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Minireview

Interaction domains: from simple binding events to complex cellular behavior

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Abstract Many of the signaling pathways and regulatory systems in eukaryotic cells are controlled by proteins with multiple interaction domains that mediate specific protein–protein and protein–phospholipid interactions, and thereby determine the biological output of receptors for external and intrinsic signals. Here, we discuss the basic features of interaction domains, and suggest that rather simple binary interactions can be used in sophisticated ways to generate complex cellular responses. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Protein–protein interactions; Phosphorylation-dependent binding; SH2 domains; Signal transduction

1. Interaction domains

The cytoplasmic proteins that convey information from cell surface receptors to their intracellular targets are commonly constructed of modular domains, that either have a catalytic function (such as protein or lipid kinase activity), or mediate the interactions of proteins with one another, or with phospholipids, nucleic acids or small molecule second messengers [1]. These latter interaction domains play a critical role in the selective activation of signaling pathways, through their ability to recruit target proteins to activated receptors, and to regulate the subsequent formation of signaling complexes at appropriate subcellular locations [2,3]. Such interaction domains may control not only the specificity of signal transduction, but also the kinetics with which cells respond to external and intrinsic cues, as discussed below, and can therefore give rise to complex cellular behaviors. Interaction domains were originally identified in the context of phosphotyrosine signaling, through the ability of Src homology 2 (SH2) domains of cytoplasmic proteins to recognize specific phosphotyrosine-containing motifs on activated receptor tyrosine kinases [4–7]. However, it is now apparent that specific protein–protein interactions play an essential role in the signaling output of a wide range of cell surface receptors, as well as in cellular events such as protein and vesicle trafficking, the cell cycle, gene expression, DNA repair, control of the cytoskeleton and targeted protein degradation. Intriguingly, closely related protein interaction domains are used repeatedly in distinct proteins to mediate a wide range of regulatory processes.

Interaction domains, not surprisingly, appear designed to recognize exposed features of their binding partners. This applies to the sizable family of domains and proteins that recognize post-translationally modified sequences, including phosphotyrosine motifs (SH2, phosphotyrosine-binding (PTB) domains) [4–10], phosphothreonine/serine-containing elements (i.e. 14-3-3 proteins; FHA, WD40 domains) [11], acetylated or methylated lysine (bromo and chromo domains) [12–14] or hydroxyproline motifs (VHL protein) [15,16]. Similarly, proline-rich sequences tend to be readily accessible, and are recognized by interaction modules such as SH3, WW and EVH1 domains [17–21], the same applies to the extreme C-termini of proteins, which are frequently bound by PDZ domains [22–25].

In addition to interaction domains that mediate protein–protein interactions, a growing family of domains bind phospholipids, especially phosphoinositides (PI) (i.e. PH, FYVE, PX, ENTH, Ferm and Tubby domains) [26,27]. Such modules bind the lipid's charged inositol headgroup in a fashion that depends on its sites of phosphorylation, and are thereby recruited to membrane regions where the relevant phosphoinositides are generated [28,29]. PH domains, for example, tend to bind PI-4,5-P_2 or PI-3,4,5-P_3 at the plasma membrane [30–34], whereas FYVE domains bind PI-3-P in the endosome compartment [35–38]. As discussed in more detail below, interaction domains can have rather flexible and multivalent binding properties; the Tubby domain for example binds PI-4,5-P_2 at the plasma membrane, but also binds DNA following PI-4,5-P_2 hydrolysis and consequent translocation of the Tubby protein to the nucleus [27]. This ability of interaction domains to mediate multiple types of interaction is especially evident for domains made up of repeated units, notably TPR repeats, HEAT motifs, ankyrin repeats, leucine-rich repeats or WD40 repeats [39–42]. The assembly of these repeated elements into larger domains can create quite extensive binding surfaces with diverse specificities. In addition folded modular domains can undergo homo- or heterotypic interactions, that result in their dimerization or oligomerization. This is a notable feature of the domains that regulate apoptotic signaling cascades (i.e. death domains, death effector domains, caspase recruitment domains) [43–45], but is also seen for modules such as SAM domains, which mediate the association of a wide range of receptors, cytoplasmic proteins, and

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transcription factors [46–48], and PDZ domains, which can associate one with another as well as with C-terminal sequences [49,50].

2. SH2 domains and phosphotyrosine recognition

The ability of interaction domains to mediate the formation of protein complexes in a fashion that depends on protein phosphorylation, is typified by the binding of SH2 domains to phosphotyrosine sites. SH2 domains are a common feature of an otherwise diverse group of cytoplasmic polypeptides (Fig. 1), that serve as intracellular targets of receptor tyrosine kinases (RTK), and more complex multi-subunit receptors such as those for antigens, cytokines and extracellular matrix components [1,51,52]. SH2 domains bind specific phosphotyrosine-containing peptide motifs, such as those found in the non-catalytic region of activated growth factor receptors, located either between the membrane and the kinase domain (juxtamembrane), in a loop inserted into the kinase domain (kinase insert) or in the C-terminal tail [53]. Such interactions link receptor autophosphorylation to the activation of specific cytoplasmic signaling pathways. A significant fraction of the binding energy for SH2 domain–phosphopeptide interactions comes from the association with phosphotyrosine, and the formation of stable SH2-mediated complexes is therefore phosphotyrosine-dependent [54,55]. However, a combination of structural, biochemical and genetic analysis has revealed that SH2 domains also recognize between three and five residues immediately C-terminal to the phosphotyrosine, in a fashion that varies from one SH2 domain to another [7,54,56–60]. The sequence of residues C-terminal to the phosphotyrosine of an SH2 domain-binding site can therefore influence the binding affinity, with 'optimal' motifs binding with approximately 100-fold higher affinity than randomized phosphopeptides (although this varies substantially from one SH2 domain to another). The sequence context of an SH2 domain-binding site can therefore have a significant effect on the specificity with which a given receptor interacts with its downstream targets [61,62]. It is important to bear in mind that the complexes that form in vivo will ultimately depend on the local concentrations of the phosphorylated receptor and relevant SH2 domain proteins, and as discussed below in the context of the cell cycle, the optimal binding motif for an interaction domain established by in vitro analysis is not invariably the one used in vivo. In addition, some physiological receptor autophosphorylation sites can associate with multiple SH2 proteins in vivo [63].

SH2 domains tend to bind their cognate peptide ligands with dissociation constants of approximately 500 nM to 1 μM, and indeed many interaction domains show similar binding affinities for their ligands, in the order of 0.5–10 μM [64,65]. These affinities appear sufficient for specific binding in vivo, especially when multiple domains are coupled together to localize interacting proteins to the same place in the cell and promote multivalent interactions. However, they also yield fairly high off-rates so that the interactions are dynamic. This is likely important in signaling processes that must be rapidly turned on and off to accommodate the shifting needs of the cell.

