

COMMENTARY

The FHA domain mediates phosphoprotein interactions

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SUMMARY

The forkhead-associated (FHA) domain is a phosphopeptide-binding domain first identified in a group of forkhead transcription factors but is present in a wide variety of proteins from both prokaryotes and eukaryotes. In yeast and human, many proteins containing an FHA domain are found in the nucleus and involved in DNA repair, cell cycle arrest, or pre-mRNA processing. In plants, the FHA domain is part of a protein that is localized to the plasma membrane and participates in the regulation of receptor-like protein kinase signaling pathways. Recent studies show that a functional FHA domain consists of 120-140 amino acid residues, which is significantly larger than the sequence motif first described. Although FHA domains

do not exhibit extensive sequence similarity, they share similar secondary and tertiary structures, featuring a sandwich of two anti-parallel β -sheets. One intriguing finding is that FHA domains may bind phosphothreonine, phosphoserine and sometimes phosphotyrosine, distinguishing them from other well-studied phosphoprotein-binding domains. The diversity of proteins containing FHA domains and potential differences in binding specificities suggest the FHA domain is involved in coordinating diverse cellular processes.

Key words: FHA domain, Phosphorylation, KAPP, Rad53p, NIPP1, RLK signaling, Checkpoint

INTRODUCTION

Cellular signaling requires constant assembly and disassembly of protein complexes. Components within an assembled complex have to collaborate to fulfill a specific biological task. A central question in the field of signal transduction is how functional protein complexes can be correctly formed temporally and spatially within a complicated cellular or extracellular environment in which many proteins are present. In the past decade, new insights about the mechanisms of protein-protein interactions have been obtained. Many signaling proteins contain small structural modules that can specifically recognize their ligands, which are often peptide sequences. Post-translational modification, such as protein phosphorylation, also plays an important role in regulating many protein-protein interactions.

The discovery of Src homolog 2 (SH2) domains, and subsequently of their binding sites, established a paradigm for phosphorylation-dependent protein interactions (Cantley et al., 1991; Koch et al., 1991; Waksman et al., 1992; Songyang et al., 1993; Marengere et al., 1994). SH2 domains are conserved sequences containing about 100 residues and are found in many signaling proteins. SH2 domains stably associate only with phosphorylated, but not unphosphorylated, targets (Mayer and Baltimore, 1993). Different SH2 domains possess different interaction specificities and fall into two groups: one prefers to bind pY-hydrophilic-hydrophilic-hydrophobic motifs, whereas

the other selectively interacts with pY-hydrophobic-X-hydrophobic peptides (Cohen et al., 1995). SH2 domains bind phosphotyrosine peptides but have little or no affinity for phosphoserine/phosphothreonine-containing sequences. Other phosphotyrosine-dependent protein interaction domains, such as protein-tyrosine-binding (PTB) domains, act as alternatives to SH2 domains (Kavanaugh et al., 1995).

In addition to the phosphotyrosine-binding domains, there are also phosphoserine/phosphothreonine-binding domains. Among them are the well-studied 14-3-3 proteins, a family of 30-kDa proteins that are ubiquitous in eukaryotic cells. Unlike SH2 domains, which are noncatalytic modules often encoded as part of a larger protein containing a catalytic domain, such as a protein kinase, 14-3-3 proteins act as independent binding domains. 14-3-3 proteins selectively interact with phosphoserine-containing proteins carrying the motifs RSXpSXP (Muslin et al., 1996), RXF/YpSXP (Yaffe et al., 1997) or RXSXpSXP (Andrews et al., 1998).

