Tandem SH2 Domains Confer High Specificity in Tyrosine Kinase Signaling

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SH2 domain proteins transmit intracellular signals initiated by activated tyrosine kinase-linked receptors. Recent three-dimensional structures suggest mechanisms by which tandem SH2 domains might confer higher specificity than individual SH2 domains. To test this, binding studies were conducted with tandem domains from the five signaling enzymes: phosphatidylinositol 3-kinase p85, ZAP-70, Syk, SHP-2, and phospholipase C-γ1. Bisphosphorylated TAMs (tyrosine-based activation motifs) were derived from biologically relevant sites in platelet-derived growth factor, T cell, B cell, and high affinity IgE receptors and the receptor subunits IRS-1 (insulin receptor substrate-1) and SHPS-1/SIRP. Each tandem SH2 domain binds a distinct TAM corresponding to its appropriate biological partner with highest affinity (0.5–3.0 ns). Alternative TAMs bind the tandem SH2 domains with 1,000- to >10,000-fold lower affinity than biologically relevant TAMs. This level of specificity is significantly greater than the 20–50-fold typically seen for individual SH2 domains. We conclude that high biological specificity is conferred by the simultaneous interaction of two SH2 domains in a signaling enzyme with bisphosphorylated TAMs in activated receptors and substrates.

SH2 domain proteins transmit intracellular signals initiated by activated tyrosine kinase receptors (1). The SH2 domains bind phosphorylated receptor tyrosines, and, since many SH2 domain proteins also contain or associate with catalytic subunits, these interactions recruit the effector enzymes to activated receptors. Tyrosine kinase signaling pathways thus gain specificity from the intrinsic binding preferences of SH2 domains for short sequences that flank phosphorytrosine.

A great deal has been learned by studying isolated, individual SH2 domains. Common mechanisms are used for phosphorytrosine recognition (2–12). Most notably, SH2 domain residues ArgoA2 and Argβ5 chelate the phosphorytrosine phosphate. The latter is within the conserved FLVRES sequence. Binding site selectivity is conferred by interactions between two variable loops within SH2 domains (EF and BG) and peptide residues COOH-terminal to phosphorytrosine. The degree of selectivity varies, but phosphopeptides derived from biologically relevant sites typically bind with 20–50-fold higher affinity than irrelevant or randomized sequences (e.g. Refs. 13–18). Although it is true that a small subset of SH2 domains shows greater selectivity (e.g. 1,000-fold for Grb2), these are exceptions and not the rule.

Nevertheless, biological specificity in intact cells is substantially greater than 50-fold, suggesting that more is involved than individual SH2 domain interactions. In fact, all SH2 domain proteins contain additional binding modules (e.g. SH2, SH3, PTB, and PH domains) or motifs. A fundamental concept may have been overlooked by the common tendency to evaluate specificity using isolated, individual domains. Simultaneous binding to multiple domains could enhance specificity through combinatorial effects, as 1) each independent domain has intrinsic linear binding specificity; 2) relative orientations between binding sites may be limited by structural constraints; and 3) the ligands for all domains must be present in the same cellular locations and properly oriented for multisite binding.

As an example, bisphosphorylated tyrosine-based activation motif (TAM)2 peptides activate the SH2 domain enzymes, phosphatidylinositol (PI) 3-kinase, SHP-1, SHP-2, and Syk, more potently than monophosphoryl peptides (19–25). Each enzyme contains two SH2 domains, suggesting that bivalent interactions might confer higher affinity, perhaps through an avidity effect. Moreover, tandem phosphorylation sites are necessary, for example, in immune cell signaling where bisphosphorylated TAMs bind the tandem SH2 domains of ZAP-70 or Syk (e.g. Refs. 26 and 27). Two solved structures of tandem SH2 domains, from ZAP-70 and SHP-2, suggest that spatial constraints on binding site orientation might play a role in higher specificity (28, 29).

Using five different proteins, we show that tandem SH2 domain interactions have substantially higher affinities than comparable single SH2 domain interactions. Furthermore, since each tandem SH2 domain binds a distinct, biologically relevant TAM with highest affinity and alternate, irrelevant TAMs with much lower affinity, we conclude that tandem domain interactions greatly enhance biological specificity in tyrosine kinase signaling pathways.

