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Phosphatidylinositol 3-Kinase p85 SH2 Domain Specificity Defined by Direct Phosphopeptide/SH2 Domain Binding†

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ABSTRACT: We have developed a competition binding assay to quantify relative affinities of isolated Src-homology 2 (SH2) domains for phosphopeptide sequences. Eleven synthetic 11–12-amino acid phosphopeptides containing YM*X*M or YV*X*M recognition motifs bound to a PI 3-kinase p85 SH2 domain with highest affinities, including sequences surrounding phosphorylated tyrosines of the PDGF, CSF-1/c-Fms, and *kit*-encoded receptors, IRS-1, and polyoma middle T antigens; matched, unphosphorylated sequences did not bind. A scrambled YM*X*M phosphopeptide or sequences corresponding to the GAP or PLC-γ SH2 domain binding motifs of the PDGF, FGF, and EGF receptors bound to the p85 SH2 domain with 30–100-fold reduced affinity, indicating that this affinity range confers specificity. Binding specificity was appropriately reversed with an SH2 domain from PLC-γ: a phosphopeptide corresponding to the site surrounding PDGF receptor Tyr1021 binds with ≈40-fold higher affinity than a YM*X*M-phosphopeptide. We conclude that essential features of specific phosphoprotein/SH2 domain interactions can be reconstituted using truncated versions of both the phosphoprotein (a phosphopeptide) and cognate SH2 domain-containing protein (the SH2 domain). SH2 domain binding specificity results from differences in affinity conferred by the linear sequence surrounding phosphotyrosine.

Activated growth factor receptor tyrosine kinases and their cellular substrates are phosphorylated on specific tyrosine residues which leads, in turn, to their association with an additional set of cytoplasmic signaling proteins having Src-homology 2 (SH2) domains (Ullrich & Schlessinger, 1990; Cantley et al., 1991; Pawson & Gish, 1992). SH2 domains are ≈100 amino acid modules which bind phosphotyrosine-containing proteins. For example, ligand-induced PDGF and EGF receptor activation is accompanied by binding of phospholipase C-γ (PLC-γ) (Kumjian et al., 1989; Margolis et al., 1989; Meisenhelder et al., 1989), *ras* GTPase activating protein (GAP) (Molloy et al., 1989; Ellis et al., 1990; Kazlauskas et al., 1990), growth factor receptor-bound protein 2 (GRB2) (Lowenstein et al., 1992), and phosphatidylinositol (PI) 3-kinase (Coughlin et al., 1989; Bjorge et al., 1990; Fantl et al., 1992). Phosphoprotein/SH2 protein interactions are thought to couple tyrosine kinases to downstream signaling pathways within the cell.

However, with many phosphoproteins and SH2 domain proteins in a cell, specificity is crucial to maintaining organization within complex sets of interactive proteins and

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divergent pathways. We are interested in a better understanding of the molecular basis for signal discrimination. Several lines of evidence suggest that SH2 domains recognize phosphotyrosine within specific sequence contexts. For example, phosphorylation of PDGF receptor tyrosines 740 and 751 initiates PI 3-kinase interactions; 771 is implicated in GAP interactions and 1009 and 1021 are implicated in associations with PLC-γ (Kashishian et al., 1992; Fantl et al., 1992; Rönnstrand et al., 1992).