Several other domains also selectively interact with phosphoserine/phosphothreonine-containing peptides. cAMP-responsive-element-binding protein (CREB)-binding protein (CBP) contains a CBP domain for phosphoprotein binding (Chrivia et al., 1993; Kwok et al., 1994; Radhakrishnan et al., 1997; Parker et al., 1996). The phosphorylation of the CREB transcription factor on serine residues is a crucial step for its interaction with CBP. Some F-box proteins, which contain protein-protein-interaction domains called WD40 repeats, can

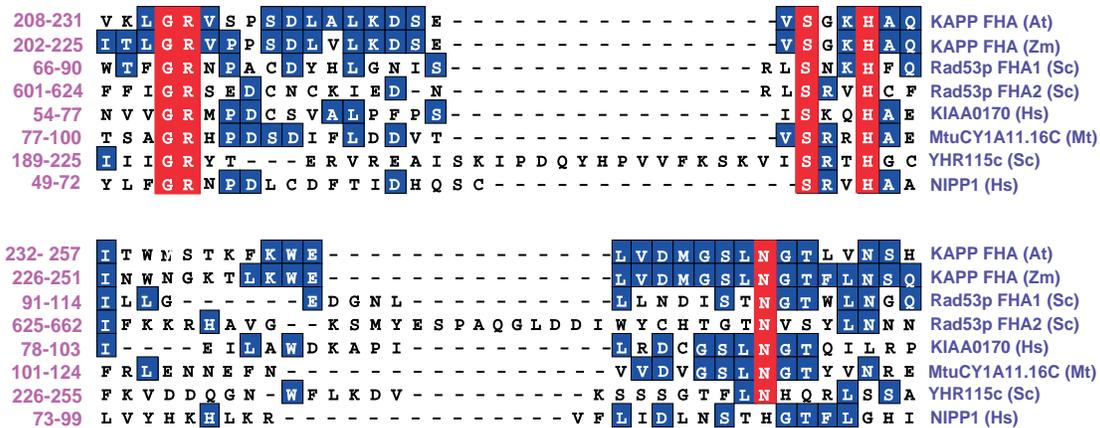


Fig. 1. Alignments of selected FHA domain homology regions that have been shown to interact specifically with phosphoprotein or phosphopeptides (Li et al., 1999; Braun et al., 1997; Durocher et al., 1999; Sun et al., 1998; Boudrez et al., 2000). The proteins are from *Arabidopsis* (KAPP FHA (At), accession number 1709235), Maize (KAPP FHA (zm), accession number AAB93832); *Saccharomyces cerevisiae* (Rad53p FHA1 and Rad53p FHA2, accession number A39616; Yhr115cp, accession number S48957), human (KIAA0170, accession number 1136400; NIPP1, accession number AF061958), and *Mycobacterium tuberculosis* (MtuCY1A11.16C, accession number 2496539). Red blocks represent the invariant residues; in NIPP1, position 92 is a histidine residue instead of asparagine residue. There is no significant homology in regions flanking the FHA domain.

recruit proteins containing either a phosphorylated serine or a phosphorylated threonine. The F-box proteins then target the captured proteins to an enzyme complex and tag them with ubiquitin, which leads to their degradation (Chung et al., 1998; Townsley and Ruderman, 1998). There are also examples of WW-domain-containing proteins that can interact with phosphorylated proteins, at least some of which may contain phosphoserine or phosphothreonine motifs (Lu et al., 1999).

Here, we focus on a unique family of proteins that contain a phosphoprotein-binding domain known as the forkhead-associated (FHA) domain. FHA domains have been found in a wide variety of proteins from both prokaryotes and eukaryotes (Hofmann and Bucher, 1995; Sun et al., 1998; Li et al., 1999; Durocher et al., 1999; Liao et al., 1999; Hammet et al., 2000). Advances in our understanding of the function and structure of FHA domains suggest they regulate many different signaling pathways through their interaction with phosphorylated protein targets.

