Phosphoserine/threonine-binding domains

Michael B Yaffe*† and Andrew EH Elia†

Phosphorylation of proteins on serine and threonine residues has traditionally been viewed as a means to allosterically regulate catalytic activity. Research within the past five years, however, has revealed that serine/threonine phosphorylation can also directly result in the formation of multimolecular signaling complexes through specific interactions between phosphoserine/threonine (pSer/Thr)-binding modules and phosphorylated sequence motifs. pSer/Thr-binding proteins and domains currently include 14-3-3, WW domains, forkhead-associated domains, and, tentatively, WD40 repeats and leucine-rich regions. It seems likely that additional modules will be found in the future. The amino acid sequences recognized by these pSer/Thr-binding modules show partial overlap with the optimal phosphorylation motifs for different protein kinase subfamilies, allowing the formation of specific signaling complexes to be controlled through combinatorial interactions between particular upstream kinases and a particular binding module. The structural basis for pSer/Thr binding differs dramatically between 14-3-3 proteins, WW domains and forkhead-associated domains, suggesting that their pSer/Thr binding function was acquired through convergent evolution.

Addresses
*Center for Cancer Research E18-580, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA
e-mail: myaffe@mit.edu
†Division of Signal Transduction, Harvard Institutes of Medicine, 10th Floor, BIDMC/330 Brookline Avenue, Boston, Massachusetts 02215, USA

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Abbreviations

FHA	forkhead-associated
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
pSer/Thr	phosphoserine/threonine
SCF	Skp1–Cdc53/Cullin–F-box

Introduction

Signal transduction events in eukaryotic cells involve the reversible assembly of large multiprotein complexes. These signaling 'machines' integrate and transmit the information that controls ion fluxes, cytoskeletal rearrangements, patterns of gene expression, cell cycle progression and programmed cell death. Assembly of protein and lipid kinases and phosphatases, along with other enzymes, adaptors and regulatory molecules, into signaling complexes is generally regulated through protein phosphorylation, allowing reversibility as the balance shifts between kinase and phosphatase activity. Historically, attention was focused on complexes regulated via tyrosine phosphorylation of transmembrane receptors, generating binding sites for proteins with modular Src-homology 2 (SH2) and phosphotyrosine binding (PTB) domains. In contrast, phosphorylation of proteins on serine and threonine residues was thought to regulate protein function largely through allosteric modifications rather than by directly mediating protein–protein interactions. Within the past few years, however, a variety of signaling molecules and modular domains have been identified that specifically bind to short motifs containing phosphoserine/threonine (pSer/Thr), resulting in the recruitment of these substrates into protein–protein signaling complexes in response to phosphorylation by serine/threonine kinases. In this review we focus on recent advances in the identification of new pSer/Thr-binding modules, including 14-3-3 proteins, WW domains, forkhead-associated (FHA) domains and WD40 repeats/LRR modules in F-box proteins, together with the structural basis for their pSer/Thr-motif recognition, their substrates and the functions that these molecules and domains perform.

14-3-3 proteins

14-3-3 proteins are a family of dimeric α-helical pSer/Thr-binding proteins present in high abundance in all eukaryotic cells. 14-3-3 proteins were the first signaling molecules to be identified as distinct pSer/Thr-binding proteins, generally recognizing the sequences R(S/Ar)XpSXP and RX(Ar/S)XpSXP in which pS denotes pSer/Thr and Ar denotes aromatic residues [1,2]. However, a few 14-3-3-binding ligands have been identified whose sequences either deviate significantly from these motifs or do not require phosphorylation for binding [3••]. Mammalian cells contain seven distinct 14-3-3 genes, whereas yeast and plants contain from two to twelve. In all cases, there is strong conservation of the residues lining the central channel of the 14-3-3 dimer that participate in phosphospecific binding [2]. Because of limited space, this review focuses on mammalian and yeast 14-3-3 binding proteins with critical roles in cell cycle control, mitogen-activated protein kinase (MAPK) activation, apoptosis, gene transcription and chromosome remodeling. Excellent recent reviews covering 14-3-3 function in plants [4] as well as in other processes within yeast and mammalian cells [3••] are available.