Clearly the specificity and affinity with which SH2 domain proteins interact with activated receptors, and cytoplasmic docking proteins, can be considerably increased by the presence of additional interaction domains. Thus, proteins with two tandem SH2 domains bind cooperatively to bisphosphorylated sites [66], and Src family kinases can potentially interact with their targets through both their SH2 domain and the covalently linked SH3 domain, which recognizes proline-rich sequences [67–69].

3. SH2 domains and interaction modules in the evolution of signaling networks

SH2 domains display features that are characteristic of the larger family of interaction domains. They are ~100 amino acids long, rather typical of this class of protein modules which are generally in the 35–150 amino acid range. They are found in a large number of proteins, which otherwise have distinct biochemical activities; we presently estimate there are 111 SH2 domains in the non-redundant set of human gene products, found in proteins with diverse functions, including regulation of protein/lipid phosphorylation, phospholipid metabolism, transcriptional regulation, cytoskeletal organization, and control of Ras-like GTPases (Fig. 1). Based on this observation, one possible explanation for the widespread use of interaction domains is that they allow for the rapid evolution of new signaling pathways, by the incorporation of a novel interaction domain into a pre-existing polypeptide. For example, yeast have no functional SH2 domains, and lack conventional tyrosine kinase activity. However, tyrosine kinase activity and the SH2 domain make a coincident appearance in metazoan organisms (even being found in a facultative metazoan such as *Dictyostelium discoideum*) [70,71]; one can speculate that tyrosine kinases evolved as a mechanism to allow communication between cells of increasingly complex organisms, and that the SH2 domain developed to couple the phosphotyrosine signal to intracellular biochemical pathways. The existence of a phosphotyrosine recognition module, such as an SH2 domain, then provides a rapid means of physically linking tyrosine kinases to new signaling pathways, by the insertion of an SH2 domain into a pre-existing enzyme or adaptor protein. The increased complexity of signaling networks in higher organisms may therefore result, in part, from increasingly numerous interconnections mediated by interaction domains [72].

In this context, SH2 domains appear optimally structured to allow their incorporation into a host polypeptide, since their N- and C-termini are close together in space, and on the opposite face of the domain from the phosphopeptide-binding surface. This is a common feature of interaction domains, which presumably facilitates their integration into surface-exposed regions of their host proteins, while preserving their ligand-binding activity. An exception to this rule involves modules which make homo- or heterotypic interactions, such as SAM domains, that involve their N- and C-termini. These domains are usually found at the ends of signaling proteins, presumably because an internal location would sterically block their ability to self-associate [46].

4. SH2 domains show flexible biochemical properties and diverse biological functions

A feature of SH2 domains noted above is their ability to act in synergy with other interaction modules, and signaling proteins commonly possess multiple protein and phospholipid

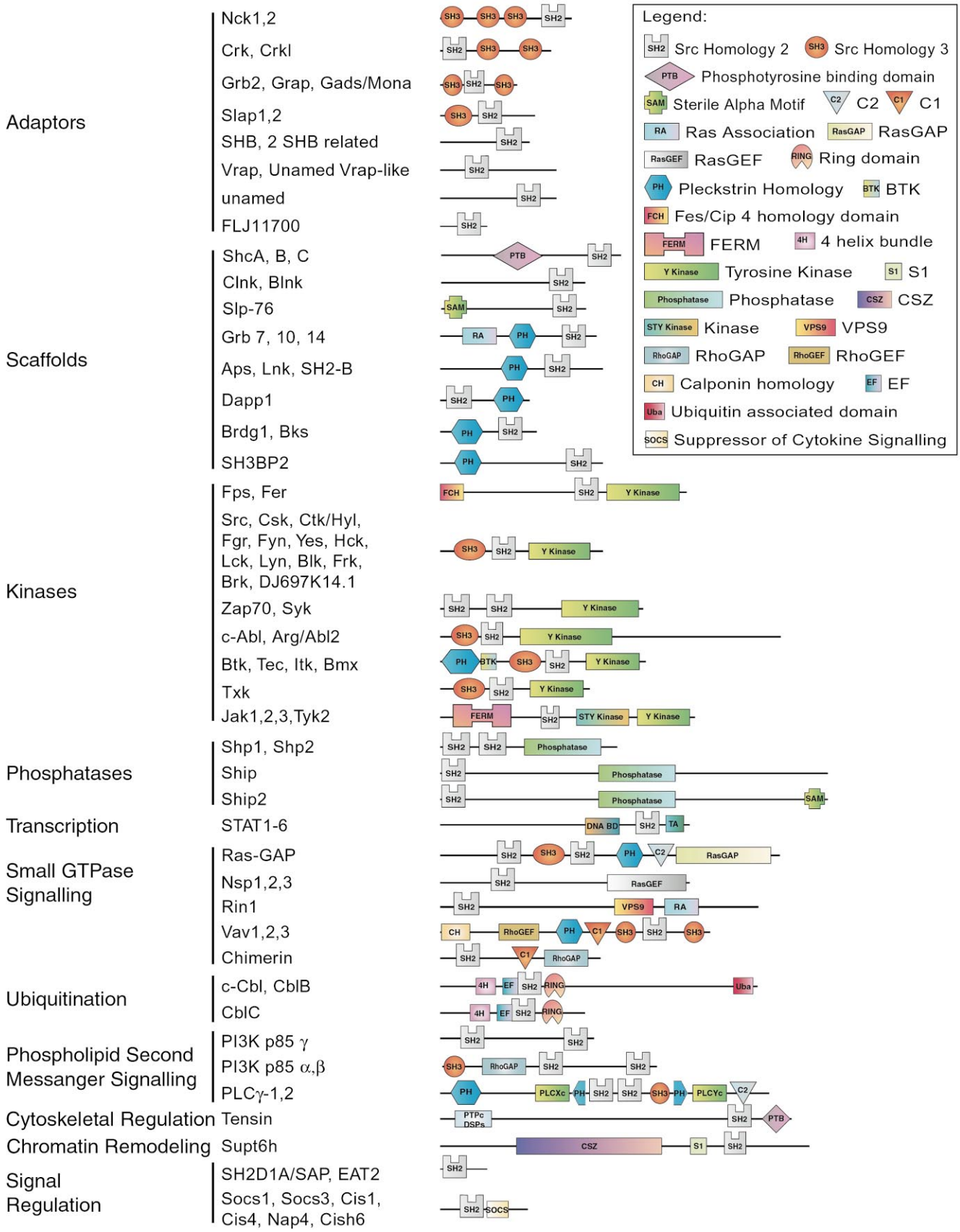

Fig. 1. The modular nature of SH2-containing signaling proteins. A comparison of the modular protein domain composition and positional organization of the non-redundant human SH2 domain-containing proteins identified by SMART (http://smart.embl-heidelberg.de/). Additional information on individual domains can be found at http://www.mshri.on.ca/pawson/research1.html and http://smart.embl-heidelberg.de/.

