Much more than the sum of the parts: structures of the dual SH2 domains of ZAP-70 and Syp

Proteins involved in signaling pathways frequently contain one or more SH2 domains. New structural information on proteins that carry two SH2 domains show, surprisingly, that the domains are closely interlinked, so the binding sites are rigidly oriented with respect to each other. Thus, only ligands with the right spacing of the phosphotyrosines will be tightly bound.

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One of the major challenges in the investigation of intracellular signal transduction is unraveling the interactions of the multiple targeting domains in pathways. In signaling involving phosphotyrosine, the kinases (adding phosphate from ATP to the tyrosine oxygen), and phosphatases (removing the phosphate from phosphotyrosine) contain other domains in addition to the catalytic one. The four kinds of additional domains now generally recognized, Src homology 2 and 3 (SH2, SH3), the 'alternate' phosphotyrosine binding domain (PTB), and the pleckstrin homology domain (PH) [1,2], can frequently be expressed as individual domains that have similar properties to those in the full length protein. Many of the enzymes and adaptor proteins (proteins that lack specific enzymatic activity) involved in signal transduction also have sites for phosphorylation that presumably bind inter- or intramolecularly to SH2 and/or PTB domains, and proline-rich segments that similarly bind to SH3 domains. This rich complexity rapidly leads to a problem of identifying physiologically significant interactions, especially since the variability in binding affinities among the individual domain types are relatively modest, of two to three orders of magnitude [3]. One way of resolving this problem of modest specificity, and defining what really interacts with what, is to use the combined specificities of more than one of the domains to achieve significantly greater specificity, expanding a lock-and-key recognition system to two (or more) locks and keys.

Two recently published structures of dual SH2 domains elegantly illustrate this point, and show some new features of such interactions that had not previously been clearly described, specifically the topological orientation of the domains, and the role of 'tied', or independent ligands. One structure is of the dual SH2 domains of ZAP-70 (zeta-associated protein of 70 kDa) kinase in a complex with a doubly-phosphorylated peptide, the immunoreceptor tyrosine-mediated activation motif (ITAM), derived from the zeta (ζ) subunit of the T-cell receptor [4]. The ZAP-70 kinase binds to the ITAMs of the T-cell receptor after they have been phosphorylated by a member of the Src family of tyrosine kinases, and its activity is required for T-cell activation [5]. The other structure is of the dual SH2 domains from the phosphatase SH-PTP2 (also known as Syp, PTP2C and PTP1D), with the two SH2 domains occupied by an 11-residue phosphopeptide derived from the platelet-derived growth factor receptor (PDGFR) [6]. The observed independence of SH2 and SH3 domains in Lck [7], Grb2 [8], and Abl [9] suggested that multiple SH2 domains in one protein might be relatively independent. The two new structures show, however, that these dual SH2 domains are in fact tightly interrelated.

ZAP-70 dual SH2 domains

In the ZAP-70 structure ([4]; Fig. 1), the carboxy-terminal SH2 domain is readily recognizable, having the features expected for a single SH2 domain — a well-defined deep binding pocket for the phosphotyrosyl side chain, a secondary 'plug' site for specific hydrophobic interactions with the ligand, carboxy-terminal to the phosphotyrosine, and a compact fold. The carboxy- and amino-terminal SH2 domains are linked by a long (65 amino acid) 'interdomain', which is highly structured, forming a coiled coil. The amino and carboxyl termini of the interdomain are close, bringing the amino- and carboxy-terminal SH2 domains into contact. The amino-terminal SH2 domain differs significantly from the expected structure for an SH2 domain, however. Most surprisingly, the normally well defined charge and hydrogen bond arrangement around the phosphotyrosine that binds to the amino-terminal SH2 is significantly different from that seen before. As usual, the phosphotyrosyl side chain is well buried in the protein, but its contacts are provided by both amino- and carboxy-terminal SH2 domains. The relevant residues from the carboxy-terminal SH2 domain are from the carboxy-terminal helix (αB; [7]), which has not previously been implicated in phosphotyrosine binding. Curiously, this well-defined interface may not exist in the absence of the ITAM ligand, since, prior to the addition of ligand, the uncomplexed dual SH2 domains of ZAP-70 apparently exist in multiple conformations based on isoelectric focusing [4]. Part of the entropic cost for this alignment on binding may come from the binding of the 'intermotif' of the ligand (NLGRREE; Fig. 1), which forms a nearly complete α-helical turn and makes multiple hydrogen bonds and charge-mediated contacts to the protein, in a fashion not previously observed in SH2 structures. The thermodynamic contribution of this interaction remains to be identified.