Early genetic studies in the fission yeast *Schizosaccharomyces pombe* identified two 14-3-3 genes, *Rad24* and *Rad25*, as critical components in the DNA damage checkpoint [5]. Both genes functioned downstream of the DNA-damage-activated protein kinase Chk1 and upstream of the mitotic regulatory cyclin-dependent kinase Cdc2, whose dephosphorylation by the protein phosphatase Cdc25 is essential for mitotic progression. The link between Chk1 and the 14-3-3 proteins became clearer when human and fission yeast Chk1 proteins were found to phosphorylate Cdc25 [6,7], generating a 14-3-3 binding site at Ser216 and leading to G_2/M checkpoint arrest [7]. 14-3-3 tethers Cdc25 within the cytoplasm [8•–11•], blocking its access to nuclear Cdc2–CyclinB and thereby preventing mitotic entry. Association between

Table 1

pSer/Thr-binding modules and motifs, where known, along with some of their ligands and ligand kinases.

Protein	Motif	Substrates	Upstream kinase(s)
14-3-3	R(S/Ar)XpSXP or RX(Ar/S)XpSXP		Basophilic kinases
14-3-3		Cdc25	Chk1
		Raf	PKCζ
		BAD	Akt, Rsk, PKA
		MSN2/4	TOR
		TAZ	
		YAP	
		Tlx-2	
		Hat1	
		Histone deacetylase 4/5	
		DNA topoisomerase II	
		TERT	
WW domains	pSP or pTP		Proline-directed kinases
Pin1		Cdc25	
		Tau	
		Wee1	
		Myt1	
		Plk1	
		Cdc27	
Ess1		RNA Pol II	
NEDD4		?	
FHA domains	XXXpTXX(D/I/S/Y)		
Rad53		Rad9	
Chk2		?	
Nbs1		?	
KAPP		Receptor-like kinases	
Ki-67		Hklp2	
Nipp1		CDC5L	
Chfr		?	
SNIP1		?	
WD40 domains of F-box proteins			
Cdc4		Sic1	Cln–Cdc28
		Far1	Cln–Cdc28
		Cdc6	Cln/Clb?–Cdc28
		Gcn4	Pcl–Pho85
β-TrCP	DpSGXXpS	IκBα, IκBβ, IκBε	IKK
		β-catenin	GSK-3β
		Vpu	
		NF-κB p105	IKK
Pop1/2		Cdc18	Cyclin–Cdc2
		Rum1	Cyclin–Cdc2
LRR domains of F-box proteins			
Grr1		Cln1/2	Cln–Cdc28
		Gic2	
Skp2		p27	Cyclin E,A–Cdk2

Only a subset of proteins containing these domains, along with selected ligands and kinases are indicated. Motifs are shown in single letter amino acid code; pS and pT denote phosphoserine and phosphothreonine, respectively; Ar denotes the aromatic residues Phe, Tyr and Trp.

14-3-3 and Cdc25 via this site normally occurs under non-DNA damage conditions, during interphase in eukaryotic cells [7,12] and extracts [13] and also during the physiological G_2 arrest of *Xenopus* oocytes [9•].

A second mechanism for 14-3-3 proteins in regulating G_2/M progression emerged from the finding that one particular isotype, 14-3-3σ, was strongly upregulated by p53 in colorectal cancer cells following exposure to ionizing radiation and DNA-damaging agents [14]. 14-3-3σ, in contrast to other 14-3-3 isotypes, does not bind Cdc25 *in vivo*, suggesting that another 14-3-3σ binding partner, perhaps Cyclin B–Cdc2 itself, is the G_2/M checkpoint target [14]. 14-3-3$\sigma^{-/-}$ somatic cells initiate but are unable to maintain a G_2/M arrest following DNA damage and die by mitotic catastrophe [15•]. Furthermore, 14-3-3σ expression is downregulated in a variety of tumor cell lines and primary breast cancers through selective gene methylation [16].