interaction domains within the same polypeptide chain. This is evident for SH2/SH3 adaptors, such as Grb2, Nck and Crk family members, each of which possesses a single SH2 domain and one to three SH3 domains, and can thereby link a single phosphotyrosine site to multiple intracellular targets with proline-rich SH3-binding sequences [2,73,74]. Interestingly, Grb2 can potentially regulate both the MAP kinase and PI 3′-kinase pathways, through its ability to engage both the Ras guanine nucleotide exchange factor Sos [75,76], and the Gab1/Gab2 docking proteins, which in turn are substrates for phosphorylation and subsequent recruitment of PI 3′-kinase [77–81]. Thus Grb2 may coordinate signals involved in growth, differentiation and survival. In contrast, the SH3 domains of Nck recruit proteins involved in cytoskeletal reorganization, such as the PAK serine/threonine kinase, the N-WASP protein that controls actin polymerization through an association with the Arp2/3 complex, and WASP-interacting protein (WIP) [82,83]. Thus SH2/SH3 adaptors potentially function to nucleate signaling complexes containing multiple proteins involved in a specific aspect of cellular regulation. Mutations of the Grb2 and Nck genes in both invertebrates and vertebrates support these biochemical observations. For example, in *Drosophila* the Nck gene (termed Dock) is required for the targeting of specific photoreceptor axons [84], while in mammalian cells, mutation of both Nck genes abrogates the ability of the Tir protein of enteropathogenic *Escherichia coli* (EPEC) to recruit N-WASP and the Arp2/3 complex to the site of bacterial attachment, and thus blocks the massive reorganization of the actin cytoskeleton normally elicited by binding the phosphorylated EPEC Tir polypeptide to the Nck SH2 domain [85].

Consistent with this multifaceted role, gene targeting experiments in the mouse have indicated that Grb2 has numerous biological activities. By analyzing various combinations of wild-type, hypomorphic and null Grb2 alleles, we have found that Grb2 is required for endoderm formation in the early mouse embryo, and thus for development of the blastocyst, for epiblast formation, for chorio-allantoic fusion and organization of the spongiotrophoblast layer of the placenta, for the survival of a subset of migrating neural crest cells, and therefore for proper formation of the branchial arches, for closure of the palate, and for negative selection in T cells [86–88]. These results indicate that a single signaling protein, and presumably the core Ras-MAP kinase pathway that it controls, is put to a wide variety of uses at various stages of embryonic development and postnatal life. The intriguing issue, then, is not so much the organization of individual pathways (important as this is), but the identification of modulatory proteins that may shape an individual cell's response to a common signaling pathway. Such proteins could be adaptors or scaffolding proteins, positive or negative regulators, or preexisting transcription factors.

A final feature of SH2 domains is worth emphasizing, namely their apparent flexibility. This is demonstrated by the SH2 domain of the SH2D1A protein (also termed SAP or DSHP). The SH2D1A protein is comprised almost entirely of a single SH2 domain, which is mutated in X-linked lymphoproliferative disease (XLP), a fatal disorder typified by an aberrant T cell response to infection with Epstein–Barr virus [89–91]. The SH2 domain of SH2D1A is unusual in the sense that it can bind not only a phosphotyrosine residue and more C-terminal amino acids, but also engages at least two residues N-terminal to the phosphotyrosine [92,93]. In this sense, SH2D1A-binding motifs, such as that found in the C-terminal tail of the SLAM/CD150 T cell receptor, can be viewed as having three 'prongs' (N-terminal residues, the phosphotyrosine and C-terminal residues) that engage the SH2 domain, as opposed to a conventional SH2 domain-binding motif with two 'prongs'. Intriguingly, as a consequence of this increased binding surface, the SH2D1A SH2 domain can bind the relevant tyrosine-based motif even in its unphosphorylated form, although phosphorylation of the tyrosine increases binding by about 5-fold [89,93]. A distinct example is provided by the mammalian Crk SH2 domain, which has a unique proline-rich insert in the DE loop which can bind directly to the Abl SH3 domain [94]. Structural analysis has revealed that this loop in the SH2 domain does indeed adopt a polyproline type II helix, which engages the SH3 domain (L. Donaldson, T. Pawson, L. Kay, J. Forman-Kay, unpublished results). Thus even in the highly conserved SH2 domain family, there is sufficient structural flexibility to bind either phosphorylated or non-phosphorylated motifs at the same surface, or an SH3 domain at a different site.

5. A family of flexible PTB domains

This inherent flexibility of interaction domains is especially evident for PTB domains, originally characterized through their ability to recognize phosphorylated Asn-Pro-X-Tyr β-turn motifs, such as those found in the RTKs for nerve growth factor (Trk), insulin or epidermal growth factor [95–97]. The PTB domains of scaffolding proteins such as Shc, FRS2 or IRS-1 bind autophosphorylated receptors, positioning these proteins for multisite phosphorylation and subsequent binding of SH2 domain targets such as Grb2 (for Shc and FRS2) or PI 3′-kinase (for IRS-1) [98–100]. Interestingly, the ability of these proteins to associate with activated receptors also appears dependent on their recruitment to the plasma membrane. This is achieved in different ways – the PTB domain of Shc binds PI-4,5-P_2 in addition to phosphopeptide, FRS2 is myristilated at its N-terminal glycine, and IRS-1 has a PH domain N-terminal to the PTB domain with the potential to bind phosphoinositides [99,101,102]. The PTB domain of FRS2, however, also binds a non-phosphorylated peptide ligand found in the juxtamembrane region of the FGF receptor, and structural analysis has revealed that this makes a much more extensive and quite different interaction with the PTB domain from that exhibited by Asn-Pro-X-pTyr motifs [103,104]. Thus a single PTB domain can bind RTKs using two different binding modes, one phospho-dependent and the other phospho-independent. Furthermore, it is now evident that PTB domains on proteins such as Numb and X11, which function in the control of asymmetric cell division and trafficking respectively, bind Asn-X-X-Tyr/Phe β-turn motifs, but in a fashion that is independent of phosphorylation [105–107]. Furthermore Numb is able to bind unrelated peptide motifs that adopt different conformations [108]. As with the SH2D1A SH2 domain, the additional binding energy forfeited by the absence of the phosphotyrosine is provided by a more extensive interface with the unphosphorylated peptide. Thus PTB domains may have originally evolved to mediate phospho-independent signaling events, involved in processes such as cell polarity and protein trafficking, and subsequently have acquired a capacity to recognize phosphotyrosine motifs and

contribute to tyrosine kinase signaling. Intriguingly, although they show no obvious sequence relationship to PTB domains, PH domains (which recognize phosphoinositides) and EVH1 domains (which bind proline-rich sequences) have the same fold as PTB domains, suggesting that this structure represents a flexible scaffold which can be adapted to mediate a wide range of protein–protein and protein–phospholipid interactions [109–112].