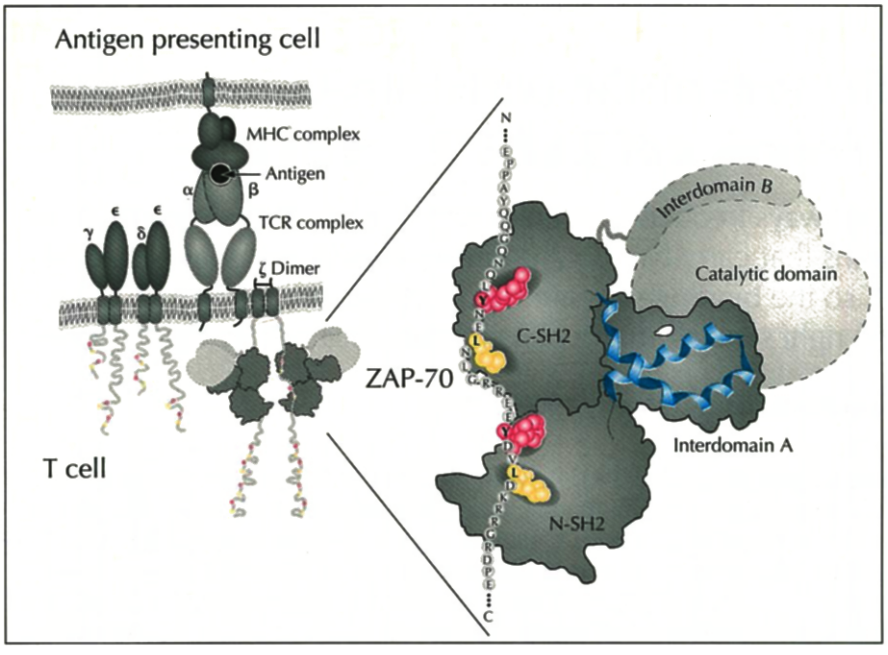

Fig. 1. Cartoon of ZAP-70 bound to the phosphorylated ζ_1 subunit of the activated T-cell receptor. Left, the T-cell receptor binds to a complex of antigen and the major histocompatibility complex (MHC) present on an antigen presenting cell. This association stimulates phosphorylation of the T-cell receptor on tyrosine residues (red) within the ITAMs (immunoreceptor tyrosine-mediated activation motif). The SH2 domains of ZAP-70 bind to the phosphorylated ITAMs. Right, a schematic of the doubly-phosphorylated ζ_1 ITAM bound to ZAP-70, based on the observed crystal structure. The structure of the catalytic domain and interdomain B of ZAP-70 have not yet been determined; these domains are shown in a lighter grey. The phosphotyrosine (red) and two leucine residues (yellow) are the principal specificity determinants. Reprinted with permission from [4].

The ZAP-70 SH2 domains give a clear indication that SH2 domain–ligand interactions can be very different from those seen in the examples previously known [10], and that some interactions are modulated by neighboring domains. Recognition of the ITAM ligand apparently requires two co-linear phosphotyrosine sites on the ligand (orientation I, Fig. 2) with a spacing that is complemented by the fixed, short separation of the binding sites in the complex.

SH-PTP2

The similarity of the structure of the dual SH2 domains of SH-PTP2 [6] to those previously determined for single SH2 domains can be summed up by the observation that the dual domain structure could be solved using molecular replacement with the previously determined single SH2 domain [11]. The two domains superimpose (with ligand bound) to a root-mean-square deviation value of 0.56 Å. There are small systematic differences between the two domains, in particular a rotation of one of the phosphates around the C–O bond of the phosphotyrosine, leading to a different network of hydrogen bonds to the phosphate. Otherwise the ligand interactions are very similar to the single domain complex [11]. Of particular note is the nature of the interface, and the constraints that this then imposes on the orientation of the binding sites. The crystal structure contains an intramolecular cystine link, even though the crystals were grown in solutions containing 10 mM dithiothreitol, pH 8.5. The authors note that cystine bridges are uncommon in cytoplasmic proteins. The view that this bridge would exist normally in the intact mammalian protein (the protein was expressed in *Escherichia coli*) is most strongly supported by the tight packing of residues around it in the interdomain interface, and the mean temperature factor of this region, which indicates that it is as well ordered as the rest of the protein.

