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Specific phosphopeptide binding regulates a conformational change in the PI 3-kinase SH2 domain associated with enzyme activation

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SH2 (*src*-homology 2) domains define a newly recognized binding motif that mediates the physical association of target phosphotyrosyl proteins with downstream effector enzymes. An example of such phosphoprotein-effector coupling is provided by the association of phosphatidylinositol 3-kinase (PI 3-kinase) with specific phosphorylation sites within the PDGF receptor, the c-Src/polyoma virus middle T antigen complex and the insulin receptor substrate IRS-1. Notably, phosphoprotein association with the SH2 domains of p85 also stimulates an increase in catalytic activity of the PI 3-kinase p110 subunit, which can be mimicked by phosphopeptides corresponding to targeted phosphoprotein phosphorylation sites. To investigate how phosphoprotein binding to the p85 SH2 domain stimulates p110 catalytic activation, we have examined the differential effects of phosphotyrosine and PDGF receptor-, IRS-1- and c-Src-derived phosphopeptides on the conformation of an isolated SH2 domain of PI 3-kinase. Although phosphotyrosine and both activating and non-activating phosphopeptides bind to the SH2 domain, activating phosphopeptides bind with higher affinity and induce a qualitatively distinct conformational change as monitored by CD and NMR spectroscopy. Amide proton exchange and protease protection assays further show that high affinity, specific phosphopeptide binding induces non-local dynamic SH2 domain stabilization. Based on these findings we propose that specific phosphoprotein binding to the p85 subunit induces a change in SH2 domain structure which is transmitted to the p110 subunit and regulates enzymatic activity by an allosteric mechanism.

Key words: growth factor receptor/insulin receptor/oncogene/signal transduction/tyrosine kinase

Introduction

Activation of receptor and non-receptor protein tyrosine kinases is accompanied by phosphorylation of specific tyrosine residues both within the kinases themselves and on cytoplasmic protein substrates (Ullrich and Schlessinger, 1990). A newly identified mechanism for signaling by activated tyrosine kinases is the direct interaction of phosphorylated sites with additional cytoplasmic proteins that contain *src* homology (SH2) domains (Cantley *et al.*, 1991; Koch *et al.*, 1991). SH2 domains are discrete modules of ~100 amino acids that bind to phosphopeptide sequences. Recent studies demonstrate, for example, that the activated, phosphorylated platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors interact directly with the SH2 domains of phospholipase Cγ (PLCγ) (Kumjian *et al.*, 1989; Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989), the GTPase activating protein (GAP) associated with *ras* (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).

Specificity in the interaction of a particular phosphoprotein and SH2 domain is derived from both partners: sequence determinants for binding certainly surround phosphotyrosine (Cantley *et al.*, 1991; Mohammadi *et al.*, 1992; Peters *et al.*, 1992; Piccione,E., Case,R.D., Domchek,S.M., Hu,P., Chaudhuri,M., Backer,J.M., Schlessinger,J. and Shuelson,S.E., manuscript submitted) and presumably also occur in variable regions of the SH2. An emerging paradigm for such recognition is provided by the interaction of PI 3-kinase with various phosphoproteins. PI 3-kinase is composed of a 110 kDa catalytic subunit (Carpenter *et al.*, 1990; Hiles *et al.*, 1992) and an 85 kDa regulatory subunit which contains two SH2 domains and one SH3 domain (Escobedo *et al.*, 1991b; Otsu *et al.*, 1991; Skolnik *et al.*, 1991). PI 3-kinase activity physically associates via SH2 interactions with growth factor (PDGF, CSF-1/c-*fms*, *kit*-encoded) receptors, a receptor substrate (IRS-1, insulin receptor substrate 1) (Sun *et al.*, 1991) and a viral transforming protein [the polyoma virus middle T antigen (mT) which associates with and is phosphorylated by c-Src]. As predicted by Cantley *et al.* (1991), phosphotyrosine residues within pTyr-Met/Val-Xxx-Met (YMXM or YVXM) sequences of these phosphoproteins direct the interactions (Courtneidge and Heber, 1987; Kazlauskas and Cooper, 1989; Talmage *et al.*, 1989; Auger *et al.*, 1992; Reedijk *et al.*, 1992; Shoelson *et al.*, 1992).

A unique feature of phosphoprotein/SH2 domain recognition is its ability to be reconstituted with isolated phosphopeptide and protein fragments. For example, expressed SH2 domains have been used to precipitate phosphorylated receptors and receptor substrates, which demonstrates that the isolated SH2 domain folds properly and retains native binding properties. Furthermore, phosphoprotein-PI 3-kinase interactions can be blocked by phosphopeptides corresponding to specific phosphorylated sequences of these phosphoproteins (Escobedo *et al.*, 1991a; Auger *et al.*, 1992; Backer *et al.*, 1992; Fantl *et al.*, 1992). In fact, isolated SH2 domains of p85, expressed as fusion proteins, retain high affinity and specificity of binding

towards phosphopeptides having the appropriate sequences (Domchek *et al.*, 1992; Felder *et al.*, 1993; Piccione *et al.*, manuscript submitted). Such a simplified model system provides an attractive opportunity to investigate molecular details of the SH2 domain–phosphoprotein interaction.

