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Conformation of an Shc-derived phosphotyrosine-containing peptide complexed with the Grb2 SH2 domain

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Summary

We have determined the structure of an Shc-derived phosphotyrosine-containing peptide complexed with Grb2 SH2 based on intra- and intermolecular NOE correlations observed by a series of isotope-filtered NMR experiments using a PFG z-filter. In contrast to an extended conformation of phosphotyrosine-containing peptides bound to Src, Syp and PLC γ SH2s, the Shc-derived peptide formed a turn at the +1 and +2 positions next to the phosphotyrosine residue. Trp^{121}, located at the EF1 site of Grb2 SH2, blocked the peptide binding in an extended conformation. The present study confirms that each phosphotyrosine-containing peptide binds to the cognate SH2 with a specific conformation, which gives the structural basis for the binding specificity between SH2s and target proteins.

Three-dimensional structures of Src homology 2 (SH2) domains complexed with phosphotyrosine-containing peptides have been determined by X-ray crystallography (Waksman et al., 1992,1993; Eck et al., 1993; Lee et al., 1994; Mikol et al., 1995; Rahuel et al., 1996) and NMR spectroscopy (Pascal et al., 1994; Narula et al., 1995; Xu et al., 1995; Zho et al., 1995; Breeze et al., 1996). These studies show that the phosphotyrosine-containing peptides bind to SH2 at several sites. The first binding site is a conserved pocket lined by consensus basic residues which accommodates the phosphotyrosine residue. The second binding site is more variant in several SH2 domains and recognizes the specific amino acid residue(s) on the C-terminal side following the phosphotyrosine residue. For example, Src family SH2 domains (Waksman et al., 1992, 1993; Eck et al., 1993; Mikol et al., 1995; Xu et al., 1995) have a specific hydrophobic pocket which accommodates a hydrophobic residue at the +3 position, thus preferring an isoleucine residue at this position. In contrast, the SH2 domains of PLCγ (Pascal et al., 1994) and the Syp tyrosine phosphatase (Lee et al., 1994) recognize at least five, primarily hydrophobic, residues which fit into an extended hydrophobic groove running over the ligand-binding surface. This suggests that the surface topography of the SH2 domains can be altered with resulting changes in specificity, while conserving the structure of the central core of the domain.

Growth factor receptor bound protein 2 (Grb2; Lowenstein et al., 1992) is an adaptor protein with a domain structure of SH3-SH2-SH3. The two SH3 domains of Grb2 bind to proline-rich sequences in the carboxyl terminal region of the nucleotide exchange factor, Son of sevenless (Sos) protein in cytosol (Buday and Downward, 1993). Upon EGF stimulation, Grb2 SH2 binds to the EGF receptor directly or indirectly through proteins such as Shc, FAK, Syp and IRS-1 by recognizing the phosphotyrosine-containing sequences and relocates Sos to interact with Ras (Rozakis-Adcock et al., 1992; Skolnik et al., 1992; Buday and Downward, 1993; Pronk et al., 1994; Schlaepfer et al., 1994). Thus, Grb2 mediates signal transduction from EGF to Ras. The consensus phosphotyrosine-containing sequence for Grb2 SH2 binding has been

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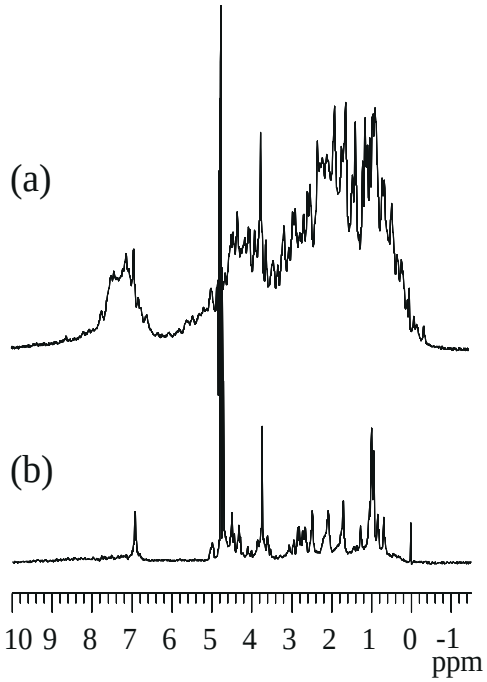