THE DISCOVERY OF FHA DOMAINS

FHA domains were first identified in a group of forkhead (FH) transcription factors (Hofmann and Bucher, 1995) and have since been identified in many signaling proteins, including protein kinases, protein phosphatases, adenylate cyclases, proteases, kinesins, zinc-finger proteins and glycoproteins (see <http://smart.embl-heidelberg.de/>) (Schultz et al., 2000). The existence of FHA domains in a wide variety of proteins suggests that they are involved in many different cellular processes. Sequence alignments show the homology region of FHA domains from different proteins comprises of 55-75 residues. This region contains three conserved blocks, which are separated by divergent sequences (Hofmann and Bucher, 1995) (Fig. 1). Serial-deletion and limited-proteolysis experiments indicate, however, that the homology region is insufficient to form a functional unit (Li et al., 1999; Hammet et al., 2000). The functional FHA domain module contains

120-140 amino acid residues and has recently been shown to be a true phosphoprotein-binding module (Sun et al., 1998; Li et al., 1999; Durocher et al., 1999; Liao et al., 1999; Yaffe and Cantley, 1999; Boudrez et al., 2000; Sueishi et al., 2000).

FUNCTIONS OF FHA-DOMAIN-CONTAINING PROTEINS

FHA domains have been identified in >100 different proteins from different organisms. Among these identified FHA-domain-containing proteins, a specific cellular target has been identified for only a few proteins. The exact roles for the others remain a mystery.

Arabidopsis KAPP

The first evidence for involvement of FHA domains in phosphorylation-dependent protein-protein interactions came from the study of the *Arabidopsis* kinase-associated protein phosphatase (KAPP) (Stone et al., 1994; Hofmann and Bucher, 1995). Stone et al. identified KAPP through interaction cloning by using an *Arabidopsis* receptor-like protein kinase as bait (Stone et al., 1994). Sequence analyses showed that KAPP contains three different functional domains: an N-terminal type I membrane anchor, a kinase interaction (KI) domain, and a C-terminal protein phosphatase type 2C domain. A KAPP fragment containing 239 residues (residues 98-336) interacts with the plant receptor-like protein kinase (RLK) catalytic domain when the latter is phosphorylated. Plant RLKs play important roles in regulating hormone perception, growth and development, floral organ abscission, and disease resistance (Li et al., 1997; Clark et al., 1997; Torii et al., 1996; Jinn et al., 2000; Song et al., 1995). The 239-residue KAPP fragment was designated as the kinase-interacting (KI) domain (Stone et al., 1994). A 52-residue region in the KI domain shares sequence identity with FHA domains (Hofmann and Bucher, 1995). Serial deletion and in vitro binding analyses demonstrated the minimal KI domain consists of 119 residues spanning residues