In addition to mediating physical association, phosphoprotein–SH2 interactions may regulate enzymatic activity. For example, the association of IRS-1 with PI 3-kinase was shown recently to stimulate enzymatic activity (Backer *et al.*, 1992). Furthermore, synthetic phosphopeptides corresponding to YMXM and YVXM motifs of IRS-1, the PDGF receptor and mT antigens were also shown to stimulate PI 3-kinase activity (Backer *et al.*, 1992; Carpenter *et al.*, 1993). These findings predict that phosphoprotein (or phosphopeptide) binding to SH2 domains of the p85 subunit of PI 3-kinase effects a change in structure that is transmitted to the 110 kDa catalytic subunit. As a first step towards testing this prediction, we have investigated possible changes in structure and stability of an isolated p85 SH2 domain which accompany binding of activating and non-activating phosphopeptides. CD, NMR and protease protection assays demonstrate that a specific conformational change occurs on high affinity binding of activating phosphopeptides which contain YM/VXM sequences. These findings, which provide a basis for understanding structural transmission of allosteric effects in SH2 domain-containing proteins, are discussed in reference to recent crystallographic (Waksman *et al.*, 1992) and NMR (Booker *et al.*, 1992; Overduin *et al.*, 1992a,b) structures of SH2 domains.

Results

Peptide design

Phosphopeptides IRS-1 pY628 and PDGFR pY740 (Table I) contain essential elements of the PI 3-kinase p85 recognition motif (along with flanking residues whose importance for SH2 domain recognition is unclear). The design strategy was based on the expectation that important features of the binding motif would be included within these sequences. Therefore, in each case at least three residues N-terminal to phosphotyrosine and four residues C-terminal to the YM/VXM site were incorporated. For phosphorylation sites which do not contain p85 recognition motifs (i.e. c-Src pY527) peptides similarly contain three residues N-terminal to and six residues C-terminal to phosphotyrosine. A truncated (5 amino acid) version of IRS-1 pY628 was also prepared (designated IRS-1 pY628–5). Residues found in the IRS-1 pY628 sequence were randomized for the scrambled peptide.

Circular dichroism

Structural effects of phosphopeptide and phosphotyrosine binding were monitored by circular dichroism (CD). In Figure 1A are shown the far UV CD spectra of the isolated N-terminal PI 3-kinase p85 SH2 domain (▲) and isolated PDGF receptor-derived phosphopeptide, PDGFR pY740 (×). The 123 residue SH2 protein domain contains primarily β-sheet with an estimated α-helix content of ~13%. Thermal unfolding (inset, Figure 1A) is cooperative, demonstrating a stably folded structure. By contrast, no evidence of ordered structure is observed in the 11 amino acid phosphopeptide.

In Figure 1B are shown the CD spectra of an equimolar complex between the SH2 domain and phosphopeptide PDGFR pY740 (●), and the phosphopeptide alone (×). Also shown is the calculated difference spectrum (○), defined as $\Delta^{\theta}(\lambda) = (\Theta_{SH2} + \Theta_{peptide}) - \Theta_{complex}$. The difference spectrum exhibits distinctive minima at 230 and 200 nm, indicative of a change in global structure either in the SH2 domain or in the phosphopeptide. This pattern is not in accord with any simple transformation among canonical secondary structures, such as random coil→α-helix or β-sheet→α-helix (Woody, 1985; Johnson, 1990). The same distinctive difference spectrum is observed on binding of phosphopeptide IRS-1 pY628 (Figure 1C). By contrast, addition of the non-phosphorylated form of the peptide (IRS-1 Y628) to the SH2 domain yields no significant difference spectrum (Figure 1D). The non-phosphorylated peptide has independently been shown not to bind the SH2 domain by biospecific interaction analysis (Felder *et al.*, 1993) and competition binding assay (Piccione *et al.*, manuscript submitted).

The truncated IRS-1 phosphopeptide was used to investigate the origin of the observed CD difference spectrum. IRS-1 pY628–5 is a five amino acid sub-fragment of IRS-1 pY628 (Table I) which retains the pYM/VXM sequence motif thought to be essential for PI 3-kinase p85–phosphoprotein interactions (Cantley *et al.*, 1991; Fantl *et al.*, 1991; Piccione *et al.*, manuscript submitted). Although the shorter phosphopeptide exhibits 60-fold weaker binding than the 12mer (Table I; Piccione *et al.*, manuscript submitted), the 5 and 12 residue peptides induce qualitatively

Table I

Peptide	Sequence	Relative affinity[a]	PI 3-kinase activation[b]	Change in structure		Change in dynamics	
				CD	NMR	NMR	Proteolysis
IRS-1 pY628	G N G D pY M P M S P K S	1.0	+	+	+	+	+
IRS-1 pY628–5	pY M P M S	0.018	ND	+	ND	ND	ND
Scrambled IRS-1 pY628	P M P N S K M D pY G G	0.016	–	–	–	–	ND
IRS-1 Y628	G N G D Y M P M S P K S	<0.0005	–	–	–	–	–
PDGFR pY740	D G G pY M D M S K D E	1.0	+	+	+	+	+
c-Src pY527	E P Q pY Q P G E N L	<0.001	–	–	–	–	–
Phosphotyrosine	pY	0.0002	–	–	–	–	ND

[a]Relative affinities for p85 SH2 binding were determined using a competition assay (Domchek *et al.*, 1992; Piccione *et al.*, manuscript submitted).
[b]PI 3-kinase enzymatic activation experiments were previously reported (Backer *et al.*, 1992). Changes in SH2 structure and dynamics were determined in this report. ND: not determined.