A unique feature of phosphoprotein/SH2 domain interactions which has facilitated analyses of specificity is the ability of protein fragments to retain function. Recombinant SH2 domains bind selectively to phosphoproteins (Anderson et al., 1990; Margolis et al., 1990; Mayer et al., 1990), and phosphopeptides corresponding to sites surrounding phosphorylated tyrosines block phosphoprotein/SH2 protein interactions (Escobedo et al., 1991; Auger et al., 1992; Fantl et al., 1992; Backer et al., 1992; Myers et al., 1992; Carpenter et al., 1992). In the current study we develop a direct binding assay between isolated SH2 domains and phosphopeptides to quantify relative affinities for these interactions. Structure–activity relationships obtained from binding analyses, in conjunction with studies of SH2 domain/phosphopeptide structure (Waksman et al., 1992, 1993; Overduin et al., 1992a,b; Booker et al., 1992; Shoelson et al., 1993; Eck et al., 1993; Williams & Shoelson, 1993), should provide a more detailed and general understanding of the requisites for high-affinity binding and specificity.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Syntheses of phosphorylated peptides were conducted on a Milligen/Biosearch 9600 synthesizer using 4-alkoxybenzyl alcohol polystyrene resin (PAC, Milligen/Biosearch) with the first residues attached at 0.35–0.37 mmol/g substitution levels. The N^{α}-Fmoc protecting group was used throughout in conjunction with standard side-chain protecting groups. N^{α}-Fmoc-*O*-(*O,O*-dimethoxyphosphoryl)-L-tyrosine (Fmoc-Tyr($OP(OCH_3)_2$)) (Kitas et al., 1991; Domchek et al., 1992) was used for incorporation of phosphotyrosine. Coupling reactions with 0.2 M N^{α}-Fmoc amino acid, 1-hydroxybenzotriazole (HOBt), and (benzotriazoyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) were conducted for 1–2 h prior to incorporation of Fmoc-Tyr($OP(OCH_3)_2$) and for 4–24 h afterward. Peptides were cleaved from the resin, and side-chain protecting groups were simultaneously removed by treatment with trifluoroacetic acid, thioanisole, ethanedithiol, and anisole (90:5:3:2) for 2 h at 22 °C. Methyl protecting groups on phosphotyrosine were removed during a second stage of deprotection with trimethylsilyl bromide (Kitas et al., 1991). All peptides were precipitated with diethyl ether (4 °C) and desalted on a column of Bio-Gel P2. Peptides were further purified using a Waters' Prep 4000 HPLC equipped with a Dynamax-300A 12-μm C8 column (41.4 × 250 mm). Amino acid analyses and results obtained from plasma desorption mass spectrometry (for selected peptides) were as expected.

SH2 Domain/GST Fusion Protein Expression. The N- and C-terminal SH2 domains of p85 and the C-terminal SH2 domain of PLC-γ were expressed as glutathione *S*-transferase (GST) fusion proteins (Hu et al., 1992; Rotin et al., 1992) and purified by affinity chromatography on glutathione–agarose (Smith & Johnson, 1988), except the fusion protein was eluted from the affinity matrix with 8.0 M urea and dialyzed against 10 mM sodium phosphate, 140 mM NaCl, and 10 mM dithiothreitol (pH 7.4). Protein concentrations were estimated by Bradford assay. Purified GST/SH2 domain fusion proteins were stored at −20 °C until needed; no loss in binding capacity was noted for >3 months.

^{125}I-Radiolabeled Phosphopeptide. Dimethylformamide (10 μL) was added to 2.0 mCi (1.0 μmol) of [^{125}]monoiodo-Bolton–Hunter reagent (ICN), benzene was removed in vacuo, and phosphopeptide IRS-1 pY628 or PDGFR pY1021 (0.5 mg, 0.33 μmol) in 20 μL of neat dimethyl sulfoxide was added (phosphopeptides are named by tyrosine site within the intact phosphoprotein). The pH_{app} was adjusted to 7.0 as necessary with *N*-methylmorpholine, and the reaction was allowed to proceed at room temperature for 24 h. The peptide was precipitated with excess ice-cold acetone, and reaction products were separated by reversed-phase HPLC. Fractions of [^{125}I]phosphopeptide that exhibited SH2 binding were combined and aliquoted. Bovine serum albumin (50 μg/aliquot) was added, and the radiolabeled peptide was lyophilized and stored at −20 °C until used.