Fig. 1. 1D NMR spectra of the complex between $^{13}C/^{15}N$-labeled Grb2 SH2 and the unlabeled Shc peptide. (a) Without PFG z-filter; (b) with PFG z-filter.

extensively studied by peptide library screening (Songyang et al., 1994) and is now accepted as pTyr-(Leu/Val)-Asn-(Val/Pro), where Asn at +2 is essential, implying that Grb2 SH2 recognizes the phosphotyrosine-containing sequence in a specific manner.

Recently, the NMR solution structure of free Grb2 SH2 was reported (Thornton et al., 1996). The phosphotyrosine binding pocket of Grb2 SH2 was similar to those of other SH2 domains. However, the side chain of Trp^{121} (EF1) in Grb2 occupied the +3 binding pocket in Src SH2, so that the phosphotyrosine-containing peptide did not seem to bind in an extended conformation. The X-ray crystal structure of Grb2 SH2 complexed with the ligand peptide derived from BCR-Abl demonstrated that the peptide formed a β-turn at positions +1 and +2 (Rahuel et al., 1996). These studies prompted us to investigate the three-dimensional structure of a phosphotyrosine-containing peptide derived from Shc (313–325; Asp-Asp-Pro-Ser-pTyr-Val-Asn-Val-Gln-Asn-Leu-Asp-Lys, abbreviated Shc peptide) complexed with Grb2 SH2. Shc is a physiologically relevant target protein for Grb2 SH2, so that we can elucidate the structural basis for recognition of this phosphotyrosine-containing peptide. Although a detailed spectral and structural analysis of the complex will be reported elsewhere, we will focus here on the conformation of the Shc peptide and its interaction mode with Grb2 SH2.

Grb2 SH2 (residues 58–159) cloned into a pGEX-4T-2 vector was expressed as a GST fusion protein in *Escherichia coli* BL21 (DE3). For preparation of the $^{13}C/^{15}N$-labeled protein, transformed cells were grown in M9 minimal medium containing $^{15}NH_4Cl$ (1 g/l) and [*ul* ^{13}C] glucose (2 g/l). GST fusion protein was purified using Glutathione Sepharose 4B beads (Pharmacia, Uppsala, Sweden) and was cleaved with trypsin. Grb2 SH2 was further purified by a Mono S column (Pharmacia) and a Superose 12 column (Pharmacia). The Shc peptide was synthesized by the solid-phase Fmoc strategy using Na-Fmoc-*O*-(*O*,*O*-dimethoxyphosphoryl)-L-tyrosine (Fmoc-Tyr($OP(OMe)_2$) (Watanabe Chem. Co. Ltd., Hiroshima, Japan). The synthesized peptide was purified by reverse-phase HPLC on a Resource RPC column (Pharmacia). Both Grb2 SH2 and the Shc peptide were characterized by mass spectrometry and amino acid sequence analysis.

NMR samples were prepared in buffers containing 20 mM potassium phosphate, 150 mM NaCl, 5 mM DTT-d_{10} and 0.5% (w/v) NaN_3 dissolved in 90% H_2O/10% D_2O or D_2O at pH 6.3 (direct pH meter reading) at a concentration of 1.0–1.5 mM, where the protein concentration was estimated by absorbance at 280 nm using a molar extinction coefficient of 14 000 $M^{-1}\,cm^{-1}$. The phosphotyrosine-containing peptide (1.5 mol equiv) was added to a 250 µl sample solution in a restricted volume NMR tube (Shigemi, Inc., Tokyo, Japan).