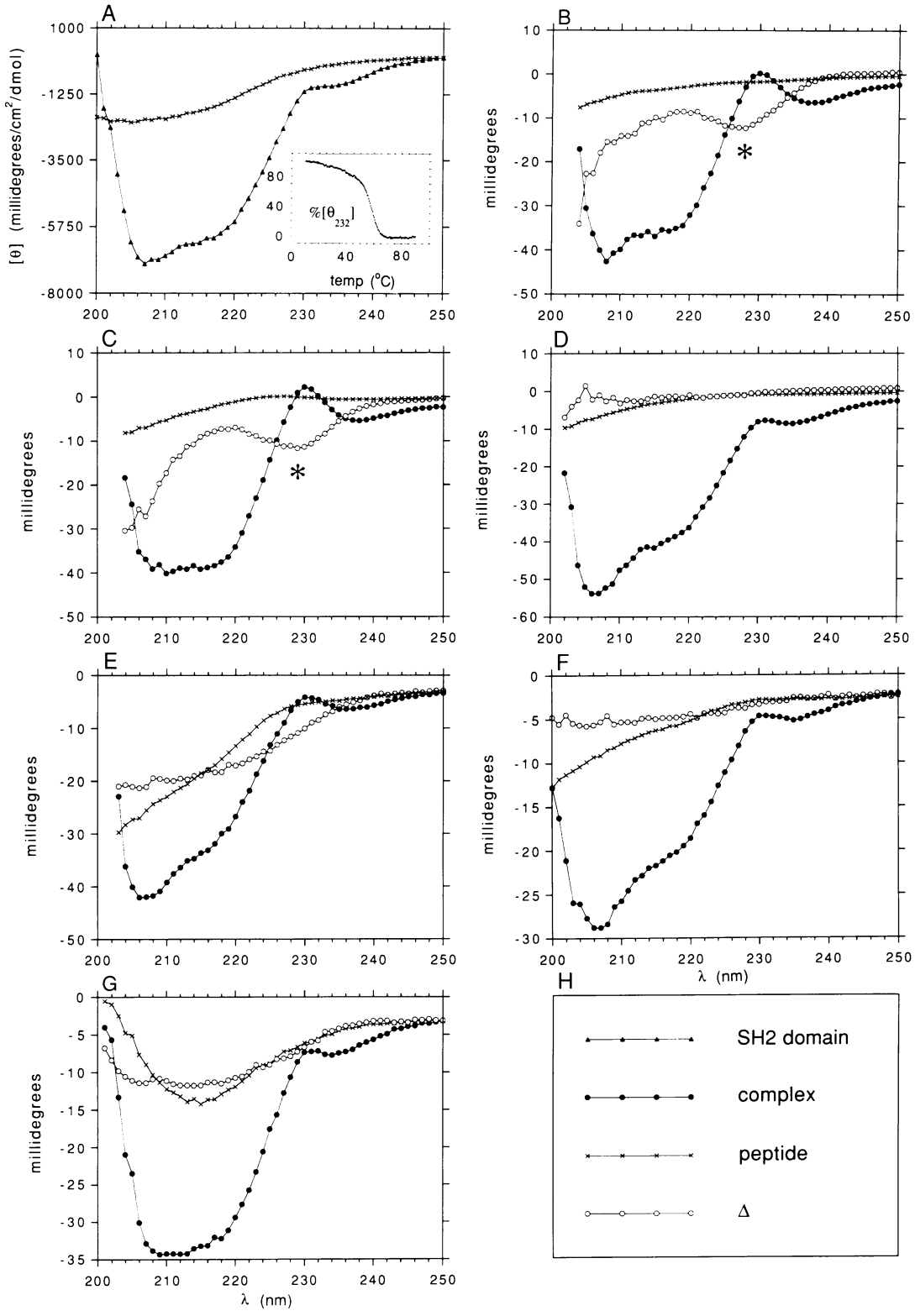

Fig. 1. Far UV CD spectra. (**A**) Isolated N-terminal p85 SH2 domain (▲; 25 μM) and isolated PDGFR pY740 (×; 75 μM). **Inset**: cooperative thermal unfolding of the isolated SH2 domain monitored at 232 nm; the refolding reaction is reversible (data not shown). In all other panels data are shown for the isolated peptide or phosphotyrosine (×) or the complex formed between SH2 domain and either peptide or phosphotyrosine (●). Calculated difference spectra (○) are defined as $\Delta^{\theta}(\lambda) = (\Theta_{SH2} + \Theta_{peptide}) - \Theta_{complex}$; asterisks denote characteristic minima in the difference spectra for specific complexes. (**B**) PDGFR pY740 (75 μM); (**C**) IRS-1 pY628 (75 μM); (**D**) IRS-1 Y628 (75 μM); (**E**) c-Src pY527 (0.4 mM); (**F**) c-Src Y527 (0.4 mM); (**G**) phosphotyrosine (0.75 mM). Note that mean residue ellipticities, [Θ], are reported on the ordinate of panel A; for all other panels raw ellipticity data are shown.

identical CD changes (data not shown). These results suggest that the specific difference spectrum reflects primarily a change in SH2 structure rather than peptide folding, since in the latter case the difference spectrum is expected to be sensitive to peptide length (the number of residues whose conformation is stabilized in the complex). This assignment

is supported by NMR experiments (below) and is in accord with comparative crystallographic and NMR structures (Booker *et al.*, 1992; Overduin *et al.*, 1992a,b; Waksman *et al.*, 1992).

To investigate the relationship between CD-sensitive changes in structure and enzyme activation, two non-activating phosphopeptides (Backer *et al.*, 1992) were similarly studied. The first, c-Src pY527, is derived from a presumably unrelated signaling pathway and contains a distinct sequence motif (PQpYQPE). The second non-activating phosphopeptide is a scrambled version of the IRS-1 sequence, obtained as a random permutation. At high concentrations both of these phosphopeptides competitively inhibit IRS-1 pY628 binding (Table I; Piccione *et al.*, manuscript submitted), strongly suggesting that they occupy the same binding site within the SH2 domain. However, CD studies of these 'inactive' complexes reveal a difference spectrum that lacks the distinctive minimum at 230 nm seen with IRS-1 pY628 and PDGFR pY740 (spectrum of the c-Src pY527 complex shown in Figure 1E; data not shown but virtually identical for the scrambled peptide). This difference spectrum is characteristic of peptide immobilization, rather than a more global change in protein structure. This spectral change is also phosphorylation-dependent, as addition of the corresponding non-phosphorylated sequence, c-Src Y527, yields no significant difference spectrum (Figure 1F).