Phosphopeptide/SH2 Domain Binding Assay. GST/SH2 domain fusion proteins (500 nM unless otherwise noted), 35 fmol of HPLC-purified, [^{125}I]monoiodo-Bolton–Hunter-treated phosphopeptide (67 nCi), and varying concentrations of unlabeled peptides were combined in a 200-μL total volume of 20 mM Tris-HCl, 250 mM NaCl, 0.1% bovine serum albumin, and 10 mM dithiothreitol, pH 7.4, and vortexed. Glutathione–agarose (25 μL of a 1:4 aqueous slurry, Molecular Probes) was added, and the samples were incubated overnight at 22 °C with constant mixing. Following centrifugation for 5 min at 12 000*g*, supernatant solutions were removed by aspiration and ^{125}I-radioactivity associated with the pellets was determined with a γ-counter.

RESULTS

Peptide Design. Phosphopeptides listed in Table I were designed to contain essential elements of the PI 3-kinase p85 recognition motifs YM*X*M or YV*X*M, when appropriate, along with flanking residues whose importance for SH2 domain recognition is unknown. At least three residues N-terminal to phosphotyrosine and four residues C-terminal to the YM/V*X*M were incorporated in every case. For phosphorylation sites which do not contain p85 recognition motifs (PGDFR pY771 and pY1021, EGFR pY992, FGFR pY766, c-Src pY527), peptides were similarly designed to contain ≥3 residues N-terminal to and ≥7 residues C-terminal to phosphotyrosine, with the expectation that important features of the binding motif would be included within these sequences. Residues found in the IRS-1 pY628 sequence were randomized for the "scrambled" peptide.

Binding Assay Development. In the presence of 100–500 nM GST/SH2 domain fusion protein 20–30% (20 000–30 000 cpm) of the added radiolabeled peptide (100 000 cpm) was bound to the glutathione–agarose beads; this value was called maximum binding. In the absence of GST/SH2 domain fusion protein or in the presence of GST/SH2 domain and excess (1.0 mM) unlabeled YM*X*M-phosphopeptide, ≈2% of the ^{125}I-labeled phosphopeptide was associated with the unwashed glutathione–agarose beads. This value was taken to be nonspecific binding (NSB). In actual competition experiments the amount of radioactivity associated with the pellets varied according to the concentration of unlabeled peptides. These values were plotted as bound (% maximum) vs log peptide concentration (Figures 1–3), where bound (% maximum) = (bound − NSB)/(maximum bound − NSB); data were best fit to the sigmoid dose–response equation, $y = a/(1 + e^{x-c}) + d$ (DeLean et al., 1978).

Table I: Relative Affinities of Phosphopeptides and Phosphotyrosine for the N-Terminal SH2 Domain of PI 3-Kinase p85 (N-p85) and the C-Terminal SH2 Domain of PLC-γ (C-PLCγ)[a]

SH2	Phosphoprotein	Name/Position	Sequence	ID_{50} (μM)
N-p85	PDGF Receptor β (human)	PDGFR pY740	D-G-G-pY-M-D-M-S-K-D-E	1.1 ± 0.09
N-p85		PDGFR pY751	S-V-D-pY-V-P-M-L-D-M-K	1.8 ± 0.36
N-p85		PDGFR pY771	S-S-N-pY-M-A-P-Y-D-N-Y	90 ± 31
N-p85		PDGFR pY1021	D-N-D-pY-I-I-P-L-P-D-P-K	45 ± 14
N-p85	Polyoma mT (hamster)	*h*mT pY298	R-E-N-E-pY-M-P-M-A-P-Q-I-H	0.9 ± 0.08
N-p85	(mouse)	*m*mT pY315	E-E-E-pY-M-P-M-E-D-L-Y	0.6 ± 0.17
N-p85	IRS-1 (rat)	IRS-1 pY608	D-D-G-pY-M-P-M-S-P-G-V	0.7 ± 0.15
N-p85		IRS-1 pY628	G-N-G-D-pY-M-P-M-S-P-K-S	1.1 ± 0.11
N-p85		IRS-1 pY658	P-N-G-pY-M-M-M-S-P-S-G	1.6 ± 0.13
N-p85		IRS-1 pY727	T-G-D-pY-M-N-M-S-P-V-G	1.2 ± 0.14
N-p85		IRS-1 pY939	S-E-E-pY-M-N-M-D-L-G-P	1.6 ± 0.13
N-p85	scrambled	IRS-1 pY628	P-M-P-N-S-K-M-D-pY-G-G	70 ± 18
N-p85	shortened	IRS-1 pY628-5	pY-M-P-M-S	60 ± 15
N-p85	CSF-1R/c-Fms	CSF-1R pY721	V-D-T-pY-V-E-M-R-P-V-S	0.5 ± 0.08
N-p85	c-Kit	c-Kit pY721	T-N-E-pY-M-D-M-K-P-G-V	0.9 ± 0.06
N-p85	EGF receptor	EGFR pY992	D-A-D-E-pY-L-I-P-Q-Q-G-F-F	35 ± 15
N-p85	FGF receptor	FGFR pY766	S-N-Q-E-pY-L-D-L-S-M-P-L-D-Q	73 ± 27
N-p85	c-Src	c-Src pY527	E-P-Q-pY-Q-P-G-E-N-L	>1,000
N-p85	Phosphotyrosine	Tyr(P)	pY	5,900 ± 940
C-PLCγ	PDGF Receptor β	PDGFR pY1021	D-N-D-pY-I-I-P-L-P-D-P-K	2.0 ± 0.3
C-PLCγ	IRS-1	IRS-1 pY628	G-N-G-D-pY-M-P-M-S-P-K-S	76 ± 49