Details about the exact role of the 14-3-3 proteins in Raf activation and MAPK signaling continue to be controversial. 14-3-3 both stabilizes the active form of Raf and maintains inactive Raf in a reactivatable conformation [17,18] but is not required for Ras-mediated recruitment of Raf to the plasma membrane [17]. 14-3-3 must dimerize both to function as a Raf cofactor [18] and to promote protein kinase C mediated Raf activation [19].

14-3-3 proteins are critical regulators of apoptosis under some conditions. Muslin and colleagues [20] found that a cardiac-specific dominant-negative 14-3-3η transgene caused increased cardiomyocyte apoptosis in response to left ventricular pressure overload. In neurons and certain IL-3-dependent cell lines, a complex between the anti-apoptotic protein Bcl-X_L and the pro-apoptotic protein BAD causes cell death in a manner prevented by BAD–14-3-3 binding [21•,22]. BAD contains two 14-3-3-binding sites, Ser112 and Ser136, of which only the latter is essential for anti-apoptotic effects *in vivo*. In addition, growth factors stimulate the phosphorylation of a third critical site, Ser155, within the BAD BH3 domain, which prevents BAD–Bcl-X_L complex formation [21•,23•–25•]. Ser155 is phosphorylated in uncomplexed BAD in the absence of Ser112 and Ser136 phosphorylation [23•–25•]. Ser155 phosphorylation of preformed BAD–Bcl-X_L complexes, however, requires Ser136 phosphorylation and 14-3-3 binding, suggesting that 14-3-3 binding alters the conformation of Bcl-X_L-bound BAD to expose the Ser155 site [21•]. The protein kinase(s) responsible for BAD phosphorylation *in vivo* remain controversial. The bulk of evidence favors the phosphatidylinositol 3-kinase regulated enzyme PKB/AKT for the Ser136 kinase [26], the MAPK-dependent kinase RSK for the Ser112 kinase [24•,25•] and the cAMP-dependent kinase protein kinase A for the Ser155 kinase [23•,25•]. Other 14-3-3-regulated activators of apoptosis include the kinase ASK1 [27] and the forkhead transcription factor FKHRL1 [28••].

In addition to MAPK activation and apoptosis, 14-3-3 proteins regulate both gene transcription and chromatin remodeling in an incompletely understood manner. The yeast transcriptional activators MSN2 and MSN4 are sequestered as cytoplasmic 14-3-3 complexes upon activation of the TOR kinase signaling pathway, blocking the ability of these zinc-finger transcription factors to drive expression of stress-related genes [29]. In mammalian cells, two transcriptional coactivators, TAZ and YAP, undergo similar 14-3-3-mediated cytoplasmic sequestration, resulting in impaired gene transcription [30]. In contrast, binding of Tlx-2, a HOX11 homeobox transcription factor family member, to 14-3-3η directly or indirectly increases the nuclear localization of Tlx-2 [31]. In *Xenopus* oocyte nuclei the Hat1 acetyltransferase holoenzyme is in an active complex with the retinoblastoma-associated protein RbAp48 and 14-3-3 [32], whereas in mammalian cells 14-3-3 binds to histone deacetylase 4 and 5, sequestering them in the cytoplasm to block transcriptional repression [33,34]. A complex between 14-3-3 and human DNA topoisomerase II with impaired TopoII DNA-binding activity *in vitro* has also been reported [35]. Seimiya *et al.* [36] showed that 14-3-3 binding to the overexpressed catalytic subunit of human telomerase (TERT) in 293 cells caused nuclear relocalization through blocking a TERT nuclear-export motif adjacent to the 14-3-3 binding site. Summarizing these divergent data on 14-3-3 binding to DNA-interacting proteins into a simple and coherent model for 14-3-3 function is not yet possible, particularly as 14-3-3 stabilizes the cytoplasmic accumulation for some and facilitates the nuclear localization for others. Identification of the relevant serine/threonine kinases together with their regulation may help in assigning specific roles for 14-3-3 as an integrator of specific signaling pathways that regulate gene expression.