6. Phosphoserine/threonine recognition domains – a familiar theme

A growing family of interaction domains have been identified as binding phosphoserine/threonine-containing motifs, suggesting that protein phosphorylation is a rather general way of regulating protein–protein interactions [11]. This was first recognized in the context of 14-3-3 proteins, which binds motifs such as Arg-Ser-X-pSer-X-Pro [113,114], and has more recently been described for FHA domains, which are found in proteins that regulate the DNA damage response (i.e. Rad53, Chk2), gene expression (Forkhead proteins) and protein trafficking (kinesins) [115,116]. FHA domains bind preferentially to phosphothreonine motifs, and recognize the +3 residue relative to the phosphothreonine in a fashion that differs from one FHA domain to another and may impart biological specificity. Intriguingly, although the structural mechanisms are quite different, this is reminiscent of SH2 domains, which generally bind phosphotyrosine and show a strong selectivity for the +3 residue. Interestingly, the FHA domain fold is very similar to that of MH2 domains, found at the C-terminus of Smad proteins, the targets of TGF β-receptor serine/threonine kinases [115,117]. Recent work has suggested that autophosphorylation of serine residues within the juxtamembrane region of the type I TGF β-receptor may provide a docking site which is recognized by the MH2 domain of regulatory (R-) Smad proteins, positioning the R-Smad for C-terminal serine phosphorylation by the activated receptor [118]. This in turn leads to its oligomerization with the common Smad (Smad4), and subsequent relocalization to the nucleus where the Smad complex participates in the regulation of gene expression.

There are conceptual similarities between this mode of regulated receptor–target interactions, and that revealed by structural analysis of the autoinhibited EphB2 RTK, a representative of the largest family of mammalian RTKs [119] (Fig. 2). In the inactive state, the juxtamembrane region of EphB2 is ordered, and folds against the kinase domain, primarily making contact with the small lobe. Two juxtamembrane tyrosine residues which are subject to autophosphorylation upon EphB2 activation are inserted into hydrophobic pockets, formed by residues from the juxtamembrane region itself as well as the catalytic domain. In the autoinhibited state, the juxtamembrane region appears to repress kinase activity by creating a kink in the αC-helix of the small lobe of the kinase domain; this movement is transmitted to the ATP-binding site, so that although ATP can bind, the phosphates are not productively positioned for phosphotransfer. In addition, the juxtamembrane region may inhibit the movement of the activation segment of the kinase domain into an active conformation. EphB2 activation leads to autophosphorylation of the juxtamembrane tyrosines, which we predict are therefore expelled from their pockets as a result of electrostatic repulsion and steric clash. We speculate that this leads to the disordering of the juxtamembrane region, freeing the kinase domain to snap into an active state; coincidentally the juxtamembrane phosphotyrosines are exposed for potential interactions with SH2 domain proteins. This model is remarkably similar to that proposed for the activation of the type I TGF β-receptor, and the subsequent interaction of its phosphorylated juxtamembrane region with R-Smad targets [118]. The principal

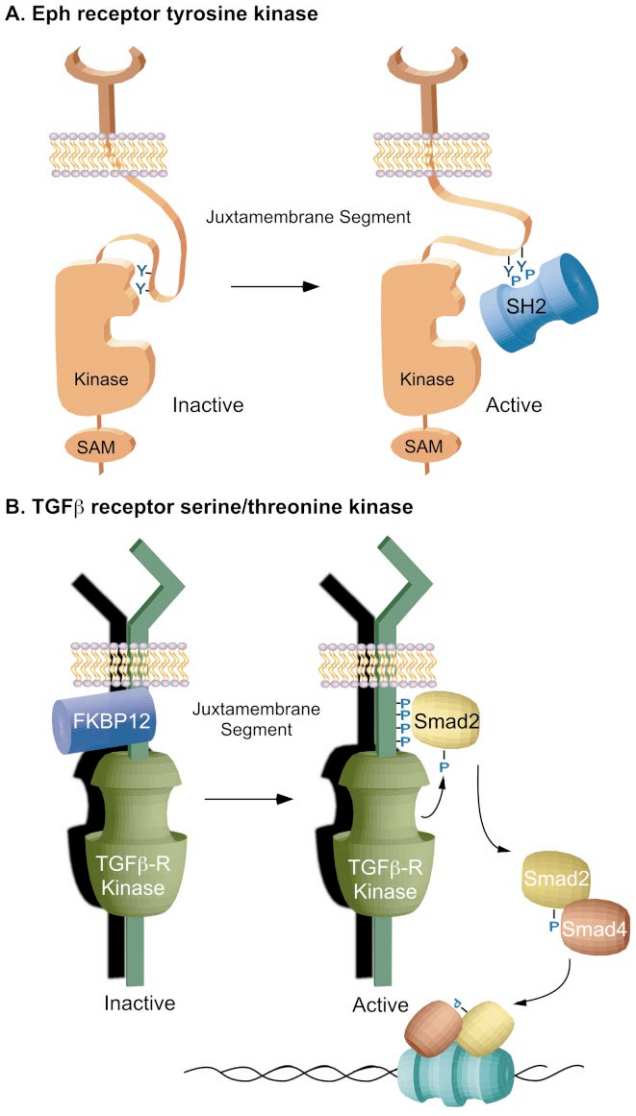

Fig. 2. Regulation of receptors by phosphorylation of juxtamembrane residues. A: The EphB2 receptor tyrosine kinase is held in an inactive conformation by an interaction between the juxtamembrane segment and residues of the small lobe of the kinase. Autophosphorylation of dual tyrosine residues in the juxtamembrane segment results in a structural reorganization that allows ATP to productively bind in the kinase active site and allows the activation segment to adopt a production conformation, resulting in an active kinase. Furthermore, the phosphorylated tyrosine residues can subsequently act as binding sites for SH2 domain-containing proteins. B: In a similar manner, the TGF β-receptor is held in its inactive conformation by FKBP12 binding to the juxtamembrane segment. Autophosphorylation of multiple serine and threonine residues in the juxtamembrane segment releases inhibition. These phosphorylated sites in turn act to recruit Smad2, which is itself phosphorylated, allowing Smad2 to bind to Smad4. The Smad2/Smad4 complex translocates to the nucleus where it acts to promote transcription.

difference involves the observation that the autoinhibited state of the TGF β-receptor involves an accessory protein, FKBP12, which is required for the folding of the juxtamembrane region and occlusion of the ATP-binding site.

7. The use of phospho-dependent protein interactions to regulate complex events in the cell cycle

Recent data have suggested that one important function of both tyrosine and serine/threonine phosphorylation is to create binding sites for E3 protein ubiquitin ligases, proteins (or protein complexes) that mediate transfer of ubiquitin from an E2 enzyme to a specific substrate, which is then recognized by cellular machines involved in proteolysis or endocytosis. c-Cbl, for example, has a variant SH2 domain at its N-terminus, which recognizes sites on activated RTKs, followed by a RING-H2 domain that binds E2 ubiquitin ligases [120–122]. Thus c-Cbl acts in essence as an adaptor to recruit an E2 ubiquitin ligase to an activated RTK, and thereby promote ubiquitination and downregulation of the receptor.