[a] Peptides were named by the position of phosphorylated tyrosine in the intact protein. Phosphotyrosine and the entire YM/V*X*M or alternative recognition motif (when present) are underlined. Each peptide was assayed three to six times; average ID_{50} values are reported.

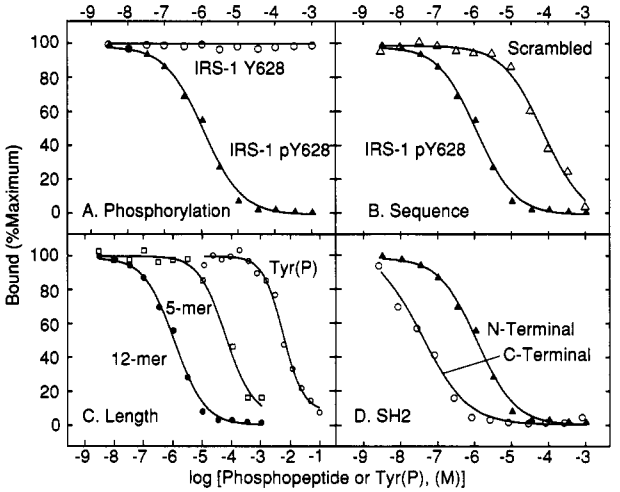

FIGURE 1: Binding assay development. Competitive binding assays with GST/SH2 domain fusion proteins were conducted as described under Experimental Procedures. (A) Phosphorylation is required for binding. Competition with phosphopeptide IRS-1 pY628 (▲) vs the identical unphosphorylated sequence, IRS-1 Y628 (○). (B) Importance of phosphopeptide sequence on relative binding affinity. Competition with IRS-1 pY628 (▲) vs a scrambled version of the same peptide (△). (C) Effect of phosphopeptide length on binding affinity. Competition with the 12-residue IRS-1 pY628 (●) vs a five-residue version of the same peptide (IRS-1 pY628-5) which retains the YM*X*M motif (□) vs free phosphotyrosine (○) (note different abscissa scale). (D) Relative affinities of C-terminal (○) vs N-terminal (▲) SH2 domains of PI 3-kinase p85.