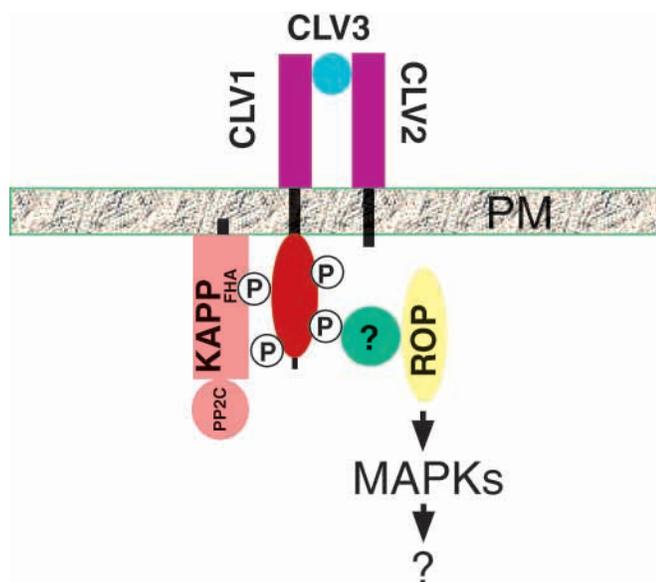


Fig. 2. Model for the role of the KAPP FHA domain in CLV1 RLK signaling pathway (Clark et al., 1997; Williams et al., 1997; Stone et al., 1998; Trotochaud et al., 1999; Jeong et al., 1999). A putative ligand, CLV3 (Fletcher et al., 1999; Trotochaud et al., 2000; Brand et al., 2000), interacts specifically with the extracellular domains of CLV1 and CLV2, which triggers conformational changes in the cytoplasmic kinase domain of CLV1 and leads to autophosphorylation of the CLV1 catalytic domain on multiple serine residues. The KAPP FHA domain selectively recognizes and interacts with a phosphoserine-containing region. The PP2C domain of KAPP then dephosphorylates CLV1 and attenuates CLV1 signaling. A Rho-GTPase-related protein, ROP, was also found in the assembled CLV1 signaling complex, which suggests that CLV1 transduces its signal through a MAPK pathway. The question marks represent unknown components.

180-298 (Li et al., 1999). Further site-directed mutagenesis of four conserved residues (G211, S226, H229 and N250) within the FHA-homology region and in vitro binding analyses indicated that this 52-residue region (residues 208-259) is essential but not sufficient for its interaction with phosphorylated RLKs (Li et al., 1999).

Recent work has revealed the role of KAPP in RLK signaling pathways in vivo. Biochemical and genetic data showed that KAPP is a negative regulator in the *Arabidopsis* CLAVATA 1 (CLV1) signal transduction pathway (Williams et al., 1997; Stone et al., 1998; Trotochaud et al., 1999). CLV1 encodes a leucine-rich-repeat RLK (Clark et al., 1997). Plants containing missense mutations in CLV1 display excess floral organs, abnormally shaped fruits and enlarged stems. Reduction of KAPP protein from the *clv1-1* mutant background by co-suppression resulted in the restoration of wild-type *Arabidopsis* plants (Stone et al., 1998). Conversely, overexpression of KAPP in wild-type *Arabidopsis* plants generated a weak *clv1* phenotype (Williams et al., 1997).

KAPP homologs have been isolated in both maize and rice and show similar phosphorylation-dependent binding to RLKs (Braun et al., 1997; van der Knaap et al., 1999). It has been hypothesized that the KAPP FHA domain brings the KAPP phosphatase close to its RLK target to attenuate receptor kinase signaling (Stone et al., 1998; Williams et al., 1997; Fig. 2).

Although in vitro binding analyses showed that the KAPP FHA domain interacts with multiple RLK catalytic domains (Braun et al., 1997; Li et al., 1999), its physiological role in other plant RLK signaling pathways has not yet been elucidated.

Saccharomyces cerevisiae Rad53p

Studies of the yeast protein Rad53p support the idea that the FHA domain is a phosphoprotein-binding module. Rad53p is a protein kinase involved in the DNA damage response and cell cycle arrest in *Saccharomyces cerevisiae*. Rad53p contains two FHA domains – an N-terminal FHA1 domain and a C-terminal FHA2 domain – separated by a central Ser/Thr kinase domain. In response to the inhibition of DNA replication or to DNA damage, a kinase cascade is triggered. Rad53p is phosphorylated by another protein kinase, probably Mec1p (Sanchez et al., 1996; Sun et al., 1996). The activated Rad53p then activates Dun1p, another yeast protein kinase that controls DNA damage response (Zhou and Elledge, 1993). Using a kinase-defective Rad53p mutant as bait in a yeast two-hybrid system to search for possible interacting proteins, Sun et al. (Sun et al., 1998) identified Rad9p. Mutational analyses of the most conservative region of FHA2 demonstrated that FHA2 is involved in the interaction between Rad53p and phosphorylated Rad9p. Phosphorylation of Rad9p is induced by DNA damage signals in a TEL1/MEC1-dependent manner and allows it to interact with Rad53p. Mutation of the FHA2 domain abolished DNA-damage-induced G₂/M cell cycle arrest, indicating the biological relevance of this domain. Durocher et al. subsequently have shown that FHA1 of Rad53p also interacts with the phosphorylated form of Rad9p (Durocher et al., 1999). The kinase(s) that phosphorylates Rad9p has not been identified. It was previously believed that the two FHA domains of Rad53p play different roles in DNA checkpoint signaling (Fay et al., 1997; Sun et al., 1998; Walworth, 1998). The finding that FHA1 of Rad53p interacts with phosphorylated Rad9p, however, suggests that there might be some functional redundancy of the two FHA domains (Durocher et al., 1999). The genetic role of the FHA1 domain of Rad53p in DNA damage signaling needs to be elucidated.