All NMR spectra were recorded at a sample temperature of 28 °C on Varian Unity-plus 600 and Unity 500 spectrometers equipped with three rf channels and a pulsed field gradient triple-resonance probe with an actively shielded z gradient coil. Proton chemical shifts were referenced to internal DSS (0 ppm). ^{13}C and ^{15}N chemical shifts were referenced indirectly to liquid NH_3 and DSS according to Bax and Subramanian (1986). In addition to the standard double and triple resonance 2D, 3D and 4D NMR experiments (Cavanagh et al., 1996), a series of isotope-filtered experiments was recorded including 2D ^{13}C-filtered COSY, ^{13}C-filtered TOCSY, [F1,F2] ^{13}C-filtered NOESY and 3D [F1] ^{13}C-edited, [F3] ^{13}C-filtered NOESY (Ogura et al., 1996), with the $^{13}C/^{15}N$-labeled protein complexed with the unlabeled peptide in D_2O solution. An [F1,F2] $^{13}C/^{15}N$-filtered 2D NOESY experiment was also performed in H_2O solution, where the S-SS spin-echo pulse sequence was used for the observing pulse. In the S-SS spin-echo sequence, a 313.8 µs S pulse (3.08 kHz strength) and a 517.2 µs SS pulse (1.23 kHz strength) were applied as 180° and 90° pulses, respectively. To avoid spin diffusion, a NOESY mixing time of 100 ms was used. The 2D homonuclear correlated spectra were processed with standard VNMR (Varian Instruments, Palo Alto, CA, U.S.A.) software. Other spectra were processed with the NMRPipe package (Delaglio et al., 1995) using linear prediction in the ^{15}N and/or ^{13}C dimensions of the 3D and 4D spectra.

The 3D structure of Grb2 SH2 complexed with the Shc peptide was calculated based on 1427 NOE-derived protein–protein distance restraints, 30 ϕ torsion angle restraints and 38 hydrogen bond restraints. In addition, a

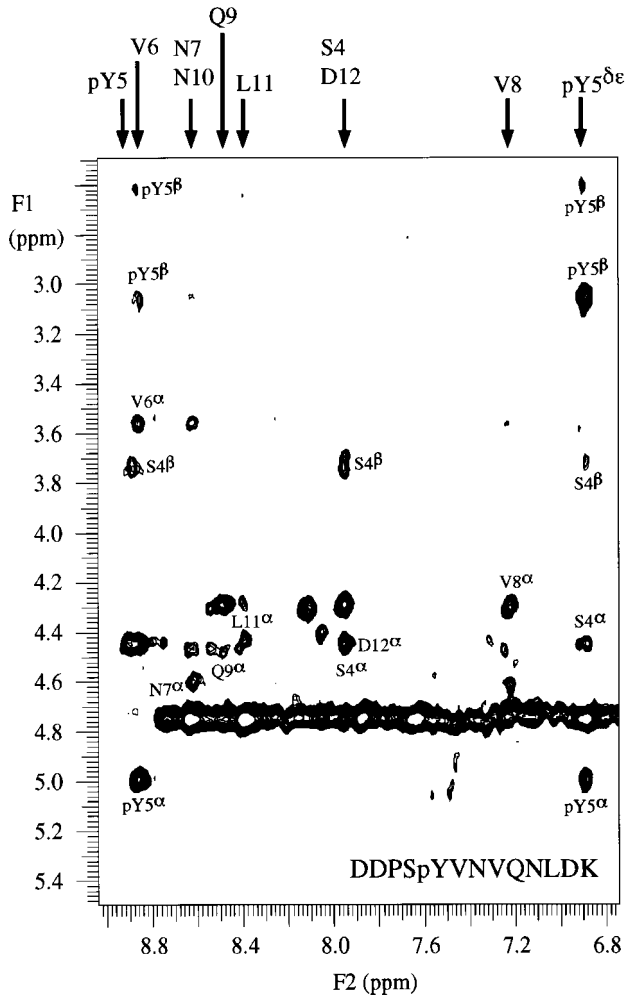

Fig. 2. $C^{\alpha}H$-NH region of the [F1,F2] $^{13}C/^{15}N$-filtered 2D NOESY spectrum recorded with the unlabeled Shc peptide complexed with the $^{13}C/^{15}N$-labeled Grb2 SH2 domain in 90% H_2O solution at pH 6.3 and 28 °C. Inter- and intraresidue NOE signals corresponding to the unlabeled Shc peptide are shown.

total of 89 intermolecular distance restraints between SH2 and the Shc peptide and 133 distance restraints within the Shc peptide were included in the calculation. Calculations with X-PLOR v. 3.1 were achieved on a Silicon Graphics Impact workstation.