As shown (Figure 1A), inhibition of ^{125}I-radiolabeled peptide binding was dependent on the concentration of phosphopeptide (IRS-1 pY628). No inhibition was observed at the lowest phosphopeptide concentrations (≤100 nM) whereas 100% inhibition occurred at the highest concentrations (≥33 μM). In between, partial inhibition was observed, and this was used to calculate values for half-maximal inhibition (ID_{50}); for phosphopeptide IRS-1 pY628 this was 1.1 ± 0.11 μM (n = 6; peptide sequences are shown in Table I). Under assay conditions SH2 domain concentrations are in excess over K_D (estimated by biospecific interaction analysis to be ≈3 nM) (Felder et al., 1993), and ID_{50} values overestimate K_D. However, relative ID_{50} values accurately reflect *relative differences* in binding affinity. SH2 domain binding was strictly dependent on phosphorylation, as matched nonphosphorylated sequences exhibited no inhibition (e.g., Figure 1A).

Specificity for the phosphopeptide/SH2 domain interaction is demonstrated by comparison of affinities of the YM*X*M peptide (IRS-1 pY628) vs a phosphopeptide having the same amino acids but in randomized order. As illustrated in Figure 1B, the scrambled version of IRS-1 pY628 binds with 63-fold lower relative affinity (ID_{50} = 70 ± 18 μM). Differences in phosphopeptide length also influence SH2 domain affinity. For example, phosphotyrosine itself binds with ≈6000-fold weaker affinity than 11–12-residue YM/V*X*M phosphopeptides (Figure 1C, Table I). Phosphotyrosine within a shortened but complete YM/V*X*M sequence (IRS-1 pY628-5) binds with 55-fold weaker affinity. In principle, such reduced affinity could result either from the absence of important contacts between the SH2 domain and residues N- or C-terminal to the motif or from destabilizing electrostatic interactions between free peptide α-amino and/or α-carboxyl groups and the SH2 domain. We cannot differentiate these possibilities from the current data. However, the effect of peptide length on SH2 domain affinity is particularly noteworthy in that 5–7-residue phosphopeptide sequences with C-terminal phosphotyrosine have been used for structural (Waksman et al., 1992) and functional characterization of SH2 interactions (Fantl et al., 1992; Escobedo et al., 1991). Elongation of the phosphopeptide over 12 residues does not increase affinity (data not shown). Although fewer assays have been conducted with the C-terminal SH2 domain of p85

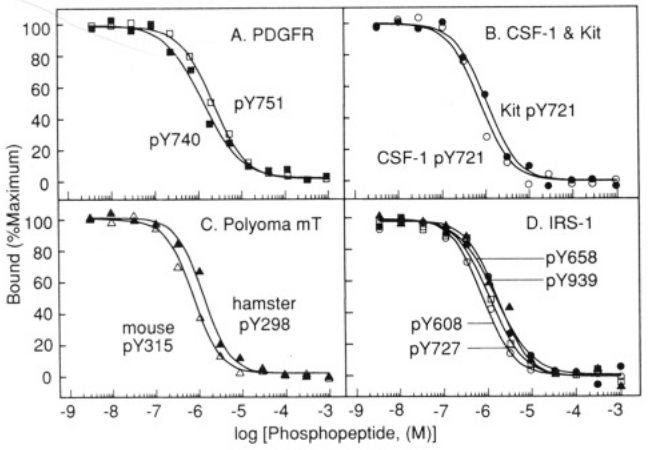

FIGURE 2: Binding of YM*X*M- and YV*X*M-phosphopeptide sequences to the N-terminal p85 SH2 domain. Competition assays were conducted with 11-amino acid phosphopeptides corresponding to sequences surrounding (A) PDGF receptor Tyr740 (■) and Tyr751 (□), (B) CSF-1 receptor/c-Fms Tyr721 (○) and Kit Tyr721 (●), (C) polyoma middle T antigens, mouse Tyr315 (△) and hamster Tyr298 (▲), and (D) IRS-1 Tyr608 (○), Tyr658 (●), Tyr727 (□), and Tyr939 (▲).

at this time, it appears to bind with ≈10-fold higher affinity (Figure 1D).