WW domains

WW domains are signaling modules of approximately 40 amino acids that fold into three anti-parallel β strands and bind short proline-rich sequences, predominantly composed of PPXY, PPLP or PPR motifs. The proline isomerase Pin1 and the ubiquitin ligase Nedd4, however, contain WW domains that specifically recognize pSer–Pro and pThr–Pro motifs [37••]. It is thought that Pin1 binding to mitotic phosphoproteins, containing pSer/Thr–Pro motifs, induces local conformational changes through proline isomerization. For two Pin1 phosphosubstrates, Cdc25C and Tau, proline isomerization facilitates their subsequent dephosphorylation by the serine/threonine phosphatase PP2A [38]. The *Saccharomyces cerevisiae* Pin1 homolog, Ess1, regulates transcription and mitosis by binding the hyperphosphorylated carboxy-terminal domain of RNA polymerase II in a region that contains 26–52 repeats of the motif YSPTSPS [39]. Verdecia *et al.* [40••] solved the structure of Pin1 complexed to the serine-phosphorylated YpSPTpSPS peptide, providing a structural framework for phosphospecific WW domain binding. In their 1.84 Å structure, all of the phosphate contacts were between the second pSer of the motif and two residues in the β1–β2 loop (Ser16 and Arg17) ,along with one in the β2 strand (Tyr23). Residues functionally equivalent to Ser16 and Arg17 are absent in the vast majority of WW domains, suggesting that the pSer/Thr-binding function has been acquired by only a select few.

Forkhead-associated domains

Forkhead-associated (FHA) domains were originally identified as 55–75 amino acid modules containing three conserved sequence blocks within a subset of forkhead-family transcription factors [41]. They were subsequently found in transcriptional control proteins, DNA-damage-activated protein kinases, cell cycle checkpoint proteins, phosphatases, kinesin motors and regulators of small G proteins. Several studies indicate that FHA domains are considerably larger than originally described, typically encompassing 130–140 amino acids [42,43•,44,45,46••]. The realization that FHA domains were pThr-binding modules emerged from work in several laboratories. Walker and colleagues [42,47] found that the *Arabidopsis* protein phosphatase KAPP FHA

domain interacted with the serine/threonine-phosphorylated forms of receptor-like kinases, whereas Stern and colleagues [48] observed that the carboxy-terminal FHA domain of Rad53p, a key cell cycle checkpoint kinase in *S. cerevesiae*, bound the serine/threonine-phosphorylated form of the DNA-damage control protein Rad9. Durocher *et al.* [49•] found that both the amino- and carboxy-terminal FHA domains of Rad53p, as well as other FHA domains, bound directly to short pThr-containing peptides. The FHA-mediated Rad53–Rad9 interaction is essential for DNA-damage responses, including Rad53p phosphorylation, G_2/M cell cycle arrest and ribonucleotide reductase gene transcription required for DNA repair [48]. Studies using peptide library screening revealed that FHA domains could be grouped into distinct classes on the basis of unique pThr-recognition motifs. Motif selection typically extends for three residues both amino-terminal and carboxy-terminal to the central pThr residue and in many, but not all, cases the pThr+3 residue provides a critical interaction [46••]. All FHA domains studies to date are pThr-specific, as pSer substitution eliminates peptide binding [46••,49].

FHA domains are often involved in processes protecting against human cancer, as evidenced by mutations in FHA-domain-containing proteins that result in tumor formation. The human Rad53p checkpoint kinase homolog, Chk2, contains a single FHA domain, as does the human p95/Nbs1 protein that is involved in double-strand break repair and telomere maintenance. Mutations resulting in premature truncation of Nbs1 occur in patients with the cancer-prone disease Nijmegen breakage syndrome, and a mutation that causes premature truncation of Chk2 cosegregates with the cancer predisposition phenotype in a family with classical Li-Fraumeni syndrome [50•]. Furthermore, a single germline Ile→Thr missense mutation within the Chk2 FHA domain was identified in a proband with variant Li-Fraumeni syndrome, and a Chk2 FHA Arg→Trp substitution was found in the human colon cancer cell line HCT15 [50•].