SCF complexes are multi-subunit E3 protein ubiquitin ligases, comprised of a scaffolding protein (Cullin), which interacts with an E2 enzyme, a RING-H2 protein, and an adaptor, Skp1 [123]. Skp1 in turn recognizes the N-terminal F-box of a targeting subunit which binds through a variable C-terminal domain, often composed of WD40 repeats or leucine-rich repeats, to the substrate for ubiquitination [124–126]. The binding of the substrate to the C-terminus of the F-box protein is frequently dependent on the phosphorylation of the target on serine or threonine, and the ubiquitination and subsequent degradation of such a protein is therefore regulated by its serine/threonine phosphorylation [127]. We have recently analyzed the phospho-dependent degradation of the Sic1 cyclin-dependent kinase (CDK) inhibitor in the yeast *Saccharomyces cerevisiae*, using a combination of biochemical and genetic tools [128]. Sic1 is a specific inhibitor of the S phase CDK, composed of the Cdc28 kinase associated with Clb-type cyclins [129]. In the G1 phase of the yeast cell cycle, the G1 CDK (Cdc28 associated with Cln-type cyclins) phosphorylates Sic1 at nine Ser/Thr-Pro motifs, and the phosphorylated form of Sic1 is then recognized by the WD40 repeat domain of an F-box protein termed Cdc4, which is part of a larger SCF E3 ubiquitin ligase complex [124,127,130] (Fig. 3). The recruitment of phospho-Sic1 to the SCF^{Cdc4} complex leads to its ubiquitination and destruction, and this relieves the inhibition of the S phase Cdc28-Clb CDK, allowing the initiation of DNA replication. Thus Sic1 is the key substrate of the G1 CDK, because Sic1 phosphorylation, and consequent recognition by the Cdc4 F-box protein, acts as a switch to promote transition for the G1 to the S phase of the cell cycle. A mutant form of Sic1 that lacks all Cdc28-Cln phosphorylation sites is stable, and therefore toxic because cells cannot replicate their DNA [131].

In trying to understand how Sic1 serine/threonine phosphorylation mediates its recognition by the Cdc4 WD40 domain, we found that no single Sic1 phosphorylation site is sufficient to mediate stable Cdc4 binding, Sic1 ubiquitination, or progression into S phase [128]. Indeed, Sic1 must be phosphorylated on at least six sites to associate with Cdc4 and allow passage through G1. Although the physiological Sic1 phosphorylation sites do not bind Cdc4 with high affinity, we observed that phosphopeptides from human cyclin E1 or

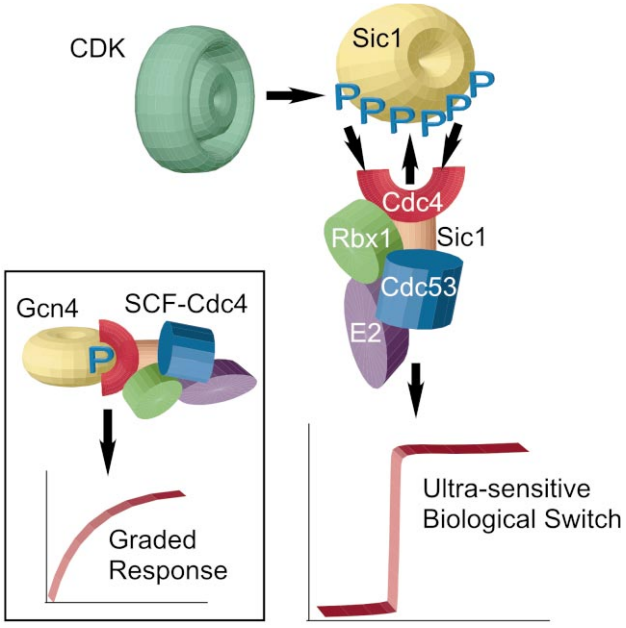

Fig. 3. Multisite phosphorylation of the CDK inhibitor Sic1 creates an ultrasensitive biological switch for the onset of DNA replication. Phosphorylation of Sic1 by the Cln1/2-Cdc28 cyclin-dependent kinase is required for the productive interaction of Sic1 with the WD40 region of the Cdc4 F-box protein. Binding of Sic1 to Cdc4 results in ubiquitination of Sic1 by the SCF^{Cdc4} E3 ubiquitin protein ligase complex and subsequent degradation of Sic1. The requirement for multisite phosphorylation results in a sigmoidal stimulus–response curve, effectively creating an ultrasensitive biological switch. By contrast, the interaction between the transcription factor Gcn4 and Cdc4 is dependent upon a single phosphorylation event resulting in a graded stimulus–response curve that obeys Michaelian kinetics (inset).

yeast Gcn4 (a transcriptional regulator that is also targeted by the SCF^{Cdc4} complex) bound to the Cdc4 WD40 domain with a K_d of $\sim$1 μM, and a Hill coefficient of 1, suggestive of a single class of binding site. Analysis of peptide SPOTS arrays yielded a consensus-binding motif for the Cdc4 WD40 repeats of Ile/Leu-Ile/Leu/Pro-pThr-Pro, with a selection against basic residues at the +2 to +5 positions. These results explain why no single Sic1 phosphorylation site binds well to Cdc4, since these sites are all sub-optimal with respect to the preferred consensus. We found that insertion of the optimal recognition motif into a Sic1 variant lacking all endogenous phosphorylation sites was sufficient for stable binding to Cdc4, and for elimination of the Sic1 CDK inhibitor. However, this variant form of Sic1 was not able to fully replace the endogenous protein, as it was degraded prematurely, leading to precocious entry into S phase, and genome instability likely due to premature firing of DNA replication origins. These and related results have suggested that the Cdc4 F-box protein has a single phosphothreonine-binding pocket, containing three essential arginine residues, which binds in equilibrium to multiple low affinity sites on Sic1. This requirement for multisite phosphorylation of Sic1 establishes a threshold of G1 CDK activity which must be surpassed for stable Cdc4 binding, and thus for Sic1 degradation and activation of the S phase CDK. Thus, Sic1 multisite phosphorylation and consequent Cdc4 recognition in essence provide a timing device for transit through the G1 phase of the cell cycle, and guard against adventitious entry into S phase in response to low level

Cdc28-Cln activity, as well as providing for a switch-like degradation of Sic1 once the appropriate level of G1 CDK activity has been reached (Fig. 3).

These results show that the phospho-dependent recognition of regulatory proteins by the SCF E3 complex is critical for cell cycle progression, and identify a phosphothreonine-binding site in the WD40 repeat domain of the Cdc4 protein. More importantly, they demonstrate that this simple binary interaction can be used to monitor levels of G1 CDK activity, and thus to elicit an ultrasensitive response to Cdc28-Cln kinase activity that ensures an orderly and timely transition into the S phase of the cell cycle.

8. Summary

Interaction domains are used to regulate many aspects of cellular function. We are starting to gain a detailed understanding of how individual interaction domains promote the formation of signaling complexes, and to draw out general rules that can be applied to many types of protein–protein and protein–phospholipid interactions [21]. A challenge for the future is to understand how interaction domains cooperate to establish the complex signaling networks that control events as fundamental and as complex as passage through the cell cycle, or organization of the neuronal or immunological synapse.