The resonance assignments of $^{13}C/^{15}N$-labeled Grb2 SH2 complexed with the unlabeled Shc peptide were made by a series of triple resonance 3D and 4D NMR experiments (Cavanagh et al., 1996). At first, the backbone resonance assignments of Grb2 SH2 were made by sequential walks in the HNCA (Kay et al., 1990; Grzesiek and Bax, 1992a), HN(CO)CA (Bax and Ikura, 1991; Grzesiek and Bax, 1992a), CBCANH (Grzesiek and Bax, 1992b) and CBCA(CO)NH (Grzesiek and Bax, 1992c; Muhandiram and Kay, 1994) spectra using home-written software (H. Hatanaka, unpublished results). The walk started at several spin systems assigned to specific amino acid types on the basis of the characteristic $^{13}C^{\alpha}/^{13}C^{\beta}$ chemical shifts. The peptide fragments connected by the walk were unambiguously assigned to specific sequences in Grb2 SH2 considering the alignments of the amino acid types. The assignments of the side-chain resonances were subsequently made by analysis of the C(CO)NH (Grzesiek et al., 1993), HN(CA)HA (Clubb et al., 1992; Seip et al., 1992), HBHA(CO)NH (Grzesiek and Bax, 1992c), 3D/4D HCCH-TOCSY (Bax et al., 1990) and 3D ^{15}N-edited NOESY (Marion et al., 1989) spectra.

The NMR resonances of the unlabeled Shc peptide complexed with labeled Grb2 SH2 were assigned using a suite of PFG-z filtered pulse sequences (Ogura et al., 1996). Figure 1 shows the 1D NMR spectra of the complex with and without isotope filter. Using the PFG z-filter together with the wide-band ^{13}C inversion pulse, both aromatic and aliphatic proton resonances directly bonded to ^{13}C were completely filtered out and the proton resonances derived from the unlabeled peptide were observed as shown in Fig. 1b, demonstrating the high filtering efficiency and the wide-band nature of the present pulse sequence. For a sample in D_2O solution, ^{13}C-filtered COSY, ^{13}C-filtered TOCSY and [F1,F2] ^{13}C-filtered 2D NOESY spectra were applied. $^{13}C/^{15}N$-filtered TOCSY and [F1,F2] $^{13}C/^{15}N$-filtered NOESY were applied for the H_2O sample (90% H_2O/10% D_2O), where the S-SS spin-echo pulse sequence (Smallcombe, 1993) was employed to eliminate the solvent-derived signal. The spin systems of individual amino acid residues in the Shc peptide were identified using ^{13}C-filtered COSY, ^{13}C-filtered TOCSY in D_2O and $^{13}C/^{15}N$-filtered TOCSY in H_2O. The sequential resonance assignments were subsequently obtained using NOE connectivities observed from [F1,F2] $^{13}C/^{15}N$-filtered 2D NOESY in H_2O. In addition to the high suppression efficiency of the filtered experiments for $^{13}C/^{15}N$-bound proton resonances, the solvent suppression by S-SS spin-

TABLE 1
PROTON RESONANCE ASSIGNMENTS (ppm) OF AN SHC-DERIVED PHOSPHOTYROSINE-CONTAINING PEPTIDE COMPLEXED WITH THE Grb2 SH2 DOMAIN AT 28 °C, PH 6.3

Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	Others
Asp[1]		4.27	2.82, 2.66	
Asp[2]		4.31	2.07, 1.75	
Pro[3]		4.96	2.77, 2.49	$C^{\gamma}H$ 2.07, 1.75; $C^{\delta}H$ 3.84, 3.72
Ser[4]	7.96	4.47	3.76, 3.72	
pTyr[5]	8.91	5.02	3.08, 2.64	$C^{\delta}H$ 6.91; $C^{\epsilon}H$ 6.91
Val[6]	8.87	3.58	0.93	$C^{\gamma}H_3$ 1.03, 0.66
Asn[7]	8.64	4.62	2.04, 1.38	
Val[8]	7.23	4.31	2.15	$C^{\gamma}H_3$ 0.96, 0.82
Gln[9]	8.50	4.52	2.19, 2.09	$C^{\gamma}H$ 2.46
Asn[10]	8.64	4.83	2.93, 2.83	
Leu[11]	8.41	4.47	1.69	$C^{\gamma}H$ 1.69; $C^{\delta}H_3$ 0.97, 0.92
Asp[12]	7.96	4.43	2.73, 2.63	
Lys[13]	7.76	4.21	1.74, 1.43	$C^{\gamma}H$ 1.87, 1.43; $C^{\delta}H$ 1.74; $C^{\epsilon}H$ 3.05

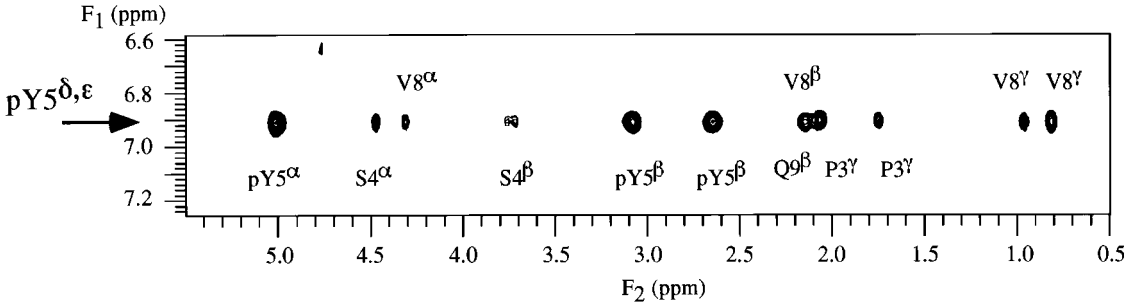

Fig. 3. Aromatic–aliphatic region of the [F1,F2] ^{13}C-filtered 2D NOESY spectrum of the unlabeled Shc-peptide complexed with the $^{13}C/^{15}N$-labeled Grb2 SH2 domain recorded in D_2O at pH 6.3 and 28 °C. Intramolecular NOE signals between aliphatic protons and the protons at 6.91 ppm are shown. These protons are assigned to H^{δ} and H^{ε} of pTyr[5].