The C-terminal region of Rad9p contains a BRCT domain – a domain found in two human proteins, BRCA1 and 53BP1, both of which have connections with human cancer (Bork et al., 1997). Homologs of both Rad9p and Rad53p have been identified in other organisms. For example, Rad9p homologs from *S. pombe*, Rhp9p and Crb2p, have functions similar to that of *S. cerevisiae* Rad9p (Wilson et al., 1997; Saka et al., 1997). Mutants lacking functional Rhp9p or Crb2p fail to arrest the cell cycle in response to DNA damage or to inhibition of DNA replication. Several other Rad53p homologs have also been studied, such as *S. pombe* Cds1p (Murakami and Okayama, 1995), *Drosophila* DMNK (Oishi et al., 1998) and human CHK2 (Matsuoka et al., 1998). All these protein kinases contain a single FHA domain at their N terminus and play a role in DNA damage checkpoint signaling. The functions of the FHA domains in these protein kinases have not been extensively studied. However, heterozygous mutations in the hCHK2 FHA domain region (I157T and R145W) cause a variant of the cancer syndrome Li-Fraumeni and a colon cancer (Bell et al., 1999). The phenotypes resulting from these heterozygous single-residue substitutions might be due to a dominant negative effect, which suggests that the

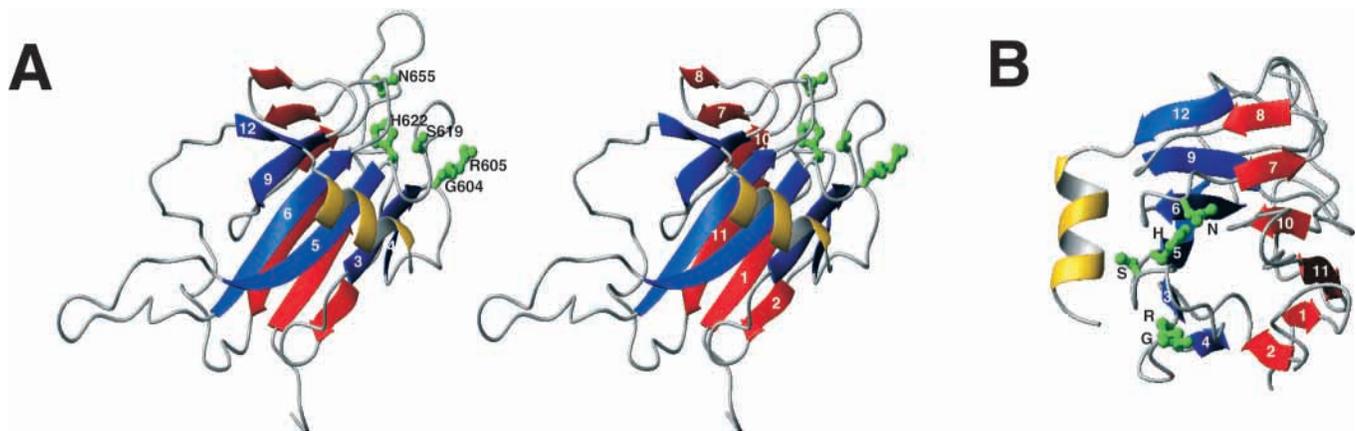


Fig. 3. Three-dimensional structure of Rad53p FHA2 with conserved residues highlighted in green. One β -sheet has been highlighted in blue, and the other in red. The figures were created by the MOLMOL program (Koradi et al., 1996), using atomic coordinates from the RCSB PDB entry 1DMZ (Liao et al., 1999). (A) Stereoview of the Rad53p FHA2 structure. (B) Topview of the Rad53p FHA2 structure

hCHK2 FHA domain is genetically involved in the G₂ checkpoint. The direct target of the hCHK2 FHA domain has yet to be identified.