References

[1] Pawson, T. (1995) Nature 373, 573–580.
[2] Pawson, T. and Scott, J.D. (1997) Science 278, 2075–2080.
[3] Kuriyan, J. and Cowburn, D. (1997) Annu. Rev. Biophys. Biomol. Struct. 26, 259–288.
[4] Sadowski, I., Stone, J.C. and Pawson, T. (1986) Mol. Cell Biol. 6, 4396–4408.
[5] Anderson, D., Koch, C.A., Grey, L., Ellis, C., Moran, M.F. and Pawson, T. (1990) Science 250, 979–982.
[6] Matsuda, M., Mayer, B.J., Fukui, Y. and Hanafusa, H. (1990) Science 248, 1537–1539.
[7] Songyang, Z., Shoelson, S.E., Chadhuri, M., Gish, G., Pawson, T., King, F., Roberts, T., Ratnofsky, S., Schaffhausen, B. and Cantley, L.C. (1993) Cell 72, 767–778.
[8] Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V. and Margolis, N. (1994) J. Biol. Chem. 269, 32031–32034.
[9] Kavanaugh, W.M. and Williams, L.T. (1994) Science 266, 1862–1865.
[10] van der Geer, P., Wiley, S., Lai, V.K.M., Olivier, J.P., Gish, G.D., Stephens, T., Kaplan, D., Shoelson, S. and Pawson, T. (1995) Curr. Biol. 5, 404–412.
[11] Yaffe, M.B. and Elia, A. (2001) Curr. Opin. Cell Biol. 13, 131–138.
[12] Owen, D.J., Ornaghi, P., Yang, J.C., Lowe, N., Evans, P.R., Ballario, P., Neuhaus, D., Filetici, P. and Travers, A.A. (2000) EMBO J. 19, 6141–6149.
[13] Marmorstein, E. (2001) Nat. Rev. Mol. Cell. Biol. 2, 422–432.
[14] Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C. and Kouzarides, T. (2001) Nature 410, 120–124.
[15] Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin Jr., W.G. (2001) Science 292, 464–468.
[16] Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, Av., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001) Science 292, 468–472.
[17] Ren, R., Mayer, B.J., Cicchetti, P. and Baltimore, D. (1993) Science 259, 1157–1161.
[18] Chen, H.I. and Sudol, M. (1995) Proc. Natl. Acad. Sci. USA 92, 7819–7823.
[19] Zarrinpar, A. and Lim, W.A. (2000) Nat. Struct. Biol. 7, 611–613.
[20] Nguyen, J.T., Turck, C.W., Cohen, F.E., Zuckermann, R.N. and Lim, W. (1998) Science 282, 2088–2092.
[21] Sudol, M. (1998) Oncogene 17, 1469–1474.
[22] Doyle, D.A., Lee, A., Lewis, J., Kim, E., Sheng, M. and MacKinnon, R. (1996) Cell 85, 1067–1076.
[23] Kim, E., Niethammer, M., Rothschild, A., Jan, Y.N. and Sheng, S. (1995) Nature 378, 85–88.
[24] Kornau, H.-C., Schenker, L.T., Kennedy, M.B. and Seeburg, P.H. (1995) Science 269, 1737–1740.
[25] Sheng, M. and Sala, C. (2001) Annu. Rev. Neurosci. 24, 1–29.
[26] Cullen, P.J., Cozier, G.E., Banting, G. and Mellor, H. (2001) Curr. Biol. 11, R882–R893.
[27] Santagata, S., Boggon, T.J., Baird, C.L., Gomez, C.A., Zhao, J., Shan, W.S., Myszka, D.G. and Shapiro, L. (2001) Science 292, 2041–2050.
[28] Hurley, J.H. and Meyer, T. (2001) Curr. Opin. Cell Biol. 13, 146–152.
[29] Hyvonen, M. and Saraste, M. (1997) EMBO J. 16, 3396–3404.
[30] Stauffer, T.P., Ahn, S. and Meyer, T. (1998) Curr. Biol. 8, 343–346.
[31] Salim, K., Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Scaife, R., Margolis, R.L., Gigg, R., Smith, C.I.E., Driscoll, P.C., Waterfield, M.D. and Panayotou, G. (1996) EMBO J. 15, 6241–6250.
[32] Klarlund, J.K., Tsiaras, W., Holik, J.J., Chawla, A. and Czech, M.P. (2000) J. Biol. Chem. 275, 32816–32821.
[33] Lemmon, M.A., Ferguson, K.M., O'Brien, R., Sigler, P.B. and Schlessinger, J. (1995) Proc. Natl. Acad. Sci. USA 92, 10472–10476.
[34] Musacchio, A., Gibson, T., Price, P., Thompson, J. and Saraste, M. (1993) Trends Biochem. 18, 343–348.
[35] Gilloly, D.J., Simonsen, A. and Stenmark, H. (2001) Biochem. J. 355, 249–258.
[36] Stenmark, H., Aasland, R., Toh, B.H. and D'Arrigo, A. (1996) J.Biol. Chem. 271, 24048–24054.
[37] Stenmark, H., Aasland, R., Toh, B.H. and D'Arrigo, A. (1996) J. Biol. Chem. 271, 24048–24054.
[38] Burd, C.G. and Emr, S.D. (1998) Mol. Cell 2, 157–162.
[39] Groves, M.R. and Barford, D. (1999) Curr. Opin. Biol. 9, 383–389.
[40] Groves, M.R., Hanlon, N., Turowski, P., Hemmings, B.A. and Barford, D. (1999) Cell 96, 99–110.
[41] Andrade, M.A., Perez-Iratxeta, C. and Ponting, C.P. (2001) J. Struct. Biol. 134, 117–131.
[42] Schulman, B.A., Carrano, A.C., Jeffrey, P.D., Bowen, Z., Kinnucan, E.R., Finnin, M.S., Elledge, S.J., Harper, J.W., Pagano, M. and Pavletich, N.P. (2000) Nature 408, 381–386.
[43] Kaufman, S.H. and Hengartner, M.D. (2001) Trends Cell Biol. 11, 526–534.
[44] Vaughn, D.E., Rodriguez, J., Lazebnik, Y. and Joshua-Tor, L. (1999) Mol. Biol. 293, 439–447.
[45] Fesik, S.W. (2000) Cell 103, 273–282.
[46] Stapleton, D., Balan, I., Pawson, T. and Sicheri, F. (1999) Nat. Struct. Biol. 6, 44–49.
[47] Thanos, C.D., Goodwill, K.E. and Bowie, J.U. (1999) Science 283, 833–836.
[48] Kim, C.A., Phillips, M.L., Kim, W., Gingery, M., Tran, H.H., Robinson, M.A., Faham, S. and Bowie, J.U. (2001) EMBO J. 20, 4173–4182.
[49] Hillier, B.J., Christopherson, K.S., Prehoda, K.E., Bredt, D.S. and Lim, W.A. (1999) Science 284, 812–815.
[50] Songyang, Z., Fanning, A.S., Fu, C., Xu, J., Marfatia, S.M., Chishti, A.H., Crompton, A., Chan, A.C., Anderson, J.M. and Cantley, L.C. (1997) Science 275, 73–77.
[51] Hunter, T. (2000) Cell 100, 113–127.
[52] Schlessinger, J. (2000) Cell 103, 211–225.
[53] Heldin, C.-H., Ostman, A. and Ronnstrand, L. (1998) Biochim. Biophys. Acta 1378, F79–F113.
[54] Piccione, E., Case, R.D., Domchek, S.M., Hu, P., Chaudhuri, M., Backer, J.M., Schlessinger, J. and Shoelson, S.E. (1993) Biochemistry 32, 3197–3202.
[55] Bradshaw, J.M., Mitaxov, V. and Waksman, G. (1999) J. Mol. Biol. 293, 971–985.