A search of the expressed sequence tag database for FHA-domain-containing proteins led to the identification of a novel prophase checkpoint protein, Chfr (checkpoint with FHA and ring finger), that prevents metaphase entry until centrosome separation is complete via an FHA-domain-dependent process [51••]. Intriguingly, Chfr was inactivated in four out of the eight human cancer cell lines examined. An FHA domain is also present within the cell-proliferation marker protein, Ki-67, originally identified as an immunogen present within nuclear extracts from a Hodgkin's-lymphoma-derived cell line. The FHA domain of Ki-67 interacts with the phosphorylated human homolog of a *Xenopus* plus-end-directed kinesin motor, Hklp2, a fraction of which colocalizes with Ki-67 on mitotic chromosomes [52]. NIPP1, a regulatory subunit of the serine/threonine protein phosphatase PP1, uses its amino-terminal FHA domain to bind phosphorylated CDC5L, a protein required for G_2/M progression and mRNA splicing [53]. CDC5L is phosphorylated in mitosis, but a role for NIPP1 in mitotic regulation has not been established.

FHA-domain proteins also participate in mRNA splicing and transcriptional regulation. NIPP1 and CDC5L colocalize in nuclear speckles with splicing factors, and the isolated NIPP1 FHA domain is sufficient to block mRNA splicing *in vitro* [53]. A nuclear SMAD-binding protein, SNIP1, was isolated in a yeast two-hybrid screen and found to suppress CBP/p300-dependent gene transcription in response to TGF-β signaling [54]. SNIP1 contains a carboxy-terminal FHA domain, though the role this domain plays in SNIP1 regulation is not known.

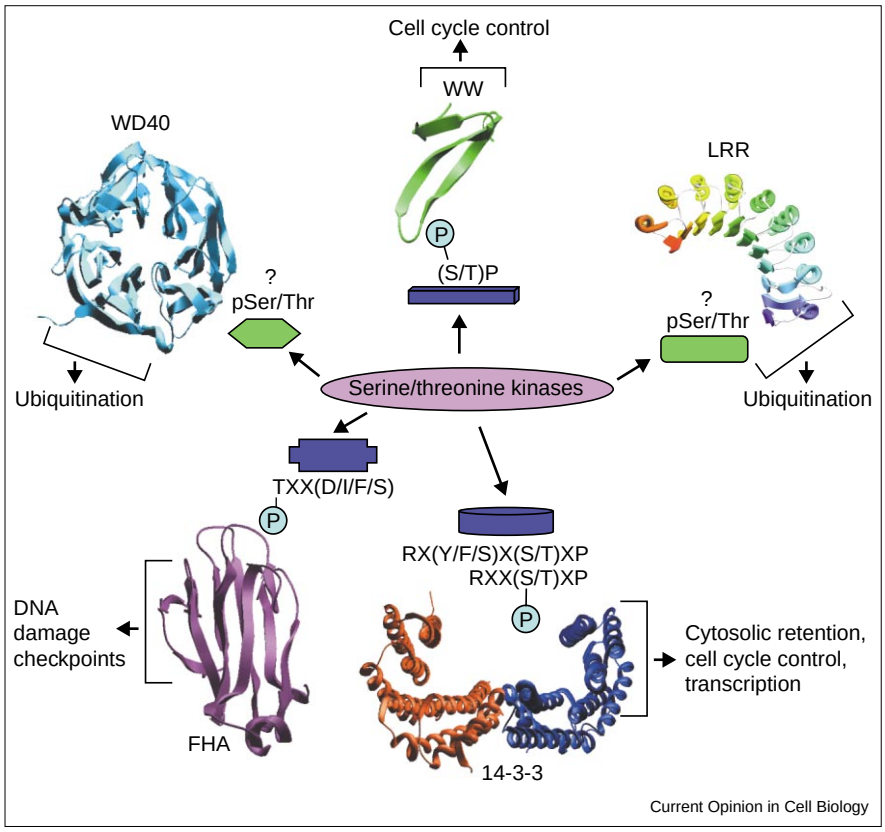
Structures of FHA domains have been determined by both NMR spectroscopy and X-ray crystallography. Tsai and colleagues [43•,45] solved the Rad53p carboxy-terminal FHA domain structure by NMR, both alone and in complex with a phosphotyrosine peptide, whereas our group, in collaboration with the groups of Smerdon and Jackson [46••], solved the structure of the Rad53p amino-terminal FHA domain in complex with a pThr peptide at 1.56 Å resolution by X-ray crystallography. Both structures reveal similar FHA-domain topologies — an 11 or 12 stranded β sandwich, with a strand topology essentially identical to that of Smad MH2 domains [46••]. The phosphopeptide-binding residues emerge from loops connecting the β strands and are highly conserved (Arg70, Ser85, Asn86 and Thr106 in the amino-terminal FHA domain, and Arg605, Ser619, Arg620 and Thr654 in the carboxy-terminal FHA domain).