Human nuclear inhibitor of PP1 (NIPP1)

Nuclear inhibitor of protein phosphatase 1 (NIPP1) is a nuclear-localized protein that interacts with a type one protein phosphatase (PP1) through a conserved PP1-interaction motif, RVTF (Trinkle-Mulcahy et al., 1999). The C terminus of NIPP1 can selectively interact with A/U-rich RNA sequences, which indicates that NIPP1 might be involved in transporting PP1 to RNA molecules (Claverie-Martin et al., 1997). Binding of NIPP to PP1 inactivates PP1 activity. The NIPP1-PP1 heterodimer, however, can be activated by phosphorylation on up to four NIPP1 Ser/Thr residues by protein kinase A and CK2. This phosphorylation abolishes the interaction between NIPP1 and PP1 (Vulsteke et al., 1997).

The N terminus of NIPP1 contains an FHA domain. The NIPP1 FHA domain is not required for interaction with PP1c or RNA. It is, however, required for association with CDC5L, a human homolog of *S. pombe* Cdc5p, that can interact with NIPP1 in a yeast-two hybrid system (Boudrez et al., 2000). The interaction between the NIPP1 FHA domain (residues 1-142) and CDC5L is phosphorylation dependent, which is consistent with other FHA domain properties. On the basis of the observation that NIPP1 is part of a complex, it was proposed that the NIPP1 FHA domain plays an important role in regulating pre-mRNA splicing and cell-cycle progression, probably by recruiting CDC5L to a functional complex (Boudrez et al., 2000). The FHA domain in NIPP1 is clearly involved in phosphoprotein interactions, but the exact function of each component in the complex needs to be established.

Human KI-67 antigen

Human KI-67 antigen is localized around mitotic chromosomes and has been widely used as a cell proliferation marker because it is expressed only in growing cells (Gerdes et al., 1984). To investigate the role of this protein in the cell cycle, an FHA domain-containing fragment of KI-67 (residues 1-99) was used as bait to search for possible interaction proteins in a yeast two-hybrid system. Sueishi et al. identified

a new protein, hKLP2 (human kinesin-like protein 2), which can interact with the KI-67 FHA domain (Sueishi et al., 2000). hKLP2 is a human homolog of Xklp2 (*Xenopus* kinesin-like protein 2), which is required in centrosome separation and for the maintenance of spindle bipolarity during mitosis in *Xenopus* egg extracts (Boleti et al., 1996). Co-immunolocalization analysis indicated that both KI-67 and hKLP2 are localized on mitotic chromosomes, which suggests that KI-67 is involved in cell proliferation.

The KI-67 FHA domain recognition sequence was mapped to hKLP2 residues 1017-1237 (Sueishi et al., 2000). Further deletion from both sides abolished the interaction, which suggests that the KI-67 FHA domain recognizes, at least partially, the 3D structure of hKLP2. Whether this interaction is phosphorylation-dependent has not been clarified. Indirect evidence suggests, however, that the target of KI-67 FHA domain is phosphorylated during mitosis.

THE STRUCTURE OF FHA DOMAINS

Rad53p FHA2 was the first FHA domain structure solved by NMR (Liao et al., 1999). The tertiary structure consists of a β -sandwich containing two twisted antiparallel β -sheets and a short α -helix at the C terminus. The α -helix may not be involved in FHA domain function, because it appears to lie outside the β -sheet structure (Fig. 3). One of the β -sheets is made up by β 1, β 2, β 7, β 8, β 10 and β 11; the other sheet consists of β 3, β 4, β 5, β 6, β 9 and β 12 (RCSB PDB entry 1DMZ; Liao et al., 1999).