[56] Reedijk, M., Liu, X., van der Geer, P., Letwin, K., Waterfield, M.D., Hunter, T. and Pawson, T. (1992) EMBO J. 11, 1365–1372.
[57] Waksman, G., Shoelson, S., Pant, N., Cowburn, D. and Kuriyan, J. (1993) Cell 72, 779–790.
[58] Eck, M.I., Shoelson, S.E. and Harrison, S.C. (1993) Nature 362, 87–91.
[59] Pascal, S.M., Singer, A.U., Gish, G., Yamazaki, T., Shoelson, S.E., Pawson, T., Kay, L.E. and Forman-Kay, J.D. (1994) Cell 77, 461–472.
[60] Kay, L.E., Muhandiram, D.R., Wolf, G., Shoelson, S.E. and Forman-Kay, J.D. (1998) Nat. Struct. Biol. 5, 156–163.
[61] Maina, F., Pante, G., Helmbacher, F., Andres, R., Porthin, A., Davies, A.M., Ponzetto, C. and Klein, R. (2001) Mol. Cell 7, 1293–1306.
[62] Lesa, G.M. and Sternberg, P.W. (1997) Mol. Biol. Cell 8, 779–793.
[63] Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G. and Comoglio, P.M. (1994) Cell 77, 261–271.
[64] Ladbury, J.E., Lemmon, M.A., Zhou, M., Green, J., Botfield, M.C. and Schlessinger, J. (1995) Proc. Natl. Acad. Sci. USA 92, 3199–3203.
[65] Mayer, B.J. (2001) J. Cell Sci. 114, 1253–1263.
[66] Ottinger, E.A., Botfield, M.C. and Sholeson, S.E. (1998) J. Biol. Chem. 273, 729–735.
[67] Kanner, S.B., Reynolds, A.B., Wang, H.-C.R., Vines, R.R. and Parsons, J.T. (1991) EMBO J. 10, 1689–1698.
[68] Nakamoto, T., Sakai, R., Ozawa, K., Yazaki, Y. and Hirai, H. (1996) J. Biol. Chem. 271, 8959–8965.
[69] Pellicena, P. and Miller, W.T. (2001) J. Biol. Chem. 276, 28190–28196.
[70] Moniakis, J., Funamoto, S., Fukuzawa, M., Meisenhelder, J., Araki, T., Abe, T., Meili, R., Hunter, T., Williams, J. and Firtel, R.A. (2001) Genes Dev. 15, 687–698.
[71] Kawata, T., Shevchenko, A., Fukuzawa, M., Jermyn, K.A., Totty, N.F., Zhukovskaya, N.V., Sterling, A.E., Mann, M. and Williams, J.G. (1997) Cell 89, 909–916.
[72] Wagner, A. (2001) Mol. Biol. Evol. 18, 1283–1292.
[73] Feller, S.M. (2001) Oncogene 20, 6348–6371.
[74] Li, W., Fan, J. and Woodley, D.T. (2001) Oncogene 20, 6403–6407.
[75] Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993) Nature 363, 83–85.
[76] Li, N., Batzer, A., Daly, R., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B. and Schlessinger, J. (1993) Nature 363, 85–88.
[77] Gu, H., Maeda, H., Moon, J.J., Lord, J.D., Yoakim, M., Nelson, B.H. and Neel, B.G. (2000) Mol. Cell. Biol. 20, 7109–7120.
[78] Lock, L.S., Royal, I., Naujokas, M.A. and Park, M. (2000) J. Biol. Chem. 275, 31536–31545.
[79] Schaeper, U.G.N.H., Fuchs, K.P., Sachs, M., Kempkes, B. and Birchmeier, W. (2000) J. Cell Biol. 149, 1419–1432.
[80] Ong, S.H., Hadari, Y.R., Gotoh, N., Guy, G.R., Schlessinger, J. and Lax, I. (2001) Proc. Natl. Acad. Sci. USA 98, 6074–6079.
[81] Ong, S.H., Dilworth, S., Hauck-Schmalenberger, I., Pawson, T. and Kiefer, F. (2001) EMBO J. 20, 6327–6336.
[82] Rohatgi, R., Nollau, P., Ho, H.Y., Kirschner, M.W. and Mayer, B.J. (2001) J. Biol. Chem. 276, 26448–26452.
[83] Moreau, V., Frischknecht, F., Reckmann, I., Vincentelli, R., Rabut, G., Stewart, D. and Way, M. (2000) Nat. Cell Biol. 2, 441–448.
[84] Hing, H., Xiao, J., Harden, N., Lim, L. and Zipursky, S.L. (1999) Cell 97, 853–863.
[85] Gruenheid, S., DeVinney, R., Bladt, F., Goosney, D., Gelkop, S., Gish, G.D., Pawson, T. and Finlay, B.B. (2001) Nat. Cell Biol. 3, 856–859.
[86] Cheng, A.M., Saxton, T.M., Sakai, R., Mbamalu, G., Vogel, W., Tortorice, C., Cardiff, R.D., Cross, J.C., Muller, W.J. and Pawson, T. (1998) Cell 95, 793–803.
[87] Saxton, T.M., Cheng, A.M., Ong, S.H., Lu, Y., Sakai, R., Cross, J.C. and Pawson, T. (2001) Curr. Biol. 11, 662–670.
[88] Gong, Q., Cheng, A.M., Akk, A.M., Alberola-Ila, J., Gong, G., Pawson, T. and Chan, A.C. (2001) Nat. Immunol. 2, 29–36.
[89] Sayos, J., Wu, C., Morra, M., Wang, N., Zhang, X., Allen, D., van Schaik, S., Notarangelo, L., Geha, R., Roncarolo, M.G., Oettgen, H., De Vries, J.E., Aversa, G. and Terhorst, C. (1998) Nature 395, 462–469.
[90] Coffey, A.J., Brooksbank, R.A. and Brandau, O. et al. (1998) Nat. Genet. 20, 129–135.
[91] Nichols, K.E., Harlin, D.P., Levitz, S., Krainer, M., Kolquist, K.A., Genovese, C., Bernard, A., Ferguson, M., Zuo, L., Snyder, E., Buckler, A.J., Wise, C., Ashley, J., Lovett, M., Valentine, M.B., Look, A.T., Gerald, W., Housman, D.E. and Haber, D.A. (1998) Proc. Natl. Acad. Sci. USA 95, 13765–13770.
[92] Poy, F., Yaffe, M.B., Sayos, J., Saxena, K., Morra, M., Sumegi, J., Cantley, L.C., Terhorst, C. and Eck, M.J. (1999) Mol. Cell 4, 555–561.
[93] Li, S.-C., Gish, G., Yang, D., Coffey, A.J., Forman-Kay, J.D., Ernberg, I., Kay, L.E. and Pawson, T. (1999) Curr. Biol. 9, 1355–1362.
[94] Anafi, M., Rosen, M.K., Gish, G.D., Kay, L.E. and Pawson, T. (1996) J. Biol. Chem. 271, 21365–21374.
[95] Zhou, M.M., Ravichandran, K.S., Olejniczak, E.F., Petros, A.M., Meadows, R.P., Sattler, M., Harlan, J.E., Wade, W.S., Burakoff, S.J. and Fesik, S.W. (1995) Nature 378, 584–592.
[96] van der Geer, P., Wiley, S., Gish, G.D., Lai, V.K., Stephens, R., White, M.F., Kaplan, D. and Pawson, T. (1996) Proc. Natl. Acad. Sci. USA 93, 963–968.
[97] Trub, T., Choi, W.E., Wolf, G., Ottinger, E., Chen, Y., Weiss, M. and Sholeson, S.E. (1995) J. Biol. Chem. 270, 18205–18208.
[98] Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Pelicci, P.G., Schlessinger, J. and Pawson, T. (1992) Nature 360, 689–692.
[99] Kouhara, H., Hadari, Y.R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I. and Schlessinger, J. (1997) Cell 89, 693–702.
[100] Backer, J.M., Myers, M.G., Shoelson, S.E., Chin, D.J., Sun, X.-J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E.Y., Schlessinger, J. and White, M.F. (1992) EMBO J. 11, 3469–3479.
[101] Dhe-Paganon, S., Ottinger, E.A., Nolte, R.T., Eck, M.J. and Shoelson, S.E. (1999) Proc. Natl. Acad. Sci. USA 96, 8378–8383.
[102] Ravichandran, K.S., Zhou, M.M., Pratt, J.C., Harlan, J.E., Walk, S.F., Fesik, S.W. and Burakoff, S.J. (1997) Mol. Cell. Biol. 17, 5540–5549.
[103] Ong, S.H., Guy, G.R., Hadari, Y.R., Laks, S., Gotoh, N., Schlessinger, J. and Lax, I. (2000) Mol. Cell. Biol. 20, 979–989.
[104] Dhalluin, C., Yan, K., Plotnikova, O., Lee, K.W., Zhen, L., Kuti, M., Mujtaba, S., Goldfarb, M.P. and Zhou, M.M. (2000) Mol. Cell 6, 921–929.
[105] Borg, J.P., Ooi, J., Levy, E. and Margolis, B. (1998) Mol. Cell. Biol. 16, 6229–6241.
[106] Zhang, Z., Lee, C.H., Mandiyan, V., Borg, J.P., Margolis, B., Schlesinger, J. and Kuriyan, J. (1997) EMBO J. 16, 6141–6150.
[107] Chien, C.T., Wang, S., Rothenberg, M., Jan, L.Y. and Jan, Y.N. (1998) Mol. Cell Biol. 18, 598–607.
[108] Zwahlen, C., Li, S.-C., Kay, L.E., Pawson, T. and Forman-Kay, J.D. (2000) EMBO J. 19, 1505–1515.
[109] Blomberg, N., Baraldi, E., Nilges, M. and Saraste, M. (1999) Trends Biochem. Sci. 24, 441–445.
[110] Forman-Kay, J.D. and Pawson, T. (1999) Curr. Opin. Struct. Biol. 9, 690–695.
[111] Prehoda, K.E., Lee, D.J. and Lim, W.A. (1999) Cell 97, 471–480.
[112] Pearson, M.A., Reczek, D., Bretscher, A. and Karplus, P.A. (2000) Cell 101, 259–270.
[113] Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Cell 84, 889–897.
[114] Tzivion, G., Shen, Y.H. and Zhu, J. (2001) Oncogene 20, 6331–6338.
[115] Durocher, D., Henckel, J., Fersht, A.R. and Jackson, S.P. (1999) Mol. Cell 4, 387–394.
[116] Durocher, D., Taylor, I.A., Sarbassova, D., Haire, L.F., Westcott, S.L., Jackson, S.P., Smerdon, S.J. and Yaffe, M.B. (2000) Mol. Cell 6, 1169–1182.
[117] Wu, G., Chen, Y.G., Ozdamar, B., Gyuricza, C.A., Chong, P.A., Wrana, J.L., Massague, J. and Shi, Y. (2000) Science 287, 92–97.
[118] Huse, M., Muir, T.W., Xu, L., Chen, Y.G., Kuriyan, J. and Massague, J. (2001) Mol. Cell 8, 671–682.