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1 G. Wolf and S. E. Shoelson, unpublished observations.
2 The abbreviations used are: TAM, tyrosine-based activation motif; PI, phosphatidylinositol; PLC, phospholipase C; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; pY, phosphorytrosine; IRS, insulin receptor substrate; Fmoc, N-(9-fluorenyl)methoxycarbonyl; HPLC, high performance liquid chromatography; TCR, T cell receptor; RU, resonance unit(s); cRU, corrected resonance unit(s).
Tandem SH2 Domain Binding

**A**

**B**

**Native TAM Sequences**

**Non-native Bisphosphoryl Peptides**

**Materials and Methods**

**Protein Expression and Purification**—cDNA fragments encoding human PI 3-kinase p85α (310–712), ZAP-70 (1–259), and Syk (1–265) tandem SH2 domains were subcloned into pGEX vectors (Pharmacia Biotech Inc.). Transformed *Escherichia coli* strain BL21(DE3) was grown at 30–37 °C; protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2–4 h. Pelleted bacteria were recrystallized in lysis buffer (50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml leupeptin, 1.0 μg/ml aprotinin, 1.0 mM benzamidine, and 5.0 mM dithiothreitol) at 25 °C. After each cycle, the chip was regenerated with 5 mM benzamidine, and 5.0 mM dithiothreitol by fast protein liquid chromatography or dialysis. Proteins were exchanged into phosphate-buffered saline/dithiothreitol by affinity chromatography (immobilized PDGFR pY1021, SHP-2 with SHPS-1/TAM, and p85α with immobilized IRS-1 pY1172, LSTpYAS-TAM1) or using 5 μg/ml anti-phosphotyrosine (Upstate Biotechnology, Inc. 05-321). BIAcore measurements were made in phosphate-buffered saline/dithiothreitol at 25 °C. After each cycle, the chip was regenerated with a 60-s pulse of 3 M NaCl and 50-s pulse of 6 M guanidine hydrochloride, pH 7.0. Neither loss of peptide or tyrosine phosphorylation (as assessed by anti-phosphotyrosine binding) nor change in baseline RU was apparent during the course of an assay.

**Surface Plasmon Resonance**—Measurements were made with a BIAcore biosensor (Pharmacia). Phosphopeptides were covalently immobilized to a biosensor chip CM5 through the ε-amino group of an Ac-Lys-Gly-Gly linker (18). Low ligand densities (50–100 resonance units (RU)) were used to minimize “bridging” between tandem SH2 domain proteins and adjacent phosphopeptides. The presence of accessible phosphotyrosine was confirmed by monoclonal antibody binding on RU using 5 μg/ml anti-phosphotyrosine (Upstate Biotechnology, Inc. 05-321). BIAcore measurements were made in phosphate-buffered saline/dithiothreitol at 25 °C. After each cycle, the chip was regenerated with a 60-s pulse of 3 M NaCl and 50-s pulse of 6 M guanidine hydrochloride, pH 7.0. Neither loss of peptide or tyrosine phosphorylation (as assessed by anti-phosphotyrosine binding) nor change in baseline RU was apparent during the course of an assay.

**Determination of KD: Values for apparent dissociation constants, KD(app), were calculated from equilibrium binding data at six or more protein concentrations. Data were fit using the Eadie-Hofstee equation,**

\[
cRU = \frac{cRU}{[protein]} = \frac{cRU_{max}}{[Inh]} + \frac{cRU_{max}}{IC50}
\]

where cRU is the response at equilibrium corrected for bulk refractive index errors using a sham-coupled flow cell, cRU_{max} is cRU when all accessible peptides on the biosensor chip are occupied, [protein] is the analyte (tandem SH2 domain) concentration, and KD is the dissociation constant. Independently purified batches of protein and independent BIAcore analyses were used for each replicate.

