are more divergent in sequence, but share a conserved structural architecture and peptide-binding mode. In both domains, there are conserved Arg, Arg/Lys, and Ser residues that are responsible for binding the phosphate moiety of pTyr (Eck et al., 1993; Zhou et al., 1995). In 14-3-3 proteins, pSer binds to two Arg residues, one Lys and one Tyr residue (Yaffe et al., 1997).

![Figure 6. Comparison of the potential phosphate binding site of FHA2 with that of SH2 domain (Eck et al., 1993) and 14-3-3 protein (Yaffe et al., 1997).](image)
In FHA domains, there are also three corresponding conserved residues: R605, R620, and S619, even though the sequence homology within this family is less than that within the SH2 family. Figure 6 shows the comparison of key binding residues in FHA2 (free), SH2 (pTyr-bound), and 14-3-3 protein (pSer-bound). The geometry of the key binding residues do not line up well in these three structures, since they are different proteins in different forms. However, the Figure suggests that it is possible for the side-chains of R605, R620, and S619 to close in to bind the phosphate moiety of a phosphopeptide. Another interesting point of the comparison is that FHA2 consists of mainly β-helices, and SH2 consists of both.

**Functional comparison with other phosphoprotein-binding domains**

Here, we address three issues that are relevant to our results and conclusions: that a different FHA domain has been shown to bind to pSer/pThr-proteins (Li et al., 1999); that Tyr phosphorylation is not generally thought to be employed as a signaling mechanism in yeast; and that the binding affinities of FHA2 to pTyr and pTyr-peptide are weak relative to SH2 and PTB domains. (a) Since we have not yet tested binding of pSer-peptides or pTyr-peptides (there are too many Ser and Thr residues on Rad9), it cannot be completely ruled out that the FHA2 domain has dual specificity for pTyr-peptides and pSer/pThr-peptides. Although this scenario is not supported by the pTyr specificity at the level of single phosphoamino acids, the latter may not be a good model for phosphopeptides. (b) It is possible that FHA domains from different proteins have different specificity, and that a particular FHA domain could have dual specificity. Dual specificity protein kinases and pTyr-specific phosphatases do exist in S. cerevisiae. (c) The well-characterized SH2 domains bind pTyr and a four residue phosphopeptide with Kd of <1 mM and 0.1-1 μM, respectively (Kuriyan & Cowburn, 1997), which are 10 to 1000-fold stronger binding than that of FHA domains. The difference could be due to a number of reasons: FHA domains may have lower affinity for ligands biologically, 826EDI(pY)YLD832 may not be the optimal linear peptide (the number of residues on either side remains to be optimized), and residues distant from one another in the primary sequence of Rad9 may be involved in the interaction with Rad53. (d) Since it has been shown that FHA2 binds to “hyperphosphorylated state” of Rad9 (Vialard et al., 1998), it is also possible that FHA2 could bind to more than one site, and the site with highest affinity has not yet been found. (e) In any case, all of our results argue against the possibility that FHA domains recognize pSer/pThr only and the observed binding to pTyr-peptide is non-specific. If FHA’s natural ligand was ABC(pS)XYZ at a different site from 826EDI(pY)YLD832, changing pS to pY alone would cause the Kd to increase by 1000-fold, from micromolar to millimolar (based on the results of SH2), and further changing the peptide sequence would completely abolish binding of the peptide.

After this work had been submitted for publication, a related paper was published (Durocher et al., 1999). This paper shows that the FHA1 domain of Rad53 prefers a pThr-peptide. Strictly speaking, their results do not contradict ours directly, since they studied mainly FHA1 and our results come mainly from FHA2, and they mainly examined pSer/pThr-peptides and we mainly examined pTyr-peptides. However, the publication of this paper further strengthens our view that FHA domains could have dual specificity, even though it remains to be established whether both specificities are involved in the actual signaling events. The available data from their paper and ours indicate that the binding affinity of FHA1 to the pThr-peptide is higher than that of FHA2 to the pTyr-peptide. However, the peptide sequences have not been fully optimized in either case. If the dual specificity of FHA domains is further established, it will represent a new class of phosphoprotein-binding domains, even if only one of the specificities is biologically relevant.

**Materials and Methods**

Fragments 549-730 and 573-730 of Rad53 were cloned into pGEX-4T vector (Pharmacia Biotech) for expression of GST fusion proteins in BL21(DE3) (Novagen). The fusion proteins were purified using glutathione agarose (Sigma). The GST tag was removed by thrombin (Sigma) digestion and gel filtration chromatography. Isotope-labeled proteins were expressed in M9 media containing 13NH4Cl, 13C-glucose, and 2H2O correspondingly.

NMR experiments were performed on a Bruker DMX-600 or DRX-800 spectrometer at 20 °C. The protein concentration was 0.2 to 0.5 mM. The samples contained 10 mM sodium phosphate, 1 mM DTT, and 1 mM EDTA in 95% H2O/5% D2O or 100% D2O at pH 6.5. Total assignments were obtained using [15N, 1H]-TROSY-HNCACB (Salzmann et al., 1999), [13N, 1H]-TROSY-HN(CO)CACB (Salzmann et al., 1999), and HNCA experiments on the 15N/13C/2H-triple labeled fragment (549-730). This paper shows that the FHA1 domain of Rad53 prefers a pThr-peptide. Strictly speaking, their results do not contradict ours directly, since they studied mainly FHA1 and our results come mainly from FHA2, and they mainly examined pSer/pThr-peptides and we mainly examined pTyr-peptides. However, the publication of this paper further strengthens our view that FHA domains could have dual specificity, even though it remains to be established whether both specificities are involved in the actual signaling events. The available data from their paper and ours indicate that the binding affinity of FHA1 to the pThr-peptide is higher than that of FHA2 to the pTyr-peptide. However, the peptide sequences have not been fully optimized in either case. If the dual specificity of FHA domains is further established, it will represent a new class of phosphoprotein-binding domains, even if only one of the specificities is biologically relevant.
involving methyl protons (Clore et al., 1987; Wagner et al., 1987). Hydrogen bond restraints ($r_{NH-O} = 1.5-2.5 \text{ Å}, r_{C-O} = 2.4-3.5 \text{ Å}$) were deduced on the basis of the secondary structure and the slowly exchanging amide protons.

Structural calculations were conducted using a simulated annealing method (Nilges et al., 1988) within X-PLOR. The simulated annealing protocol consists of two stages: in the first stage, the simulated annealing structures were determined based on the experimental inter-proton distance restraints. The resulting structures were then used as initial structures for the second stage of simulated annealing calculations where, in addition to the distance restraints, the structures were refined against secondary $^{13}C^2/^{13}C^3$ chemical shift restraints. A total of 32 structures were generated using the protocol. The structures were analyzed by X-PLOR, PROCHECK (Laskowski et al., 1993), and MOLMOL (Koradi et al., 1996) and visualized by Insight II (Molecular Simulations Inc.) and MOLSCRIPT (Kraulis, 1991). The final 15 structures with lowest energy among the converged structures were selected.

Ligand binding experiments were performed by recording a series of 2D $^{15}N$-HSQC spectra on uniformly $^{13}N$-labeled protein samples with different concentrations of ligands. The pH of the samples was maintained at 6.5 throughout the titrations.

**Accession number**

Coordinates have been deposited in the RCSB PDB (accession number 1Q5) and will be released upon publication of this work.

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**References**


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