An additional non-specific control is provided by phosphotyrosine itself. The phosphoamino acid binds to the SH2 domain, albeit weakly (Table I). Competitive inhibition of IRS-1 pY628 binding is observed at 6000-fold higher concentration than that of the phosphopeptide. Under conditions of nearly complete occupancy, the CD difference spectrum shares features of the non-specific c-Src pY527 and scrambled peptide complexes, but not the distinct features of the specific IRS-1 pY628 or PDGFR pY740 complexes.

^{1}H-NMR

In Figure 2 (upper panel) are shown the upfield-shifted aliphatic resonances of the isolated SH2 domain in the absence (spectrum A) and presence (spectra B–F) of phosphotyrosine and various phosphopeptides and corresponding non-phosphopeptides. This region of the p85 SH2 spectrum is qualitatively similar to that of the Abl SH2 reported by Mayer and coworkers (1992). The marked dispersion of aliphatic resonances in both cases demonstrates the presence of ordered structure. This region of the ^{1}H-NMR spectrum is sensitive to structural and electronic influences, in particular the contributions of aromatic ring currents. No peptide resonances are observed in the 0.9 to −0.3 p.p.m. regions of the spectra (Figure 2, upper panel, B–F), providing a window in which SH2 resonances are resolved. No perturbations are observed in this region of the SH2 spectrum upon addition of non-phosphorylated IRS-1 Y628 peptide (Figure 2, upper panel, B), consistent with no interaction between SH2 and peptide.

Significant perturbations in numerous aliphatic resonances do, however, accompany specific binding of either IRS-1 pY628 (E) or PDGFR pY740 (F). These spectra are essentially identical to one another, in accord with the CD results (above). The physical origins of these changes in chemical shift are unclear but in principle may reflect the

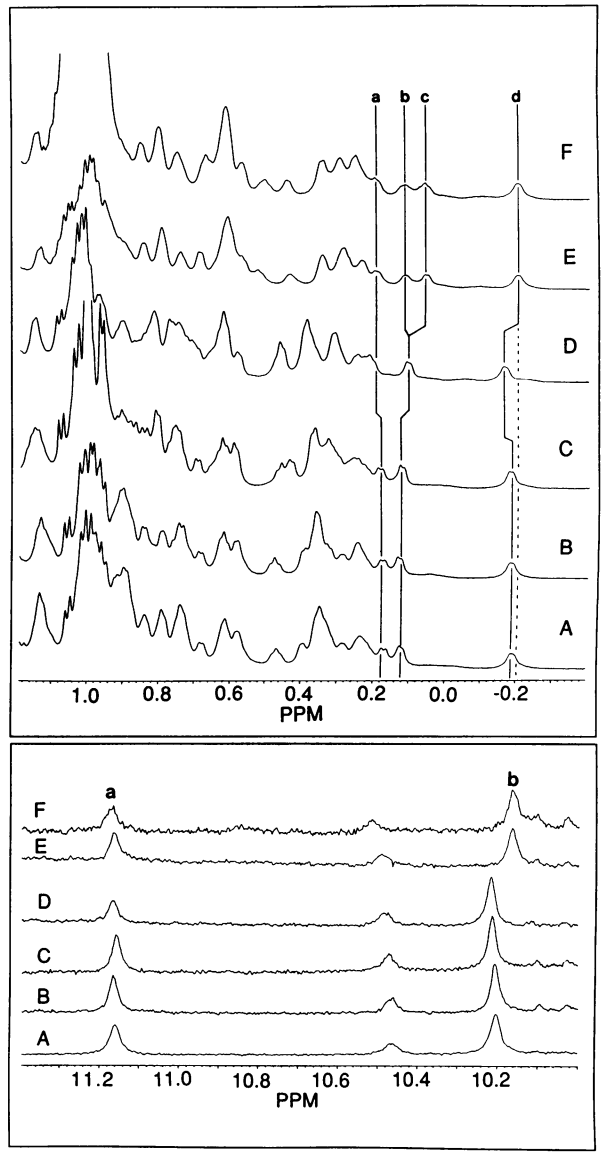

Fig. 2. Upper panel: upfield shifted aliphatic ^{1}H-NMR resonances split in 'active' complexes. SH2 (0.24 mM) (**A**) alone or in mixture with (**B**) IRS-1 Y628 (0.72 mM), (**C**) c-Src pY527 (13.9 mM), (**D**) phosphotyrosine (12.2 mM), (**E**) IRS-1 pY628 (0.72 mM) or (**F**) PDGFR pY740 (0.72 mM). **Lower panel**: ^{1}H-NMR resonance of an SH2 tryptophan shifts in 'active' complexes. SH2 (**A**) alone or in mixture with (**B**) IRS-1 Y628, (**C**) c-Src pY527, (**D**) phosphotyrosine, (**E**) IRS-1 pY628, or (**F**) PDGFR pY740. The upfield tryptophan $N_\epsilon H$ resonance (labeled b) is assigned to Trp1 and the downfield resonance (a) to Trp3 (Booker *et al.*, 1992) (concentrations as listed above).