WD40 and leucine-rich repeat domains of F-box proteins

F-box-containing proteins recognize substrates of Skp1–Cdc53/Cullin–F-box (SCF) ubiquitin ligases, targeting them for phosphorylation-dependent ubiquitination and proteasomal degradation [55•,56]. Binding of F-box proteins to Skp1 links these substrate-recognition proteins to the SCF core ligase constituents, Cdc53/Cul-1 and Hrt1/Rbx1/ROC1. In addition to F-boxes, most of these proteins also contain a WD40 or a leucine-rich repeat (LRR) domain that presumably functions as a pSer/Thr-binding module, although at least one F-box-protein–substrate complex, Skp2–cyclin E, appears to be phosphoindependent [57]. In contrast to 14-3-3, WW and FHA domains, structural proof for the direct binding of purified WD40 or LRR domains to pSer/Thr peptides is lacking. However, a large body of compelling biochemical data, some of which is summarized here, supports phosphospecific association with substrates.

Four F-box proteins, Cdc4, β-TrCP, Grr1 and Skp2, have been definitively shown to require prior substrate phosphorylation for *in vitro* binding. The yeast proteins Cdc4 and Grr1 bind, respectively, to the Cdk inhibitor Sic1 and the G_1 cyclin Cln1 *in vitro* only after their phosphorylation by a Cln–Cdc28 complex [58,59]. Similarly, *in vitro* binding of the mammalian β-TrCP protein to the NF-κB inhibitor, IκBα [60–63], and to unprocessed NF-κB p105 itself [64•], requires phosphorylation by the cytokine-inducible kinase IKK. β-TrCP binding to the transcription factor β-catenin *in vitro* relies on phosphorylation by GSK-3β [65,66] and

Figure 1



An overview of pSer/Thr-binding modules, motifs and structures. Ribbon diagrams indicate the actual structures of 14-3-3ζ [2], the WW domain of Pin1 [40], the amino-terminal FHA domain of Rad53p [46] and the LRR domain of the F-box protein Skp2 [84••]. In contrast, the β-propellor structure shown for WD40 repeats is inferred from the amino-terminal domain of clathrin. Structures of WD40 and LRR domains from F-box proteins bound to pSer/Thr peptides have not yet been solved.

in vitro interaction of the mammalian Skp2 protein with the Cdk inhibitor p27 requires phosphorylation of p27 by cyclin E–Cdk2 [67••]. For these and other F-box proteins, evidence for phosphospecific association also comes from less definitive experiments, showing either loss of substrate binding or loss of substrate ubiquitination/degradation upon mutation of potential serine/threonine phosphoacceptor sites. For example, mutation of Cdk phosphorylation sites in the replication protein Cdc18 in *S. pombe* disrupts its interaction with the F-box protein Pop2/Sud1 *in vivo* [68] and inhibits Cdc18 degradation [69].