**Determination of IC50**—Each tandem SH2 domain protein was paired with its highest affinity ligand for competition studies: ZAP-70 with immobilized TCRγ; TAML, Syk with PεCRIγ TAML, PLC-γ1 with PDGF 1009/1021, SHP-2 with SHPS-1/SIRP TAM, and p85α with immobilized PDGF 740/751. Solutions of tandem SH2 domain proteins (5 nM for p85α, 10 nM for all others) were pre-mixed with varying concentrations of competing (soluble) peptide and injected (2 μl/min for p85α, all others at 5 μl/min flow rates) over the appropriate immobilized peptide until an equilibrium in binding was achieved. Inhibition of binding was measured as a decrease in cRU as a function of peptide concentration. Experimental data were analyzed according to the equation

\[
cRU_{exp} = cRU_{max} - cRU_{max}(IC50)/IC50 \times (1 + IC50/[Inh])
\]

where cRU_{exp} is maximum binding in the absence of inhibitor, cRU_{max} is minimum binding (complete inhibition), cRU_{max} is binding at inhibitor concentration [Inh], k is the slope factor, and IC50 is the inhibitor concentration that reduces binding by one-half. Peptide concentrations were determined independently for each experimental replicate.

**Results**

Tandem SH2 Domains Bind Bisphosphorylated TAMs with Nanomolar Affinities—Binding analyses were conducted with native tandem SH2 domain sequences derived from five well characterized signaling proteins: PI 3-kinase p85, ZAP-70, Syk, SHP-2, and PLC-γ1 (Fig. 1A). Interactions were with natural...
ligands, including TAMs derived from biologically relevant sites in the PDGF receptor (31–33), T and B cell and FcεRI receptors (27, 34, 35), and the substrates IRS-1 and SHPS-1/SIRP (36–40) (Fig. 1B). Affinities were measured using surface plasmon resonance under carefully controlled conditions. Tandem SH2 domain proteins were affinities purified using phosphotyrosine-agarose to ensure that all molecules were properly folded and capable of interacting with phospholigands. Ligand densities on the sensor chips were low (<50 RU) to prevent interactions between tandem SH2 domain proteins and adjacent phosphopeptides, termed bridging. 

A typical series of binding measurements is displayed in Fig. 2. Equilibrium values were determined 10 s prior to the end of each protein injection. Scatchard plots of cRU versus RU [SHP-2] were linear (inset). This suggests that binding data can be treated as a second order, bimolecular process with a single value for $K_p(app)$. Each tandem SH2 domain binds a bisphosphorylated TAM with low nanomolar or subnanomolar affinity (Table I). As additional controls, the monophosphoryl forms of the SHPS-1/SIRP TAM and TCR$_z$ TAM1 peptides were synthesized and immobilized to sensor chips under identical conditions. The tandem SH2 domains of SHP-2 and ZAP-70, respectively, bind with micromolar affinities (data not shown), similar in magnitude to the micromolar affinities of individual SH2 domains toward monophosphorylated TAMs (Table I). As additional controls, the monophosphoryl forms of TAM1 were bound for competition studies with ZAP-70, TCR$_z$ TAM1 with high affinity (IC$_{50}$ = 8.3 nM), whereas the two PDGF receptor TAMs and the SHPS-1/SIRP TAM bind with 1,000- to 10,000-fold lower affinity (panel A). In contrast, the PI 3-kinase p85 tandem SH2 domains bind PDGF FR 740/751 TAM with high affinity (IC$_{50}$ = 1.5 nM). TCR$_z$, SHPS-1/SIRP, and PDGF FR 1009/1021 TAMs bind with more than 1,000-fold lower affinity (Fig. 4B). SHP-2 binds the SHPS-1/SIRP TAM with high affinity (IC$_{50}$ = 8 nM), but in this case the T cell and PDGF receptor TAMs bind with 1,000- to more than 10,000-fold lower affinity (Fig. 4C). These differences are significantly greater than the 20–50-fold windows of selectivity typically seen when individual SH2 domains bind specific versus non-specific or randomized motifs (Fig. 3) (13, 16–18, 41, 42).

ZAP-70 and Syk Selectivity for Immunoreceptor TAMs—MHC-bound antigens activate the T cell receptor, leading to its tyrosine phosphorylation and recruitment of the non-receptor tyrosine kinase, ZAP-70 (27). Tandem SH2 domains of ZAP-70 were passed over the chip. Binding sensograms were recorded and equilibrium measurements (RU) were used to generate binding curves.