magnetic effect of the phosphotyrosine ring current, electrostatic effects of the tyrosyl phosphate, or a more global change in SH2 domain structure. The first two mechanisms are less likely to predominate, as indicated by control studies with SH2/c-Src pY527 and SH2/phosphotyrosine complexes (Figure 2, upper panel, spectra D and C respectively). In each of these non-specific complexes similar local magnetic and electrostatic effects are presumably introduced by phosphotyrosine occupancy of the SH2 binding site. As in the CD studies, the patterns of ^{1}H-NMR perturbations induced by 'non-activating' ligands is qualitatively distinct from those of 'activating' complexes. Qualitative differences in the SH2/phosphotyrosine spectrum, compared with the

other complexes, may reflect additional electrostatic effects of the free α-amino and α-carboxylate groups of phosphotyrosine. It is also possible that differences in the spectrum of the SH2/c-Src pY527 complex reflect unfavorable interactions with flanking hydrophilic residues (rather than hydrophobic residues of the YM/VXM motif) which may alter the geometry of the phosphotyrosine moiety in the SH2 binding site.

Tryptophan $N_\epsilon H$ aromatic resonances provide additional indicators of perturbations in SH2 structure which accompany phosphopeptide binding [Figure 2, lower panel; peak b (10.21 p.p.m.) is assigned to Trp1†; peak a (11.16 p.p.m.) is assigned to Trp3)] (Booker *et al.*, 1992). None of the peptides contain tryptophan, and no peptide amide resonances occur in this region (spectra B–F). Of the two SH2 tryptophans, Trp1 is invariant and Trp3 is generally conserved as an aromatic or hydrophobic residue. In the three-dimensional structures the Trp1 side chain is buried and participates in anchoring the first β-strand, and would be expected to be sensitive to the orientation of α-helix 1 (Phe172 and Val174 of the highly conserved FLVR sequence pack against Trp148 in the Src SH2–phosphopeptide complex; Waksman *et al.*, 1992). Neither tryptophan is predicted to contact bound phosphopeptide directly. Nevertheless, a perturbation is observed in the Trp1 $N_\epsilon H$ resonance (labeled b) in the specific SH2/IRS-1 pY628 and SH2/PDGFR pY740 complexes (Figure 2, lower panel, spectra E and F, respectively), whereas no significant change is observed in the Trp3 $N_\epsilon H$ resonance (labeled a). No such perturbation is observed in the SH2 spectrum upon addition of phosphotyrosine (D), c-Src pY527 (C), nonphosphorylated IRS-1 Y628 (B), or the scrambled sequence (data not shown). As both tryptophan residues appear to be distant from the phosphopeptide binding pocket (Waksman *et al.*, 1992), specific perturbations observed with the IRS-1 and PDGFR phosphopeptides probably reflect transmitted conformational changes.

Amide proton exchange

Local and global changes in protein dynamics are conveniently monitored by the rates of amide proton exchange in freshly prepared D_2O solutions. Such exchange in the isolated SH2 domain occurs within minutes at 45°C and pD 6.0 (direct meter reading), as shown in Figure 3, lower panel, spectrum A. No change in exchange rate is seen on addition of non-phosphorylated IRS-1 Y628 (B) or c-Src pY527 (C). In contrast, addition of IRS-1 pY628 retards the exchange of ~30% of the SH2 domain amide resonances (Figure 3, upper panel). Remarkably, complete exchange requires >20 h in the bound state, yielding a >200-fold difference in exchange rates for these resonances in the free versus bound states. This marked retardation involving >30 residues cannot be entirely accounted for by the limited number of direct backbone or side chain contacts made by the phosphopeptide. Such a dramatic difference in exchange behavior between the specific complex and either the isolated SH2 or non-specific complexes with phosphotyrosine or c-Src pY527 demonstrates that specific complex formation is accompanied by non-local stabilization of amide protons involved in hydrogen bonds or otherwise buried in the complex.