SH2 Domain Specificity. On the basis of findings presented in the previous section, we conducted comparative competition assays with a large number of 11–13-residue phosphopeptides under controlled conditions of high binding (500 nM GST/SH2 domain). Phosphopeptides corresponding to known or putative recognition motifs of p85 include sequences surrounding the following phosphorylation sites: human PDGF receptor Y740 and Y751, CSF-1 receptor Y721, Kit Y721, mouse and hamster polyoma virus middle T antigens Y315 and Y298, respectively, and rat IRS-1 Y608, Y628, Y658, Y727, and Y939. Notably, all of these peptides contain phosphotyrosine within a YM*X*M or YV*X*M sequence, and each was bound by the N-terminal SH2 domain of p85 with similar relative affinity (Figure 2). Each peptide was assayed three to five times in duplicate, and average ID_{50} values were determined. All YM*X*M- or YV*X*M-phosphopeptides that were 11 or 12 amino acids in length bound within a 4-fold range of affinity (0.5–1.8 μM). As sequences N- or C-terminal to the phosphorylated YM*X*M or YV*X*M sites vary, these residues do not appear to confer specificity. However, residues N- and/or C-terminal to the motif are necessary for high affinity (Figure 1C).

To further assess sequence specificity of p85 SH2 domain interactions, we determined binding affinities with sequence motifs predicted to interact with alternative SH2 domains. Tyr771 of the PDGF receptor is predicted to interact preferentially with the *ras* GAP SH2 domain (Fantl et al., 1992; Kashishian et al., 1992). A corresponding 11-amino acid phosphopeptide (PDGFR pY771) binds to the N-terminal p85 SH2 domain with an ID_{50} value of 45 μM (Figure 3A), which is 40-fold weaker than YM/V*X*M phosphopeptide binding (Table I). When phosphorylated, Tyr527 of c-Src reportedly undergoes an intramolecular association with the c-Src SH2 domain (Cooper et al., 1986; Roussel et al., 1991). A phosphopeptide corresponding to the C-terminal tail of c-Src (c-Src pY527) binds to the p85 SH2 domain weakly (Figure 3A; ID_{50} >1 mM). Several phosphorylation sites have been predicted for SH2 domain-mediated interactions of PLC-γ, including PDGF receptor Tyr1021, FGF receptor Tyr766, and Tyr992 of the EGF receptor (Mohammadi et al., 1991,

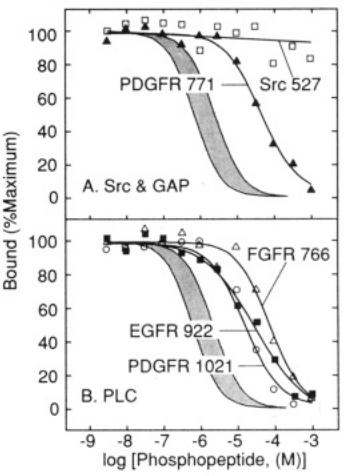

FIGURE 3: Binding of the N-terminal p85 SH2 domain with phosphopeptides corresponding to motifs which are recognized by alternative SH2 domains. Shaded zones represent the span of binding for all YM/V*X*M phosphopeptides shown in Figure 2. (A) Binding of phosphopeptides corresponding to motifs thought to interact with the c-Src SH2 domain, c-Src pY527 (□), or the *ras* GAP SH2 domain, PDGFR pY771 (▲). (B) Binding of phosphopeptides thought to interact with PLC-γ SH2 domains, including FGFR pY766 (△), EGFR pY922 (■), and PDGFR pY1021 (○).

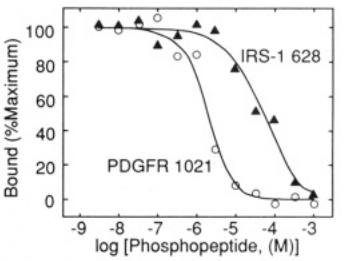

FIGURE 4: Competitive binding studies with the C-terminal PLC-γ SH2 domain. ^{125}I-Radiolabeled PDGFR pY1021 and the indicated concentrations of unlabeled PDGFR pY1021 (○) or IRS-1 pY628 (▲) were assayed with the PLC-γ SH2 domain as described under Experimental Procedures.