Each tandem SH2 domain binds a bisphosphoryl TAM derived from its biological target with greatest affinity (Table II). Alternative bisphosphoryl TAMs bind with 1,000- to >10,000-fold lower affinity. In the examples shown (Fig. 4), ZAP-70 binds TCR$_z$ TAM1 with high affinity (IC$_{50}$ = 8.3 nM), whereas the two PDGF receptor TAMs and the SHPS-1/SIRP TAM bind with 1,000- to 10,000-fold lower affinity (panel A). In contrast, the PI 3-kinase p85 tandem SH2 domains bind PDGF FR 740/751 TAM with high affinity (IC$_{50}$ = 1.5 nM). TCR$_z$, SHPS-1/SIRP, and PDGF FR 1009/1021 TAMs bind with more than 1,000-fold lower affinity (Fig. 4B). SHP-2 binds the SHPS-1/SIRP TAM with high affinity (IC$_{50}$ = 8 nM), but in this case the T cell and PDGF receptor TAMs bind with 1,000- to more than 10,000-fold lower affinity (Fig. 4C). These differences are significantly greater than the 20–50-fold windows of selectivity typically seen when individual SH2 domains bind specific versus non-specific or randomized motifs (Fig. 3) (13, 16–18, 41, 42).
proteins (TCR, PDGFR 740/751) bind bisphosphorylated TAMs in the TCR diamonds TAM1; and T cell receptor signaling. The ing mutations in either SH2 domain abolish association (44). phoryl peptides were passed over the immobilized peptides (SHPS-1/SIRP, PI 3-kinase p85 (panel A), or SHPS-1/SIRP TAM (panel B), or SHP-2 tandem SH2 domain proteins (panel C, 10 nM) containing varying concentrations of bisphosphoryl peptides were passed over the immobilized peptides (TCR TAM1; diamonds, PDGFR 740/751; circles, PDGFR 1009/1021; squares, SHPS-1/SIRP.

bind bisphosphorylated TAMs in the TCRζ subunit (43). Blocking mutations in either SH2 domain abolish association (44) and T cell receptor signaling. The ζ-chains contain three TAMs (TCRζ TAM1, TAM2, and TAM3) having the consensus sequence (pYXXI/LX₆₋₈pYXXI/IL) (Fig. 1B). Although ZAP-70 binds all three TCR TAMs (Table II), affinity is highest for TCRζ TAM1 and TAM2. Interestingly, this matches the order of in vitro phosphorylation by the Src-like kinase, Lck, consistent with an ordered assembly of tetrameric ζ-chain-ZAP-70 complexes. ZAP-70 also binds the T cell receptor e-chain (TCR e) and the common immunoreceptor γ-chain TAM (FceRIγ TAM) with high affinity (IC₅₀ = 18 and 25 nM, respectively). γTAM-1 binding correlates with the ability of ZAP-70 to reconstitute defective B cell receptor signaling in Syk-deficient cells through interactions with the B cell receptor γ-chain (45). Combined, these data illustrate a strong preference for ZAP-70 binding to conserved immunoreceptor TAMs over alternative TAMs.

Syk and ZAP-70 have homologous sequences and similar architectures, although Syk primarily mediates signaling through B cell, the high affinity IgE (FceRI), and other antigen receptors containing the common immunoreceptor γ-chain (46). The pattern of TAM selectivity is similar for Syk and ZAP-70, as Syk binds immunoreceptor TAMs (FceRIβ and γ TAMs and TCR e and ζ TAMs 1, 2, and 3) with high or moderate affinity. Alternative, non-immunoreceptor TAMs (e.g. PDGFR 740/751, PDGFR 1009/1021, and SHPS-1) bind with much lower affinity (Table II). Of the high affinity IgE receptor TAMs, Syk prefers γTAM-1 over βTAM-1 (IC₅₀ = 23 versus 420 nM, respectively). This correlates with reported interactions between Syk and γ-subunits but not the β-subunit (23, 47).

To determine whether side chains of the amino acids in the linker region between paired pYXXI motifs contribute to binding affinity, 7 residues of γTAM-1 were replaced with alanine, Ac-DGVpYTGL(A)₈pYETLK-NH₂. Both Syk and ZAP-70 bound the Ala, peptide with minor changes in affinity (IC₅₀ = 10 nM for Syk and IC₅₀ = 50 nM for ZAP-70), indicating that side chain chemistry within the linker sequence has little role in affinity or selectivity.