[119] Wybenga-Groot, L., Baskin, B., Ong,.S.H., Tong, J., Pawson, T. and Sicheri, F. (2001) Cell 106, 745–757.
[120] Meng, W., Sawasdikosol, S., Burakoff, S.J. and Eck, M.J. (1999) Nature 398, 84–90.
[121] Joazerio, C.A., Wing, S.S., Huang, H., Leverson, J.D., Hunter, T. and Liu, Y.C. (1999) Science 286, 309–312.
[122] Waterman, H., Levkowitz, G., Alroy, I. and Yarden, Y. (1999) J. Biol. Chem. 274, 22151–22154.
[123] Tyers, M. and Jorgensen, P. (2000) Curr. Opin. Genet. Dev. 10, 54–64.
[124] Bai, A., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W. and Elledge, S.J. (1996) Cell 86, 263–274.
[125] Winstop, J.T., Koepp, D.M., Zhu, C., Elledge, S.J. and Harper, J.W. (1999) Curr. Biol. 9, 1180–1182.
[126] Cenciarelli, C., Chiaur, D.S., Guardavaccaro, D., Parks, W., Vidal, M. and Pagano, M. (1999) Curr. Biol. 9, 1177–1179.
[127] Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J. and Harper, J.W. (1997) Cell 91, 209–219.
[128] Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F., Mendenhall, M.D., Sicheri, F., Pawson, T. and Tyers, M. (2001) Nature 414, 516–523.
[129] Schwob, E., Bohm, T., Mendenhall, M.D. and Nasmyth, K. (1994) Cell 79, 233–244.
[130] Feldman, R.M., Correll, C.C., Kaplan, K.B. and Deshaies, R.J. (1997) Cell 91, 221–230.
[131] Verma, R., Annan, R.S., Huddleston, M.J., Carr, S.A., Reynard, G. and Deshaies, R.J. (1997) Science 278, 455–460.