1992, Peters et al., 1992; Rönnstrand et al., 1992; Rotin et al., 1992). N-Terminal p85 SH2 domain binding affinities with phosphopeptides corresponding to each of these motifs were reduced 30–66-fold relative to YM/V*X*M phosphopeptides (Figure 3B, Table I).

As an additional control for binding specificity, related studies were conducted with alternative SH2 domains. For example, the C-terminal SH2 domain of PLC-γ binds with high affinity for a phosphopeptide corresponding to PDGF receptor Tyr1021 (2.0 μM) and with 38-fold reduced affinity for IRS-1 pY628 (a YM*X*M motif) (Figure 4, Table I). Similar findings obtained with Src and Lck SH2 domains (Payne et al., 1993) suggest that the observed 30–100-fold differences in relative affinity may confer specificity to SH2/phosphopeptide interactions in general.

DISCUSSION

Activation of tyrosine kinase receptors is accompanied by phosphorylation of specific tyrosine residues within the receptor itself (autophosphorylation) as well as on cytoplasmic substrates. Recently it has been realized that these phosphorylations are accompanied by the assembly of macromolecular complexes comprising the receptors and/or their substrates and additional cytoplasmic signaling molecules containing SH2 domains. While many details are unknown, the ubiquity of phosphoprotein/SH2 domain protein interactions suggests

an important role in tyrosine kinase signal transduction. However, potential complexity of a network of hundreds of interacting phosphoproteins and SH2 domain proteins is enormous, particularly so when many of these proteins are present in the same cell and have the capacity for multiple interactions. Much of this complexity appears to be resolved by the specificity inherent in phosphoprotein/SH2 interactions.

Previous studies have showed that mutagenesis of targeted tyrosines can block specific SH2 domain interactions. For example, mutation of human PDGF receptor residues Tyr740 and Tyr751 prevents association with PI 3-kinase, whereas mutation of Tyr771 blocks GAP interactions and mutation of Tyr1021 blocks PLC-γ interactions (Kazlauskas & Cooper, 1989; Kazlauskas et al., 1990; Fantl et al., 1992; Kashishian et al., 1992; Rönnstrand et al., 1992). Analyses of the interactions between the FGF receptor and PLC-γ also suggest that phosphorylation at a particular tyrosine residue serves as an on/off switch for association (Mohammadi et al., 1991, 1992; Peters et al., 1992). Such an all-or-nothing concept for SH2 interactions gained further support from studies in which selected phosphopeptides reportedly do or do not block phosphoprotein/SH2 domain protein interactions (Escobedo et al., 1991; Auger et al., 1992; Fantl et al., 1992). Nevertheless, SH2 domains bind to phosphotyrosine itself (Mayer et al., 1992) and can form complexes with phosphopeptides indiscriminately. Data presented in this report rationalize all of these findings. Using a competition assay with an isolated SH2 domain of PI 3-kinase, we show that specificity in phosphoprotein/SH2 domain interactions derives from relative affinity of a particular SH2 domain for phosphotyrosine within a given sequence. In fact, phosphopeptides corresponding to 11 known and putative PI 3-kinase p85 recognition sites from six different proteins all bind with nearly the same affinity to the p85 SH2 domain (Figure 2). By contrast, phosphopeptides corresponding to recognition sites for alternative SH2 domains bind with 30–>1000-fold reduced relative affinity (Figure 3), and a randomized motif, which should estimate the relative affinity of a nonspecific interaction, binds with ≈64-fold weaker affinity (Figure 1B). As a class, shorter peptides having phosphotyrosine at the N-terminus bind with lower affinity(e.g., Figure 1C) but appear to retain specificity (Escobedo et al., 1991; Fantl et al., 1992).