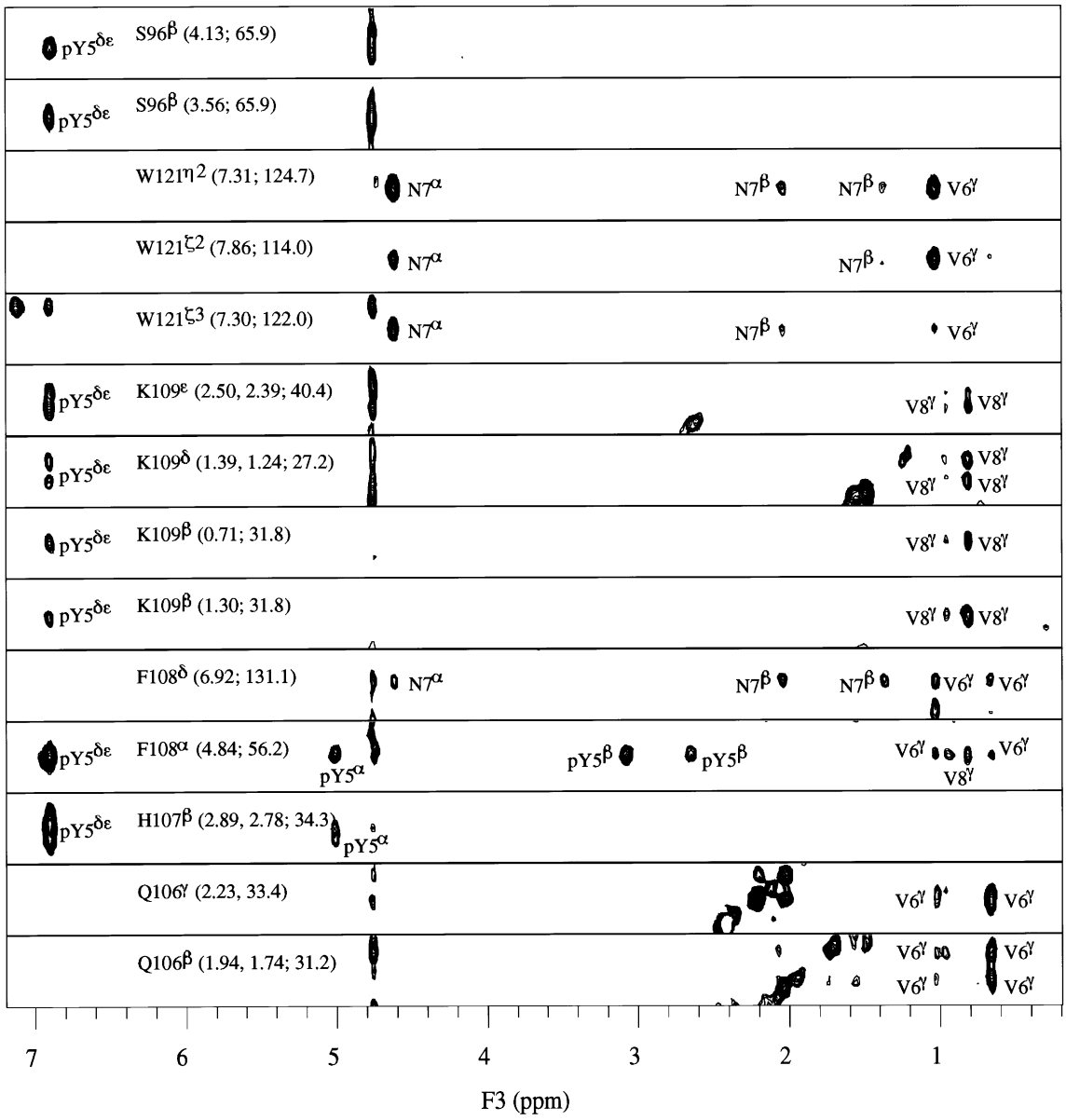

Fig. 4. Strip plots of ^{1}H-^{1}H planes extracted from the [F1] ^{13}C-edited, [F3] ^{13}C-filtered 3D NOESY spectrum of the unlabeled Shc peptide complexed with $^{13}C/^{15}N$-labeled Grb2 SH2 recorded in D_2O at pH 6.3 and 28 °C. Signals at 4.7 ppm on F3 arise from the solvent. The resonance assignments, and corresponding ^{1}H and ^{13}C chemical shifts are given in each strip. Intermolecular NOE correlations between the Shc peptide and Grb2 SH2 are annotated.