Many of the phosphotyrosine phosphatases contain SH2 domains, and it is has often been suggested that the interaction between the SH2 domains and phosphotyrosines on the ligand may protect those residues leading to a selective pattern of remaining phosphotyrosines while other sites are dephosphorylated. The occupancy of the SH2 domains also apparently increases the enzymatic activity of the phosphatase. In the case of SH-PTP2, the inhibitory effects of the SH2 domains are not mediated by intramolecular binding to phosphorylation sites on SH-PTP2 itself, as protein from *E. coli*, which is presumably not post-translationally modified, still shows the inhibition effect [12,13]. The view of the dual domains of SH-PTP2 does not offer any obvious clues as to how activation (disinhibition) might proceed, although the tight interaction between the dual SH2 domains raises the possibility that they might move as a single unit.

The two SH2 sites for phosphopeptides in SH-PTP2 [6] are occupied in the structure by two molecules of the same ligand (Fig. 3). It is observed [6] that in synthetic ligands with two phosphotyrosyl motifs, the topology and the separation of the two motifs critically affects the level of activation of SH-PTP2 enzymatic activity. The maximally effective activator has an expected distance, for an extended chain, of about 41 Å between the phosphotyrosines, closely matching the crystallographically determined distance between the two phosphotyrosines (of individual ligands) in the structure of the dual SH2 domains.

As with ZAP-70, the binding sites are on one face of the protein. In contrast to the colinear ensemble (Fig. 2, orientation I) of ZAP-70, the sites in SH-PTP2 are more nearly antiparallel to each other (Fig. 2, orientation VI).

Figure 2 compares the binding orientations observed or expected for domains facing in the same direction.

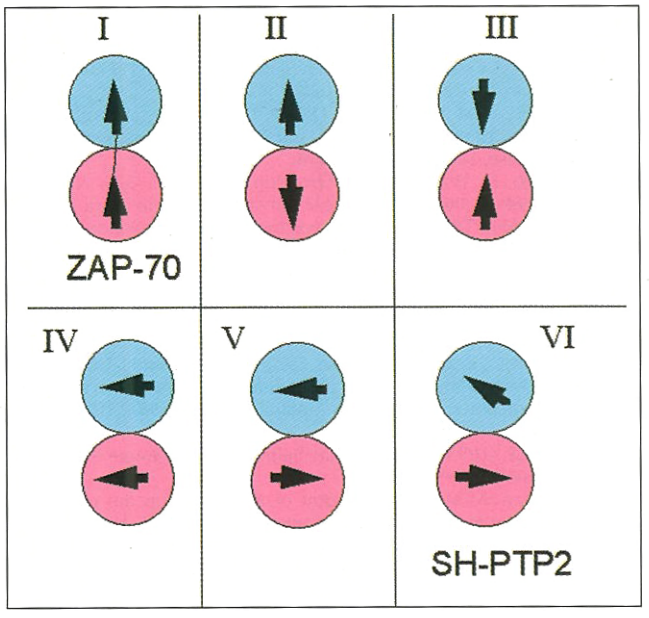

Fig. 2. Possible orientations of ligands bound to dual SH2 domains. I: Colinear orientation, with a bis-epitopic ligand occupying both sites and an inter-ligand linker between them, as seen in the ZAP-70 structure [4]. II, III: Two other colinear orientations. IV: Parallel orientation. V: antiparallel orientation. VI: approximate orientation in SH-PTP2 [6].

Orientation I is superficially the simplest for dual domains binding to a ligand with two epitopes, in that the linker between the two binding sites of the ligand is shorter than any other. The shorter the interdomain linker, the lower the likelihood that two domains from separate molecules might recognize the signal ligand. Of course, things are rarely so simple, as evidenced by the varied grouping of phosphotyrosines in other proteins, and the helical inter-ligand linker observed in ZAP-70.