More recently, two other research groups have solved the secondary structure of the KAPP FHA domain (G. Lee et al., unpublished result) and the tertiary structure of Rad53p FHA1 (Durocher et al., 2000). It is interesting that, although the primary sequences of these three FHA domains are divergent, their secondary or tertiary structures are remarkably similar (Fig. 4). The structure of the FHA domain is clearly different from those of other phosphoprotein-binding domains, such as the SH2 domain and 14-3-3. 14-3-3 proteins consist mainly of α -helices, FHA domains consist mainly of β -sheets, and SH2 domains contain both. Unlike the other domains, FHA domains

have five conserved residues involved in binding, which are found in the loop structure between β -sheets (Liao et al., 1999; Wang et al., 2000; Durocher et al., 2000). Another interesting discovery is that FHA domains share a β -sheet topology with the MH2 domains of Smad family proteins, a group of tumor suppressor molecules (Liao et al., 1999; Durocher et al., 2000). The potential significance of this finding needs to be investigated further.

BINDING SPECIFICITY

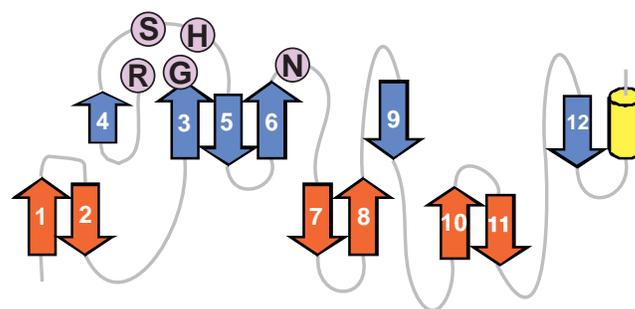
The ability of the KAPP FHA domain to bind denatured/linearized RLKs in both far-western and surface plasmon resonance analyses suggests the hypothesis that it directly binds a phosphorylated peptide in RLKs (Stone et al., 1994; Li et al., 1999). However, the finding that the KAPP FHA domain interacts with phosphorylated RLKs and that Rad53p FHA2 binds phosphorylated Rad9p does not eliminate the possibility that phosphorylation is involved indirectly in binding by FHA domains. For example, indirect effects of phosphorylation might be to alter the conformation of the target protein (Durocher et al., 1999). Durocher et al. (Durocher et al., 1999; Durocher et al., 2000) tested the binding of FHA domains from a wide spectrum of prokaryotic and eukaryotic organisms to a series of synthetic peptides. They demonstrated that FHA domains from different sources have similar interaction determinants, which are all phosphopeptides. However, the specificity of each different FHA domain appears to be distinct. There appear to be at least three different subgroups of the FHA domain, which differ in their preferences for binding partner, especially at the +3 position of the phosphothreonine peptides (numbered from the phosphothreonine). FHA1 of Rad53p selectively interacts with pTXXD-containing peptides and has almost no affinity for pTXXI/L peptides, whereas FHA2 of RAD53 preferentially interacts with pTXXI/L peptides. The KAPP FHA differs in having a high affinity for pTXXS/A peptides (Durocher et al., 2000).

The structural studies of FHA domains have shed light on the structural details of phosphopeptide binding (Liao et al., 1999; Wang et al., 2000; Durocher et al., 2000). Three of the highly conserved residues in Rad53p FHA1 (R70, S85 and N107) form hydrogen bonds or salt bridges to the phosphate of the peptide. The structure of the binding site appears to be stabilized by the hydrogen bond between the conserved H88 and S85 residues and van der Waals interaction between G69 and H88 (Durocher et al., 2000). Although the conserved residues (G69, R70, S85, H88 and N107) may be essential for the interactions with the ligand, interaction with the phosphopeptide backbone cannot confer the binding specificity. In Rad53p FHA1, non-conserved residues R83 and S82 could be critical for determination of ligand specificity. They can form hydrogen bonds with the pT+3 and pT+1 residues, respectively. Site-directed mutagenesis has confirmed that interaction between R83 and pT+3 is crucial for

binding specificity (Durocher et al., 2000). Both R83 and S82 are non-conserved residues in the FHA family members. Conserved residues therefore appear to be involved in direct binding to the ligand backbone and phosphate, whereas the adjacent non-conserved residues might determine the binding specificity.