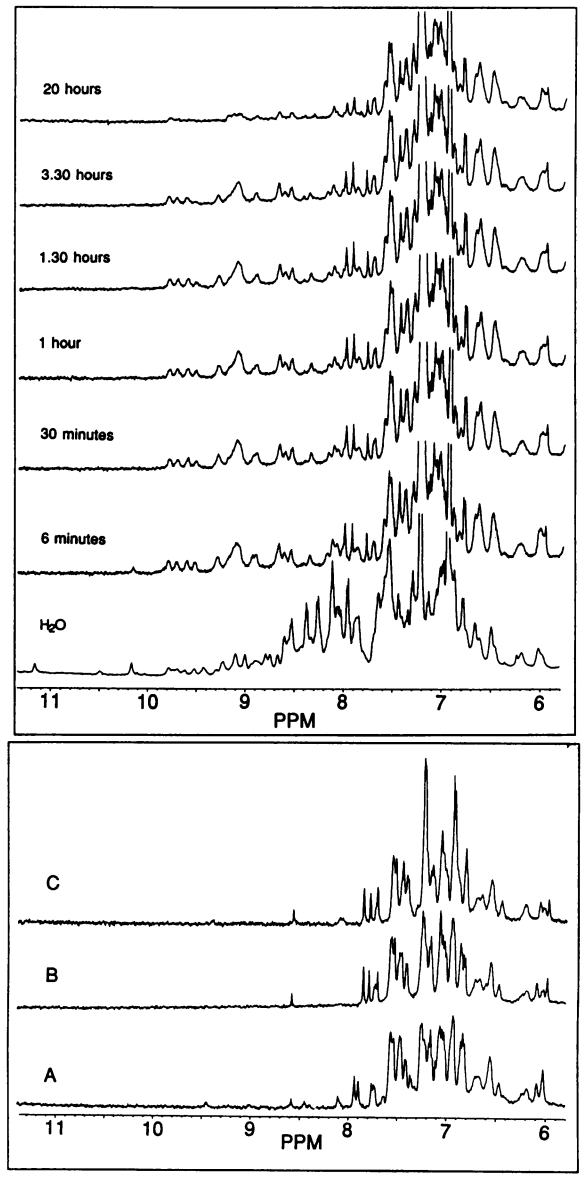

Fig. 3. Lower panel: rapidly exchanging amide protons. ^{1}H-NMR spectra of the SH2 alone (**A**) or in mixture with c-Src pY527 (**B**) or phosphotyrosine (**C**). SH2 domain, phosphotyrosine and phosphopeptide were lyophilized from H_2O and redissolved in D_2O. Spectra were obtained at 45°C within 6 min of dissolution in D_2O. **Upper panel**: retarded rate of amide proton exchange in an 'active' complex. ^{1}H-NMR spectra of the IRS-1 pY628/SH2 complex in H_2O (lowermost spectrum) or D_2O for varying times as labeled (concentration as listed in the legend to Figure 2).

Protease protection

The intact 14 kDa N-terminal SH2 domain of p85 is cleaved by 1.0 μM trypsin at 22°C within minutes into 5 and 9 kDa fragments, which undergo further proteolytic degradation. Electrophoretic separation of the intact SH2 and fragments is demonstrated in Figure 4A (the 5.4 kDa fragment is poorly visualized with Coomassie blue stain). Quantitative data obtained by densitometric scanning of the wet gels are plotted logarithmically in Figure 4C. A $t_{1/2}$ value for the first-order

†To facilitate comparisons between SH2 domains, numbering starts with the residue that defines the N-terminus of all SH2 domains, Trp1, even though the isolated protein used in this study contains 15 residues N-terminal to Trp1

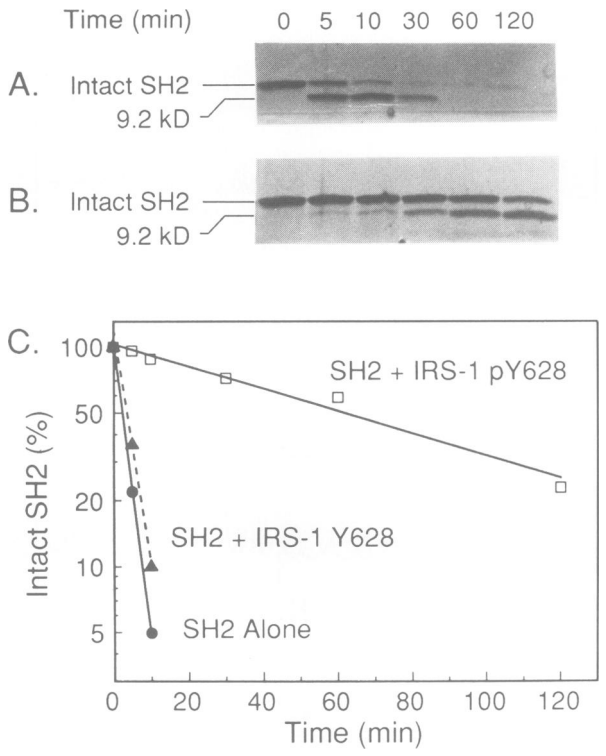

Fig. 4. Specific phosphopeptide binding protects the SH2 from proteolytic digestion. (**A**) Time course for proteolytic digestion of the N-terminal p85 SH2 by trypsin. The intact SH2 was exposed to 1.0 μM trypsin at 22°C. At the indicated times aliquots of the reaction were removed and added to Laemmli sample buffer; proteins were separated on 4–20% SDS–PAGE gels and visualized with Coomassie Blue stain. (**B**) The identical experiment was performed in the presence of 0.2 mM IRS-1 pY628 phosphopeptide. (**C**). The same experiment was performed with the matched non-phosphopeptide IRS-1 Y628 and in each case, proteins in the wet gels were quantified by densitometry. Data are plotted as percentages of initial intact SH2 domain versus time. The ordinate scale is logarithmic to allow direct calculations of first-order half-lives.

loss of intact SH2 domain in the absence of peptide was calculated to be 2.1 min. The initial site of cleavage was determined to be C-terminal to Lys31. By contrast, the identical experiment conducted in the presence of 0.2 mM IRS-1 pY628 demonstrated a dramatic protection of the SH2 domain (Figure 4B). This is reflected by the $t_{1/2}$ for proteolytic digestion of 62 min, which is ~30-fold slower than in the absence of peptide (Figure 4C). The site of initial cleavage was the same as in the absence of phosphopeptide. The corresponding matched, non-phosphorylated peptide (IRS-1 Y628) had little or no effect on inhibiting proteolysis (Figure 4C), which verifies that protection is due to SH2 binding and not a nonspecific inhibitory effect of the peptide on trypsin. Therefore, we can conclude that specific peptide binding either (i) induces regional stabilization to decrease trypsin accessibility or reactivity, or (ii) physically masks the susceptible Lys31–Met32 tryptic site. In the recently published NMR structure of the N-terminal p85 SH2 domain (Booker *et al.*, 1992), Lys31 is within a highly flexible loop between β-strands 1 and 2 (Figure 5). Such striking regional flexibility in the absence of bound phosphopeptide, in conjunction with dramatic changes observed in both amide proton exchange rate (above) and protease sensitivity following phosphopeptide binding, strongly suggests that the