Comparison of relative phosphopeptide affinities might be used to define SH2 domain specificity in greater detail. For example, the FGF receptor Tyr766 site within a YLDL motif binds to the p85 SH2 domain with ≈66-fold reduced relative affinity. There are no obvious negative determinants N- or C-terminal to the motif, and Asp is acceptable at the +2 position as it is found in PDGF receptor and Kit YM*X*M motifs (Table I). Therefore, the apparently conservative Met → Leu substitutions at the +1 and +3 positions, relative to phosphotyrosine, are responsible for reduced affinity. This finding demonstrates that hydrophobicity itself is insufficient for high-affinity interaction, and side-chain flexibility is key, as suggested previously for efficient insulin receptor kinase catalysis (Shoelson et al., 1992). Additional studies with two alternative PLC-γ recognition motifs further underscore the negative influence of reduced flexibility at the +3 position. Sequences surrounding PDGF receptor Tyr1021 and EGF receptor Tyr992 are within YIIP and YLIP motifs, respectively (Table I). Corresponding phosphopeptides (having Pro in place of Met at the +3 position) bind to the p85 SH2 domain with 30–40-fold reduced affinity. Although phosphopeptides having YLDL or YI/LIP motifs may bind to the N-terminal p85 SH2 domain with similar low affinities, and both are predicted to bind PLC-γ, these motifs may be recognized differentially by N- and C-terminal SH2 domains of PLC-γ (Songyang et al., 1993). Presumably, the +3 position of YM*X*M phosphopeptides must insert into a deep, complementary pocket in the SH2 domain, as has been seen for Src and Lck SH2 domain interactions (Eck et al., 1993; Waksman et al., 1993), and chain branching may inhibit insertion.

Analysis of the PDGF receptor GAP-binding site (Tyr771) within a YMAP motif further demonstrates the negative influence of Met → Pro substitutions at the +3 position; binding affinity of a corresponding phosphopeptide is reduced ≈80-fold, compared to YMXM phosphopeptides. Binding of a phosphopeptide corresponding to the c-Src tail (Y527) to the p85 SH2 domain provides another interesting comparison. In this case, phosphotyrosine occurs within a Glu-Pro-Gln-Tyr(P)-Gln-Pro-Gly-Glu sequence which is more hydrophilic than the YM/V*X*M motif, and at the +3 position a side chain is completely lacking. These differences, reflected in very weak affinity for the p85 SH2 domain, perhaps best demonstrate that, in addition to the lack of positive influences on binding, potential exists for negative or destabilizing effects on SH2 domain binding.

Inasmuch as phosphopeptide/SH2 domain interactions are appropriate models for interactions between the intact proteins, these findings predict that in vivo specificity is derived from differences in relative binding affinity. The narrow window between specific and nonspecific binding found here (30–70-fold in most cases) suggests that a modest increase in in vivo concentrations of a phosphoprotein or an SH2 domain-containing counterpart could force an interaction that does not normally occur, with potentially pathologic consequences. Moreover, these findings urge caution in interpreting results from experiments in which phosphoprotein and/or SH2 domain protein concentrations are abnormally high, such as with overexpression in transfected cells or in vitro interactions between isolated proteins. Corresponding interactions between intact proteins may have higher affinity and greater relative specificity, but appropriate quantitative analyses are needed.

Whereas most data in this report are limited to phosphopeptide interactions with PI 3-kinase p85 and SH2 domains, the approach is applicable to other SH2 domains. For example, a PLC-γ SH2 domain demonstrates appropriate reversals in orders of binding affinities (Figure 4). However, this approach does require previous knowledge of a tight-binding motif, and for most SH2 domains these are unknown. Differential binding of phosphopeptides suggested that SH2 domains might be used to affinity-purify their own recognition motifs from phosphopeptide mixtures. Partly on the basis of the findings presented here, degenerate phosphopeptide libraries have been generated recently and found to conveniently map previously unrecognized binding motifs (Songyang et al., 1993). Such motifs were found in relevant signaling proteins and, as phosphopeptides, they bind tightly to the appropriate SH2 domains (e.g., Src and Lck SH2 domains bind tightly to a YEEI motif; Payne et al., 1993). These findings demonstrate that phosphopeptide/isolated SH2 domain interactions can be used to analyze and predict in vivo interactions between the corresponding intact proteins.

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