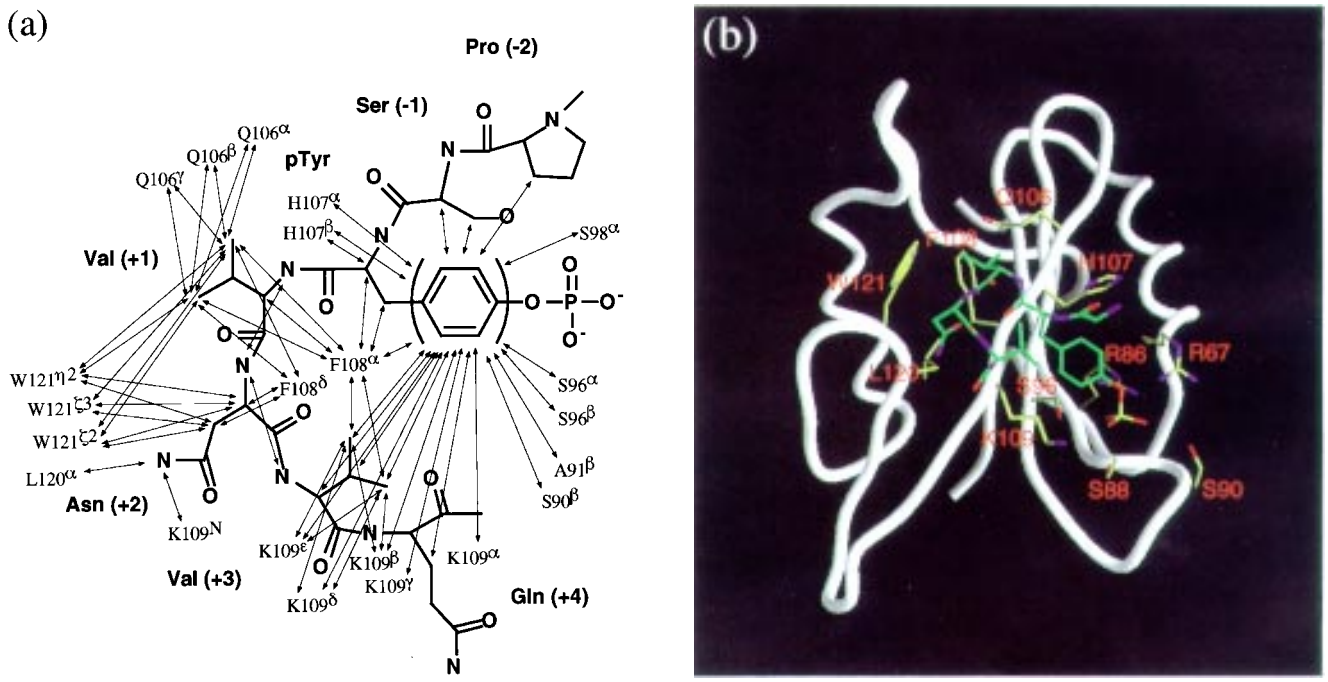

Fig. 5. (a) Schematic drawing of the intermolecular NOE interactions observed between Grb2 SH2 and the Shc peptide. The peptide residues from Pro^3 (–2) to Gln^9 (+4) are shown. A bracket on the aromatic ring of $pTyr^5$ means that the chemical shifts of $pTyr^5$ H^8 and $pTyr^5$ H^e are degenerate. The observed NOEs are shown as arrows. (b) The conformation of the Shc peptide (green) bound to Grb2 SH2 (white). The side chains of the amino acid residues in Grb2 SH2 that interact with the Shc peptide are shown in yellow. The figure was prepared using GRASP (Nicholls et al., 1991).

echo pulse sequence was sufficient to observe the NMR resonances of the unlabeled Shc peptide even in H_2O solution. Figure 2 shows the $C^{\alpha}H$-NH cross peak region of the [F1,F2] $^{13}C/^{15}N$-filtered 2D NOESY spectrum recorded on the unlabeled Shc peptide complexed with labeled Grb2 SH2 in H_2O solution, where we observe intramolecular NOE correlations in the Shc peptide and establish its sequential connectivity. Thus, sequential resonance assignments of the Shc peptide bound to Grb2 SH2 were accomplished (Table 1).

Since we have established the NMR resonance assignments of the Shc peptide and Grb2 SH2 in the complexed form, we can now investigate in detail the conformation of the Shc peptide bound to Grb2 SH2 both from intramolecular and intermolecular NOE correlations. Figure 3 shows NOE correlations from $pTyr^5$ $C^{\delta,\varepsilon}H$ resonances (overlapped) in the [F1,F2] ^{13}C-filtered 2D NOESY spectrum. We found several intramolecular NOE correlations from $pTyr^5$ $C^{\delta,\varepsilon}Hs$ to Pro^3 $C^{\gamma}H$, Ser^4 $C^{\alpha,\beta}H$, Val^8 $C^{\alpha,\gamma}H$ and Gln^9 $C^{\beta}H$ resonances, showing that the aromatic ring of $pTyr^5$ is surrounded by several residues located on both the N- and C-terminal sides. In contrast to the absence of NOE correlations between $pTyr^5$ $C^{\delta,\varepsilon}Hs$ and Val^6 $C^{\gamma}H$ (+1), strong NOE peaks observed between $pTyr^5$ $C^{\delta,\varepsilon}Hs$ and Val^8 $C^{\gamma}H$ (+3) suggested that the Shc peptide forms a turn at $pTyr^5$, Val^6 (+1), Asn^7 (+2) and Val^8 (+3).