**PI 3-Kinase p85** Binds Tandem YM/VXM Motifs—PI 3-kinase plays a prominent role in cells that have been activated by a wide variety of hormones, growth factors, cytokines, and antigens. The regulatory p85 subunit of PI 3-kinase contains two SH2 domains that bind YM/VXM motifs in activated receptors and their substrates (Fig. 3) (13, 16, 31, 48). Three alternative regulatory subunits (p55, AS53, and AS53I) have

TABLE II

| TAM          | ZAP-70 | Syk | p85 | SHP-2 | PLC-γ1 |
|--------------|--------|-----|-----|-------|--------|
| PDGFR 740/751| 94 kM  | 83 kM | 1.5 kM | >100 kM | 15 kM   |
|               | (4,100)| (3,600)| (1.0) | (>12,000)| (64)   |
| PDGFR 1009/1021| 21 kM | 34 kM | 5.0 kM | 11,000 kM | 240 kM |
|               | (3,700)| (1,500)| (3,300)| (1,400) | (1.0)  |
| TCRζ TAM1     | 8.3 ± 0.4 | 12.0 ± 6.0 | 3.3 ± 28 | >100 kM | >100 kM |
|               | (1.5) | (5.0) | (2.1) | (>12,000) | (>400) |
| TCRζ TAM2     | 5.7 ± 0.2 | 24 ± 3.5 | 35.000 ± 7500 | >100 kM | >100 kM |
|               | (1.0) | (1.0) | (23,000) | (>12,000) | (>400) |
| TCRζ TAM3     | 23 ± 1.0 | 29 ± 2.1 | 14,000 ± 2200 | >100 kM | >100 kM |
|               | (4.0) | (1.3) | (9,300) | (>12,000) | (>400) |
| TCR ε TAM1    | 18 ± 0.9 | 280 ± 29 | 79,000 ± 13,000 | >100 kM | >100 kM |
|               | (3.2) | (12) | (53,000) | (>12,000) | (>400) |
| FcεRIγ TAM     | 25 ± 2.5 | 23 ± 1.5 | 18,000 ± 150 | >100 kM | 62,000 ± 3,000 |
|               | (4.4) | (1.0) | (12,000) | (>12,000) | (260) |
| FcεRIβ TAM     | 297 ± 3.0 | 420 ± 25 | 29,000 ± 8,500 | >100 kM | 19,000 ± 1,600 |
|               | (52) | (18) | (19,000) | (>12,000) | (79) |
| SHPS-1 TAM     | 3,600 ± 230 | ND | 87,000 ± 24,000 | 8.3 ± 0.23 | >100,000 |
|               | (630) | | (58,000) | (1.0) | (>400) |

a Values are IC₅₀ ± S.E. (relative ratio is in parentheses).
b ND, not determined.

A. Proudfoot, personal communication.
identical or closely related SH2 domain sequences and predicted specificities (49, 50). Two tyrosines (740 and 751) in the human PDGF receptor, within YM/VXM motifs, are involved in PI 3-kinase signaling. The motifs are separated by 7 residues (pYVM/VXMK-pYM/VXM), resembling the immunoreceptor TAMS. Mutations in either motif impair signaling (31), suggesting a potential two-site mode for binding. Our results show that the tandem SH2 domains from PI 3-kinase p85 and the bisphosphoryl PDGFR 740/751 TAM bind with very high affinity ($K_p = 0.6 \text{ nM}$, Table I). A second PDGFR receptor TAM (PDGFR 1009/1021) and all of the other natural TAM sequences used in these studies bind with much lower affinity ($>2,000$-fold).

Unlike ZAP-70 and Syk, where spacings between phosphotyrosines are critical, distances between p85-binding YM/VXM motifs can be reduced significantly (21). A bisphosphoryl peptide composed of IRS-1-derived tetrapeptide motifs connected by a 2-residue spacer (Ac-pYMPMSSpYMPMS) retains full binding affinity ($IC_{50} = 2.6 \pm 0.8 \text{ nM}$ compared with 1.5 nM for PDGFR 740/751, Table II). The ability of p85 to bind YM/VXM motifs separated by such dissimilar distances suggests that spacing is not an important determinant for p85 specificity and that spatial relationships between the two domains may be flexible and able to change orientations relative to one another. This is in marked contrast to the constraints of orientation observed for tandem ZAP-70 and SHP-2 domains (see below) (28, 29).