The minimal domain for phosphospecific substrate recognition has been determined for a few F-box proteins. The WD40 domain of β–TrCP is sufficient for the phosphospecific association with IκBα [62] and NF-κB p105 [64•], whereas it has been proven both necessary and sufficient for β-catenin [65,66,70] and Vpu [71] binding. For Sic1, the WD40 domain is both necessary and sufficient for its interaction with Cdc4 [55•,59], whereas the LRR domain of Grr1 is necessary for Cln2 binding [72].

Does the phosphorylation-dependent interaction of substrates with F-box proteins result from global changes in protein conformation or from the generation of linear phosphopeptide motifs to which the WD40 or LRR domain binds? For β-TrCP, evidence supports the latter theory as a phosphopeptide corresponding to a site of IKK phosphorylation in IκBα (DpSGLDpS), but not its unphosphorylated counterpart, inhibits IκBα ubiquitination [73] and β-TrCP/IκBα binding [60]. In addition, immobilized peptides containing DpSGXXpS motifs from both IκBα and β-catenin bind β-TrCP in cell lysates [74•]. The DpSGXXpS motif also mediates β-TrCP binding to other IκB isotypes (IκBα, IκBε) [75] and to the HIV protein Vpu [71]. Both of the phosphoserines and the aspartate within this motif are necessary for binding [73,76]. For one other F-box protein, Skp2, phosphospecific binding to a peptide, derived from p27, has been demonstrated [67••,77•], although a consensus motif has not been defined.

For some F-box proteins, such as Cdc4 and Grr1, interaction appears to require substrate phosphorylation at multiple sites. Disruption of Cdc4-mediated Sic1 and Cdc6 ubiquitination [78,79], and disruption of Grr1-stimulated Cln2 degradation [80] requires mutation at numerous Cdk phosphorylation sites. For Sic1 it has been proposed that each of its Cdk phosphorylation sites bears only limited homology to an optimal Cdc4-binding motif and functions cooperatively in binding multimeric Cdc4 via an avidity effect (M Tyers, T Pawson, personal communication).

The structural basis for WD40 and LRR interactions with phosphopeptides is unknown, though inferences can be drawn from structures of other LRR-containing or WD40-containing proteins bound to substrates (Figure 1). The LRR

domain of bovine RNase inhibitor forms multiple repeats of single α-helix/β-strand units that align to form a large surface area for interaction with RNase [81]. How such a structure in F-box proteins might recognize short pSer/Thr peptides is unclear. In contrast, WD40 domains adopt a β-propeller structure, which has precedence for binding linear peptide motifs within the grooves formed by adjacent propeller blades [82•]. The β-TrCP WD40 domain may bind the DpSGXXpS motif in a similar manner. The structural contribution of other components within the ubiquitination complex to phosphospecific binding is also unknown. Notably, ubiquitination of p27 requires the formation of a trimeric complex with cyclin and Cdk2 [83], whereas the SCF component Skp1 stabilizes the interactions of Cdc4 with Sic1 [58,59] and β-TrCP with IκBα [63]. Whether Skp1 makes direct contacts with the phosphorylated substrate and/or stabilizes a particular F-box/WD40 conformation awaits cocrystallization studies.

Conclusions

The identification of several families of pSer/Thr-binding modules within the past four years presents new paradigms for how cell signaling events are regulated by serine/threonine phosphorylation. The optimal motifs recognized by different pSer/Thr-binding modules show partial overlap with the optimal phosphorylation motifs for particular protein kinases (Table 1). This allows the assembly of specific signaling complexes to proceed through combinatorial interactions between specific protein kinases and selective binding modules. The structural diversity manifested by these domains suggests that their phosphobinding functions evolved independently. The challenge for the future is to place these domains into relevant signaling pathways, between upstream kinases and downstream effectors.

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