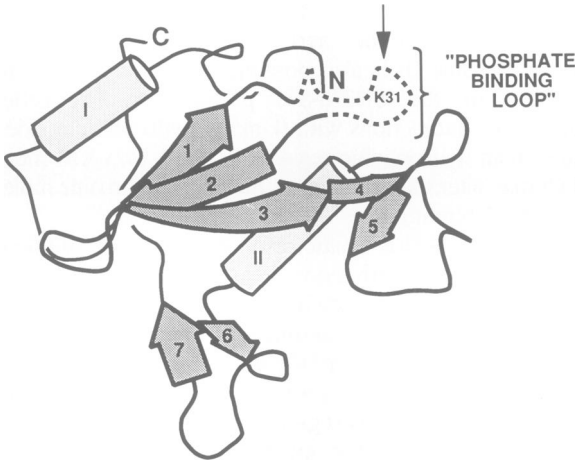

Fig. 5. Schematic representation of the N-terminal p85 SH2 domain backbone and secondary structures (Booker *et al.*, 1992). N- and C-termini are denoted by N and C, respectively; α-helices are lightly shaded cylinders numbered I and II, and β-strands are darkly shaded broad arrows numbered 1–7. The highly flexible loop between strands 1 and 2 is dashed; the position of tryptic cleavage (Lys31) is noted with an arrow.

loop between β-strands 1 and 2 is stabilized by phosphopeptide binding. Therefore, our findings support the role of this loop in phosphopeptide binding, as had been predicted for the analogous region of the Abl SH2 domain (Mayer *et al.*, 1992).

Discussion

Signal transduction by growth factor receptors and non-receptor tyrosine kinases is initiated by phosphorylation of specific tyrosine residues, both within the kinases themselves and on additional cytoplasmic substrates (Ullrich and Schlessinger, 1990). It has recently been recognized that such phosphorylations are accompanied by assembly of specific macromolecular complexes mediated by phosphoprotein–SH2 domain interactions (Cantley *et al.*, 1991; Koch *et al.*, 1991). However, an organizational problem is posed by the expression in one cell of many tyrosine kinases, substrates and possible SH2 partner proteins. Segregation of discrete signaling pathways may be accomplished through the divergent specificities of different SH2 domain–phosphoprotein pairs, as would occur if each class of SH2 domain bound selectively to a set of preferred phosphopeptide sequences. Therefore, to map distinct signaling pathways it is of critical importance to understand the key structural features that confer binding specificity. *In vitro* reconstitution of such signaling complexes for biophysical analyses is difficult, however, because the signaling molecules are typically large, unstable and membrane-associated. Fortuitously, essential features of SH2-mediated phosphoprotein assembly may be mimicked by a model system consisting of an isolated SH2 domain (~100 amino acids) and an isolated phosphopeptide (5–12 residues). In this paper we have described such a model for the interaction between PI 3-kinase and physiologically relevant partner phosphoproteins. An interesting feature of this system—in addition to directing physical association—is the regulation of PI 3-kinase enzymatic activity by

phosphoprotein coupling, an effect that can also be mimicked by corresponding phosphopeptides (Backer *et al.*, 1992; Carpenter *et al.*, 1993).

The present spectroscopic and biochemical studies were designed and performed in the absence of a known SH2 three-dimensional structure. However, while this manuscript was in preparation, four such structures were described which allow us to interpret our results further. Two structures, determined by X-ray crystallography, depict complexes between the v-Src SH2 domain and low affinity phosphopeptides (Waksman *et al.*, 1992); the structure of the Src SH2 domain in the absence of bound ligand has not been reported. Solution structures of the SH2 domains derived from the Abl oncoprotein and PI 3-kinase p85 have also been determined in the absence (but not presence) of phosphopeptides (Booker *et al.*, 1992; Overduin *et al.*, 1992a,b). The four structures share overall similarities and define a novel protein fold with comparable elements of secondary structure (seven or eight β-strands and two α-helices, e.g. see Figure 5). The α-helix content derived from these structures is $\sim 17-18\%$ of the SH2 domain, consistent with the present CD results ($\sim 13\%$ of the extended 123 residue polypeptide). CD difference curves observed in the present study cannot be ascribed to transitions between canonical secondary structures, a result which is also in accord with comparisons of liganded and unliganded structures. Nevertheless, significant structural differences are also observed between reported structures (including in the phosphate binding site).

The present study, although of low resolution, provides the first direct comparison between the same SH2 domain in the absence and presence of a bound, high affinity phosphopeptide. Evidence is presented for a specific conformational change and global dynamic stabilization induced by binding of specific phosphopeptides. We speculate that this conformational change involves, at least in part, swinging of α-helix 1 over the phosphotyrosine binding site. This appears to be a key difference between the available liganded and unliganded structures (Booker *et al.*, 1992; Overduin *et al.*, 1992a,b; Waksman *et al.*, 1992) and would rationalize transmission of an NMR perturbation to an internal tryptophan (Trp1; located N-terminal to α-helix 1) which we report here. Furthermore, protease protection and amide proton exchange assays localize a marked change in protein dynamics upon phosphopeptide binding to the loop between β-strands 1 and 2 (Figure 5), the region which exhibits greatest flexibility in the unbound p85 SH2 domain (Booker *et al.*, 1992). The corresponding loop of the Abl SH2 contains residues important for phosphoprotein binding (Mayer *et al.*, 1992). Our findings strongly support an important role for this loop in phosphoprotein binding by the p85 SH2 domain as well, which is reflected by dramatic changes in protein structure and dynamics. Moreover, comparisons between solved crystallographic (Src SH2–phosphopeptide complex) and solution (Abl SH2) structures suggest a general role for this loop in phosphotyrosine binding (J.Kuriyan and D.Cowburn, personal communication).