SHP-2 Binds TAMS with Widely Spaced Phosphotyrosines—
SHP-2 and the closely related enzyme SHP-1 are critical mediators of signals stemming from many tyrosine kinase-linked receptors. Nevertheless, their catalytic targets and cellular mechanisms of action have been difficult to define fully (51). SHPS-1 is one member of a newly identified family of membrane proteins (SIRPs) which are phosphorylated and bind SHP-2 in insulin, growth factor, and adhesion protein-activated cells (38–40). Phosphotyrosines within SHPS-1/SIRP TAMS are separated by 23–25 residues, much greater spacing than the 9–11 residues between paired immunoreceptor phosphotyrosines. SHP-2 binds the SHPS-1 TAM1 with highest affinity, $K_p = 1.3 \text{ nM}$, of the bisphosphoryl TAMs tested (Table I). Alternative immunoreceptor and PDGF receptor TAMS all bind with 1,000- to >10,000-fold lower affinity (Table II).

SHP-2 also binds IRS-1 in insulin-activated cells (37), but the two relevant phosphotyrosine binding sites, 1172 and 1222 (52), are 49 residues apart (Fig. 3). To determine whether the region between recognition motifs contributes to the interaction or simply acts as a flexible spacer, we replaced 40 amino acids (IRS-1 1179–1218) with a tether composed of four amine-alkanoic acids (22). The IRS-1 1172/Aha$_4$1222 peptide binds the tandem SH2 domains of SHP-2 with high affinity, $K_p = 3.0 \pm 0.6 \text{ nM}$. Since 40 residues can be replaced with a spacer that lacks peptide side chain and backbone chemistry, flexibility between motifs appears to be a critical feature of SHP-2 binding selectivity.

Although SHP-2 is known to bind the PDGFR receptor $\beta$-subunit, neither of the native PDGFR peptides (740/751 or 1009/1021) binds the tandem SH2 domains with high affinity (Table II). This is despite evidence that phosphorylation at Tyr$^{1009}$ is required and sufficient for in vivo association between SHP-2 and the PDGFR receptor (33, 53). Moreover, both NH$_2$- and COOH-terminal SH2 domains independently bind PDGFR 1009 peptides ($IC_{50} = 5–10 \mu\text{M}$, Fig. 3). Affinity remains relatively low ($IC_{50} = 3.5 \mu\text{M}$) even when two PDGFR 1009 sites are linked to form the PDGFR 1009/1009 peptide (Ac-SV-LpYTAVQPNEGSLpYTAVQPNE-NH$_2$). With 11 residues between phosphotyrosines, spacings for the PDGFR 1009/1009 $\beta$-subunit resemble native PDGF receptor and immunoreceptor TAMS. Presumably, spatial constraints prevent simultaneous interaction between two SHP-2 SH2 domains and two phosphotyrosine motifs of the 1009/1021 or 1009/1009 peptides. This view is supported by the crystal structure of the tandem SH2 domains of SHP-2 bound to two unlinked PDGFR 1009 peptides (29). Binding sites are widely spaced. Together, the biochemical and structural data suggest that SHP-2 binds bisphosphorylated TAMS with unusually long, flexible spacers that permit “looping” for simultaneous interaction with the two antiparallel binding sites, or it binds monophosphorylated sites within two adjacent proteins. SHP-2 binding to two identical Tyr$^{1009}$ sites in activated PDGF receptor homodimers may be an example of the latter mode.

**PLC-\(\gamma\) Binding Is Biphasic**—The tandem SH2 domains from PLC-\(\gamma\)-1 bind PDGFR 1009/1021 peptide with high affinity (Table I), whereas alternative native sequences bind with much lower affinity (Table II). This is consistent with the required phosphorylation at Tyr$^{1021}$ for in vivo association between PLC-\(\gamma\)-1 and the PDGFR receptor (32) (Fig. 3). Two $K_p$(app) values were derived from the Scatchard plots, 0.65 ± 0.06 nM at low protein concentrations and 2.2 ± 0.6 nM at higher protein concentrations. The biphasic appearance of these plots could be an intrinsic feature of the PLC-\(\gamma\)/PDGFR interaction. Alternatively, this could result from mixtures of proteins or peptides used in the analyses (e.g. a mixed populations of monomers and dimers, a partly folded protein, or full-length versus truncated peptides, etc.). We tried to eliminate the last possibilities by carefully characterizing both binding partners: 1) numerous repetitions with different batches of protein give identical results; 2) protein samples were monodisperse by light scattering and monomeric by fast protein liquid chromatography gel filtration (data not shown); and 3) peptides are ≥ 93% pure by HPLC and have the expected mass and sequence as determined by electrospray mass spectrometry and amino acid analysis.