When recognizing ligands that are close together but not colinear several other arrangements are possible. Ligands may be clustered by, for example, their presence in the carboxyl-termini of dimerized growth factor receptors. In this case, the C_2 symmetry of the dual ligand might be reflected in the pseudo-C_2 symmetry of orientations II, III, V and VI of Figure 2. Of these, orientations II and III would require fairly close contact between the ends of the ligands. Orientation V, similar to that seen for SH-PTP2 (VI) requires the least ligand–ligand contacts.

Can we rationalize why both ZAP-70 and SH-PTP2 have both SH2 binding sites on one side of the respective proteins? One clue may be that interest in the specific recognition sites has distracted attention from the likelihood that intracellular signaling complexes involve very many protein–protein (and possibly other) interactions, and therefore all the other faces of the dual domains may be occupied. Such 'large complex' models would generally require that coordinated interaction of dual domains use a common face.

Therapeutic directions

ZAP-70 is a target for intervention to block pathologic T-cell activation in autoimmune disease, or to immunosuppress generally. Phosphatase-resistant ITAM analogs can block ZAP-70 association with the activated receptor in permeabilized cells [14] (and reported in [15]). While it may be difficult to find cell-permeable phosphotyrosine analogs, the ZAP-70 structure may permit design of other peptidomimetics by mimicking the ITAM binding augmented by other contacts, such as those of the 'intermotif'. Alternatively, the unusual amino- and carboxy-terminal SH2 interface might be blocked to prevent the formation of the second phosphotyrosine binding pocket.

SH-PTP2 is not itself a current target for therapeutic intervention, but it serves as an important model for phosphatases, which are likely to be. For example, there is increased interest [16] in gene products whose expression is necessary, but not sufficient, for malignant transformation. Phosphatases are part of the critical series of 'on-off' switches controlling progression through the cell cycle, and phosphatase inhibitors are effective inducers at the mitotic entry checkpoint [17]. Effectors based on dual occupancy of the SH2 domains of the

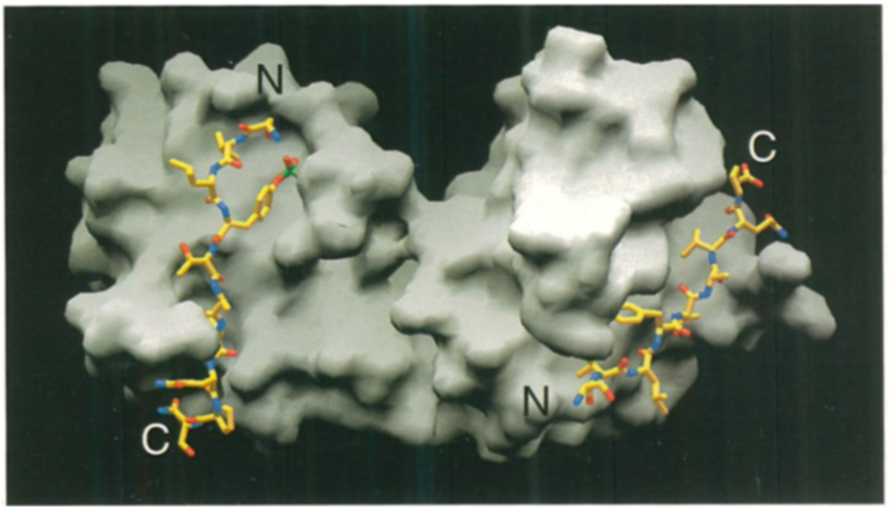

Fig. 3. GRASP [18] surface of the tandem SH2 domains of SH-PTP2 with bound peptides shown in stick representation. The phosphopeptides are widely spaced and in a roughly antiparallel orientation; the two domains have a substantial interface between them. Reprinted with permission from [6].

dual SH2 phosphatases may be significant reagents for investigational and therapeutic research in these areas.

In conclusion, these two structures and their comparison significantly extend our views of how SH2 domains are involved in signaling processes and what the role of multivalent ligands may be.

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