Intermolecular NOE correlations between the Shc peptide and Grb2 SH2 were subsequently analyzed. The [F1] ^{13}C-edited, [F3] ^{13}C-filtered 3D NOESY experiment was quite helpful for this purpose, where intermolecular NOEs were observed between ^{13}C directly bonded protons and ^{12}C directly bonded protons. In this experiment, the starting proton on the labeled protein specified by the chemical shifts of a ^{13}C (F2)-^{1}H (F1) pair and the destinating proton on the unlabeled peptide specified by a ^{1}H chemical shift (F3) are correlated with each other (Fig. 4). For example, $pTyr^5$ $C^{\delta,\varepsilon}Hs$ of the Shc peptide (6.91 ppm) showed strong intermolecular NOE correlations with Ser^{96} (βC3), Lys^{109} (βD6), Phe^{108} (βD5) and His^{107} (βD4) of Grb2 SH2. Considering the conserved structure of the phosphotyrosine binding pocket and the observed intermolecular NOEs, $pTyr^5$ was expected to bind to a similar pocket as elucidated for other SH2 domains. Intermolecular NOESY correlations were identified between the residues on the βD strand and the Shc peptide. Gln^{106} (βD3) had strong NOE correlations withVal^6 (+1). Phe^{108} (βD5) showed NOEs to $pTyr^5$, Asn^7 (+2) and Val^8 (+3), as well as Val^6 (+1). These NOE correlations confirmed that the Shc peptide forms a turn at $pTyr^5$, Val^6 (+1), Asn^7 (+2) and Val^8 (+3). The aromatic side chain of Trp^{121} (EF1) had strong intermolecular NOE correlations with Val^6 (+1) and Asn^7 (+2) but not with Val^8 (+3), which seemed to block the binding of the peptide in an extended conformation. In fact, Val^8 (+3) had NOE correlations with Lys^{109} (βD6), indicating that the Shc peptide does not form an extended conformation. The intermolecular NOE

correlations between the peptide and Grb2 SH2 are summarized schematically in Fig. 5a.

The structure of the complex was calculated based on the distance restraints derived from NOE correlations, dihedral angle and hydrogen bond restraints. The structure of Grb2 SH2 was found to be quite similar to those of other SH2 domains reported so far. However, the Shc peptide binds to SH2 not in an extended conformation, but in a bent conformation at $pTyr^5$, Val^6 (+1), Asn^7 (+2) and Val^8 (+3) (Fig. 5b). Except for these residues, the structure of the peptide is not well defined, as we could not observe intermolecular NOE correlations for N-terminal residues prior to Pro^3 (–2) and C-terminal residues succeeding Gln^9 (+4). $pTyr^5$ is located on the positively charged pocket lined by the conserved basic residues such as Arg^{67}, Arg^{88} and Lys^{109}. Trp^{121} (EF1) of Grb2 SH2 occupies the (+3) binding site, which blocks the binding of the Shc peptide in an extended conformation. Furthermore, the side-chain amide protons of Asn^7 (+2) form hydrogen bonds with the backbone carbonyl groups of Lys^{109} and Leu^{120}. Thus, the presence of Trp^{121} at the EF1 site in Grb2 SH2 and Asn^7 at the +2 position in the Shc peptide make it possible to form a specific interaction which results in the turn conformation. The Src SH2 domain with replacement of Thr at EF1 by Trp was reported to switch its peptide binding affinity to resemble that of Grb2, suggesting that Trp^{121} (EF1) was a key residue which determined the specificity toward the phosphotyrosine-containing peptides (Marengere et al., 1994). In the present study, we have been able to explain the important roles of Trp^{121} (EF1) and Asn^7 (+2) for the specific recognition between Grb2 SH2 and the cognate peptide. The present results also demonstrate the wide-band nature and high filtering efficiency of the PFG-z filtered pulse scheme.

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