Biphasic Scatchard plots could also result from binding in two orientations (head-to-head and tail-to-tail); or the tandem SH2 domains could bridge adjacent, immobilized 1009/1021 TAMS by simultaneously interacting either with two 1009 or two 1021 sites. In the latter case, peptides in solution should compete less effectively, behaving more like monophosphopeptides without the entropic advantage (avidity effect) of two-site binding. In fact, calculated IC$_{50}$ values are lower in competition experiments where PDGFR 1009/1021 peptides are in solution (240 nM, Table II) compared with $K_p$(app) values determined in equilibrium binding studies with immobilized peptides (0.65 and 2.2 nM). A bisphosphoryl peptide corresponding to tandem Tyr$^{1021}$ sites (PDGFR 1021/1021, Ac-DNDpYIPLKDNDpYIPLDPK-NH$_2$) binds with slightly higher affinity (IC$_{50} = 75 ± 4.0 \mu\text{M}$, consistent with Tyr$^{1021}$ being within the preferred binding motif (Fig. 3)(13, 14, 32). Perhaps high affinity ligands for tandem PLC-\(\gamma\) SH2 domains might be generated by flexible linkers between two Tyr$^{1021}$ sites, as was shown above for SHP-2 interactions.

DISCUSSION

Although individual cells contain numerous receptor and non-receptor tyrosine kinases and substrates, each stimulus generates a distinct collection of signals and a unique biological response. Specificity in signaling pathways is critical for normal cell biology, but the biochemical mechanisms underlying specificity are incompletely understood. Even though inherent selectivity of individual peptide binding domains (e.g. SH2, SH3, PTB, PDZ, WW) is very important, this alone cannot account for the exquisite specificity seen in cells. Since all SH2 domain proteins contain additional binding modules and/or motifs, we wondered whether biological specificity might be a...
consequence of multisite interactions among multiple domains within two or more proteins of a signaling complex. Studies with tandem SH2 domains test this question.

Tandem SH2 domains from five signaling enzymes (PI 3-kinase, ZAP-70, Syk, SHP-2, and PLC-γ1) bind bisphosphoryl TAM peptides with much higher affinity (0.5–2.6 nM) than corresponding single domain interactions (0.2–1.0 μM). Each tandem SH2 domain binds a distinct, biologically relevant TAM with highest affinity, suggesting that these interactions are selective. Competition studies further showed that specificities between tandem SH2 domains and bisphosphorylated TAMs are high, ranging from >1,000-fold to >10,000-fold. These are much greater differences than the 20–50-fold typically seen with individual SH2 domains. We conclude that the significantly higher affinities seen with tandem SH2 domains compared with their single domain counterparts translate into high specificity. We have considered possible mechanisms for enhanced affinity and selectivity.

Interactions between tandem SH2 domains and bisphosphoryl ligands must occur in discrete steps. Once the first site has bound, the effective concentration of unbound partners is exceedingly high and promotes binding at the second site. Following the principle of microscopic reversibility, dissociation occurs in identical, discrete steps. Once one site of a bivalent complex has dissociated, it may reassociate rapidly, before dissociation at the second site. This entropic advantage to bivalent binding provides the so-called “avidity effect.” These considerations explain why association rates (k_{on}) for the tandem interactions are only slightly slower than for single SH2 domains, whereas dissociation rates (k_{off}) are significantly slower (>1,000-fold).4 Since K_D reflects a ratio between dissociation (k_{off}) and association (k_{on}) rates (K_D = k_{off}/k_{on}), dramatically slower dissociation rates account for the observed, markedly higher affinities.

At least three potential mechanisms contribute to the increased specificity. 1) Numerous studies have characterized the peptide binding specificities of individual SH2 domains (see the Introduction and Fig. 3). This is retained in each of the individual domains of tandem SH2 domain proteins. 2) Peptide lengths between TAM binding motifs and amino acid side chain chemistry of these linker regions may contribute to selectivity. 3) An additional constraint on binding may be imposed by the spatial relationship of two SH2 domain binding sites, relative to one another.