A novel aspect of the present study is our ability to compare the effects of phosphopeptides that do or do not stimulate enzymatic activity. As shown in Table I, phosphopeptides that correspond to known recognition motifs for PI 3-kinase (i.e. YMXM or YVXM sequences) bind most tightly to the N-terminal p85 SH2 domain and the same peptides stimulate enzymatic activity. We now show that identical peptides induce structural and dynamic changes in the isolated SH2 domain as monitored by CD, NMR and protease protection assays. By contrast, phosphopeptides which do not correspond to known PI 3-kinase recognition motifs (or phosphotyrosine itself) bind to the SH2 with lower affinity, do not stimulate enzymatic activity, and do not induce the characteristic changes in SH2 structure and dynamics. Nevertheless, both activating and non-activating phosphopeptides bind within the same site (or an overlapping site) on the SH2 domain, as all phosphopeptides tested compete with one another for binding. Therefore, whereas occupancy per se is accompanied by some changes in structure (e.g. formation of amino–aromatic interactions with bound phosphotyrosine), such binding may not be sufficient for induction of the more global changes in structure and dynamics associated with high-affinity binding by activating phosphopeptides. These results have implications for signal transduction. We propose that activating phosphoproteins induce a change in p85 SH2 domain structure which is transmitted in turn to the p110 catalytic subunit. Such a mechanism would extend the role of the SH2 domain from physical association to allosteric coupling.

Materials and methods

Peptide synthesis

An Fmoc-based strategy for sequential peptide synthesis was used in conjunction with standard side chain protecting groups as described by Domchek *et al.* (1992), Kitas *et al.* (1991) and Piccione *et al.* (manuscript submitted). N^{α}-Fmoc-O-(*O,O*-dimethoxyphosphoryl)-L-tyrosine [Fmoc-Tyr($OP(OCH_3)_2$)] was used for incorporation of phosphotyrosine (Kitas *et al.*, 1991). Peptides were purified by ether precipitation, gel filtration and preparative reversed phase HPLC (Domchek *et al.*, 1992). Analytical HPLC demonstrated that the purified products were a single component; amino acid analyses and results obtained from plasma desorption mass spectrometry were as expected.

SH2 domain–GST fusion protein expression and generation of isolated SH2 domain

Residues 321–440 of human p85 were expressed as a glutathione S-transferase (GST) fusion protein as described by Hu *et al.* (1992). Transformed *Escherichia coli* JM101 were grown to a high density (A_{600} > 1.5) in LB medium and stimulated for 12 h with 1.0 mM IPTG. The fusion protein was isolated and purified by affinity chromatography on glutathione–agarose as described by Smith and Johnson (1988), except that the fusion protein was eluted from the affinity matrix with 8.0 M urea and dialyzed against 50 mM NH_4HCO_3. To separate the GST and SH2 domain portions of the fusion protein, a 1.0 mM solution of GST–SH2 domain was reacted with 10^{-7} M TPCK-treated trypsin at 4°C. After 40 min the reaction was stopped and the mixture was separated by gel filtration on an S100HR (Pharmacia) column (1.4×100 cm) which had been equilibrated in 50 mM ammonium acetate. Fractions corresponding to pure SH2 domain (assessed by analytical HPLC) were combined and lyophilized. N-terminal sequencing (18 cycles), amino acid composition and electrospray mass spectrometric analyses were as expected for the isolated SH2 domain (Williams and Shoelson, 1993); the sequence of the SH2 domain used in these studies was: GIPGM^{-11}-NNNMSLQNAE^{-1}-WYWGDISREE^{10}-VNEKLRDTAD^{20}-GTFLVRDA-ST^{30}-KMHGDYTLTL^{40}-RKGGNNKL-IK^{50}-IFHRDGKYGF^{60}-SDPLTFS-SVV^{70}-ELINHYRNES^{80}-LAQYNPK-LDV^{90}-KLLYPVSKYQ^{100}-QDQVVKED.

Circular dichroism spectra

Ultraviolet CD spectra were recorded on an Aviv model 60H spectropolarimeter using a 1.0 mm pathlength cuvette. Samples (25 μM SH2) were dissolved in 20 mM Tris–HCl, 50 mM sodium chloride, pH 7.0. Wavelength scans were conducted at 4°C and individual data points were collected at each wavelength (nm) for 10 s. Temperature scans were conducted from 0 to 95°C; individual data points were averaged for 30 s at each temperature (°C) following a 3.0 min equilibration. Mean residue ellipticity [Θ] was calculated based on an extinction coefficient of 17 726 L mol^{-1} cm^{-1} and a calculated molecular weight of 14 172 daltons.

^{1}H-NMR

Spectra were recorded at 500 MHz at Harvard Medical School as described by Weiss *et al.* (1991). Samples were prepared in 15 mM sodium deuteroacetate, 50 mM sodium chloride, pH 5.6 or pD 6.0 (direct meter reading) and spectra were obtained at 45°C. Chemical shifts are defined relative to the methyl resonance of acetic acid, assumed to be 2.03 p.p.m.

Protease protection assays

Isolated N-terminal p85 SH2 (10 μM) was treated with 1.0 μM trypsin (Cooper) in the presence and absence of 0.2 mM IRS-1 pY628 or IRS-1 Y628 at 22°C. At the appropriate times, aliquots were removed and reactions were terminated by addition of Laemmli sample buffer. Proteins were separated by SDS–PAGE (4–20%), visualized with Coomassie Blue dye and quantified by densitometry. The site of cleavage was identified by N-terminal protein sequencing.

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Note added in proof

Similar changes in the p85 SH2 domain structure associated with phosphopeptide binding were inferred by spectroscopic studies of Panayotou *et al.* [(1992) *EMBO J.*, **11**, 4261–4272].