Interestingly, the Rad53p FHA2 can weakly bind ($K_d \approx 0.1$ mM) to phosphotyrosine peptide from Rad9p (Liao et al., 1999). Although the biological relevance of this observation still needs to be investigated, it does have significant implications: FHA domains might have dual specificity, and some FHA domains might selectively interact with phosphotyrosine-containing targets. Tsai and co-workers have substantiated this by using combinatorial library and NMR analyses. They have identified high-affinity pY peptides, and determined the structure of a phosphotyrosine peptide complex with Rad53p FHA2 (Wang et al., 2000). They have also elucidated pT peptide consensus sequences for both FHA1 and FHA2 of Rad53p and have identified Thr192 (phosphorylated form) of Rad9p as the likely biological binding site of FHA1 (Liao et al., unpublished results). In addition, we have shown that a phosphoserine-containing peptide in CLV1 might be biologically relevant to its interaction with the KAPP FHA domain, since CLV1 is autophosphorylated only on serine residues (Stone et al., 1998). Together, these observations suggest that different FHA domains vary in their binding specificities. It is likely that FHA domains selectively interact with phosphothreonine, phosphoserine or phosphotyrosine. If this is true, FHA domains may represent a new class of dual specificity phosphoprotein-binding domain as proposed by Liao et al. (1999). Determination of the binding specificities of new FHA domains will be an important test of this hypothesis.

Rad53p FHA2



KAPP FHA

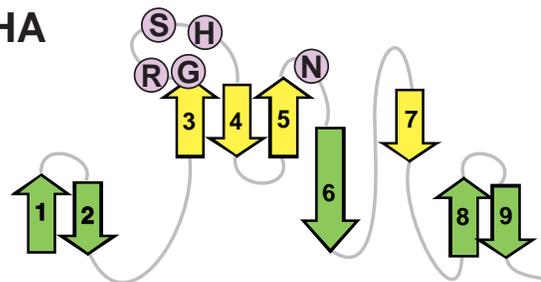


Fig. 4. The secondary structures of the Rad53p FHA2 (RCSB PDB entry 1DMZ (Liao et al., 1999)) and KAPP FHA domains (Lee et al., unpublished). Both domains consist of a pair of antiparallel β -sheets, which form a β -sandwich. Circles represent the conserved residues in FHA domains. Arrows represent β -strands, the cylinder represents an α -helix, and lines represent random coils.

CONCLUSION

FHA domains represent a new class of phosphorylation-dependent protein-protein interaction domains. Like SH2 domains, they are found in a wide variety of different signaling proteins from both prokaryotes and eukaryotes and might have evolved early. These domains might play an important role in many signal transduction pathways, because they are present in many diverse signaling proteins. Several studies have shown that FHA domains are involved in DNA damage response and cell cycle arrest. However, FHA domains also play important roles in many other signaling pathways. For example, KAPP is clearly involved in plant RLK signaling pathways on the inner face of the plasma membrane, and NIPPI is thought to be involved in control of RNA splicing. Future studies concentrating on deciphering the biological functions of other FHA-containing proteins, especially those localized in cell compartments other than the nucleus, will contribute to our understanding of the role of the FHA domains in cellular signaling. The debate about whether FHA domains display dual specificity is ongoing. The identification of new ligands of FHA domains and mapping of biologically relevant FHA-domain-binding sites on these ligands will help clarify the importance of the binding specificity. In turn, this will help us to understand the dynamics of interactions between signaling components.

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