Our data show that various tandem domains require different spacings between phosphotyrosines of bound TAMs. ZAP-70 and Syk bind linear TAMs found in immunoreceptor subunits. 9–11 residues separate phosphotyrosines in these TAMs. This distance is optimal for ZAP-70 and Syk binding, as affinities drop when residues are added or subtracted.5 The collinear mechanism portrayed in the ZAP-70 tandem domain structure, with two binding motifs aligned end-to-end in “series,” is probably the only acceptable mode of binding for the ZAP-70 or Syk domains (28). Potential mechanisms for SHP-2 binding are distinct. Binding sites in the tandem SHP-2 SH2 domain structure are spaced widely and oriented oppositely (29). We now know that the tandem SH2 domains in the intact enzyme are oriented differently, spaced widely and perpendicular to one another, and that binding site orientations are less constrained than originally suspected.6 In both cases, bisphosphoryl TAMs (such as SHPS-1/SIRP sequences) must change direction to bind both sites. The 23 intervening residues (including 4 or 5 prolines) in SHPS-1/SIRP TAMs clearly accommodate this turn. We have optimized distances with flexible spacers that form a somewhat shorter loop (−70 Å between phosphotyrosines compared with >85 Å for SHPS-1/SIRP), but these distances remain greater than the 9–11 residues that are optimal for ZAP-70 and Syk binding. Notably, this mode of binding sets no maximum limit on loop size. Providing that potential gains in entropy are compensated, phosphotyrosines can be separated by virtually any larger loop (or intervening structure). For example, IRS-1 tyrosines 1172 and 1222 are separated by 49 residues. With this mode of binding there is no structural reason why both sites even need to be on the same peptide chain. It appears that the widely spaced and oppositely oriented SH2 domain binding sites of SHP-2 are ideally poised to span identical Tyr1009 sites in activated PDGF receptor homodimers.

Even in the absence of a solved structure for the tandem PI 3-kinase p85 domains, the available data show that modes of binding are distinct from ZAP-70 or SHP-2. Whereas p85 binds phosphotyrosines that are 10 residues apart (PDGFR 740/751), it also binds sites that are more closely spaced. In fact, tandem YMYM motifs separated by only 2 residues bind with high affinity. This implies that p85 SH2 domain binding sites must be able face one another, as this is required for binding such closely spaced motifs. Although we do not know whether PI 3-kinase binds closely spaced sites in nature (and kinases may not phosphorylate both sites efficiently), there are potential examples of more widely spaced motifs. Of the eight YXXM motifs in ErbB3, three are separated by 12–24 residues, and IRS-1 contains nine YXXM motifs that may be separated by as few as 19 residues. It is also possible that PI 3-kinase binds more distant sites in the same chain or on distinct peptides chains. For example, the CSF-1 and c-Kit receptors each have single copies of the optimal YM/VXM motif, yet both bind and activate PI 3-kinase much as the PDGFR receptor. Since spatial requirements for p85 appear to be flexible, it too could span identical sites in activated receptor homodimers.

In conclusion, SH2 domains mediate protein-protein interactions. One important outcome is a change in the subcellular localization of the recruited SH2 domain protein. Since the receptors are (or associate with) kinases, binding also increases the likelihood that an SH2 domain protein will be phosphorylated. Phosphorylation activates some enzymes (e.g. PLC-γ1), whereas other phosphorylated SH2 domain proteins recruit additional effectors (e.g. Src binds Grb-2/Sos). SH2 domains also act as allosteric regulators of enzymatic activity. The SH2 domain enzymes PI 3-kinase, SHP-1 and -2, ZAP-70, and Syk are inhibited in the cytosol and activated by SH2 domain recruitment. A structural rearrangement occurs in SHP-2 and possibly in other enzymes (the recently solved x-ray crystal structure of SHP-2 shows how this occurs). Activated enzymes thus cluster around activated receptors to produce high local concentrations of catalytic products. Through these mechanisms, the high affinity and high specificity seen with tandem SH2 domains must contribute in a major way to biological specificity in signal transduction.

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4 E. A. Ottinger, M. C. Botfield, and S. E. Shoelson, unpublished observation.
5 M. C. Botfield, unpublished result.
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