The Solution Structure and Intramolecular Associations of the Tec Kinase Src Homology 3 Domain*

Tec is the prototypic member of a family of intracellular tyrosine kinases that includes Txx, Bmx, Itk, and Btk. Tec family kinases share similarities in domain structure with Src family kinases, but one of the features that differentiates them is a proline-rich region (PRR) preceding their Src homology (SH) 3 domain. Evidence that the PRR of Itk can bind in an intramolecular fashion to its SH3 domain and the lack of a regulatory tyrosine in the C terminus indicates that Tec kinases must be regulated by a different set of intramolecular interactions to the Src kinases. We have determined the solution structure of the Tec SH3 domain and have investigated interactions with its PRR, which contains two SH3-binding sites. We demonstrate that in *vitro*, the Tec PRR can bind in an intramolecular fashion to the SH3. However, the affinity is lower than that for dimerization via reciprocal PRR-SH3 association. Using site-directed mutagenesis we show that both sites can bind the Tec SH3 domain; site 1 (155KTLPPAP161) binds intramolecularly, while site 2 (163KRRPPPPIP174) cannot and binds in an intermolecular fashion. These distinct roles for the SH3 binding sites in Tec family kinases could be important for protein targeting and enzyme activation.

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Tec is an intracellular tyrosine kinase belonging to a family that includes Btk, Itk, Txx, and Bmx (1–4). Tec family kinases are modular proteins consisting, from the N terminus, of a pleckstrin homology domain, a Tec homology domain, comprising a zinc-binding Btk motif (5) and an adjacent proline-rich region (PRR), a Src homology (SH) 3 domain, a SH2 domain, and a kinase domain (6). The SH3, SH2, and kinase domains of Tec are very similar to those in the Src family kinases, while the pleckstrin homology, Btk motif, and PRR are unique to Tec family kinases (5, 6). In addition, there are several other significant differences. Unlike Src, Tec kinases contain N-terminal fatty acid attachment sites responsible for localizing the proteins to the cytoplasmic membrane (7). The pleckstrin homology domains of Tec kinases could function in a similar fashion by targeting them to the membrane in response to environmental conditions (8). Src kinases also possess a C-terminal regulatory tyrosine, which binds in an intramolecular fashion to its SH2 domain in the inactive form of the kinase, and this feature is absent in Tec family proteins.

There are two major isoforms of the Tec kinase: the full-length TecIV and a version, TecIII, generated by alternate splicing of exon 8 (9). Tec III protein lacks the C-terminal 22 amino acids of the SH3 domain, which is likely to compromise its ligand binding ability. The biological significance of this protein is unknown but it is of note that a comparable deletion in Btk causes the disease X-linked agammaglobulinemia (10). Expression of Btk and Itk is restricted to B-cells and T-cells, respectively, and Tec has been suggested to function in an equivalent manner in myeloid cells (11). However, Tec is expressed in a broad range of tissues in embryonic and adult mice (9). Tec has been shown to bind a number of proteins, including gp130 and c-Kit, implicating Tec in signaling downstream of these receptors as well as in the growth and differentiation of hematopoietic cells (12, 13).

The three-dimensional crystal structures of the Src family kinases c-Src and Hck indicate that they are maintained in an inactive form via two intramolecular interactions (14–16). Phosphorylation of the residue equivalent to Tyr527 of avian Src allows it to form an intramolecular association with the SH2 domain. The second interaction involves the SH2 kinase linker region forming a polyproline type-II helix, which binds to the SH3 domain. While the interaction between the SH2 kinase linker and the SH3 domain may be conserved in the Tec family (17), the Tec family kinases cannot be regulated by an interaction involving a C-terminal phosphoryrosine. Studies on the Tec family member Itk (18) demonstrated an intramolecular association between the PRR and the adjacent SH3 domain. As shown in Fig. 1, all Tec family members have at least one potential class I (+XXPXXP) SH3 ligand consensus sequence within their PRRs. Btk has two potential class I binding sites in its PRR: 196KPLPTPT192 (peptides containing this sequence bind Lyn, Fyn, and Hck (19, 20) and Btk SH3 (21)) and...

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Tec SH3 Domain Structure and Intramolecular Ligand Binding

FIG. 1. Comparison of Tec family PRR sequences. A, Sequence alignment of the PRR of the 5 Tec family members in both humans and mouse, showing the presence or two SH3-ligand consensus sequences (PRS-1 and PRS-2). The residue numbers for each sequence are shown in parentheses. The key residues for a class-I SH3-ligand (K/R)xPxFxF are shown as white text on black. The asterisks highlight that the BMX PRS-2 sequences represent an incomplete match to the consensus and binding to SH3 domains has not been experimentally confirmed. The alignment was generated using the program MOLMOL (29). B, Alignment of the N-terminal portion of the mouse Tec PRR-SH3 proteins used in this study. The N-terminal Gly-Ser are additional residues remaining after thrombin cleavage of the GST fusion partner. PRR-SH3 wt represents the wild type mouse sequence, PRR-SH3A1 represents a single P158A mutation disrupting the PRS-1 consensus. PRR-SH3A2 represents four P168A/P169A/P170A/P171A mutations disrupting the PRS-2 consensus. PRR-SH3A12 represents a combination of both these mutants. The mutated Ala residues are highlighted by white text on black. SH3 represents the N terminus of the protein sample used to generate the three-dimensional structure.

200KPLPPEP206 for which surprisingly, no SH3 binding has been demonstrated. It has been proposed that an intramolecular association between the Btk SH3 domain and the PRR sequesters the Tec homology domain, restricting its availability to other cellular ligands and ensuring basal enzyme activity (21). Recently, it has been shown that the PRR of Btk mediates dimer formation, however, the question of which motif mediates self-association was not addressed (22). Murine Tec PRR contains one isolated proline-rich sequence: 155KTLPPAP161 (PRS-1) and two additional class I sequences: 165KRRPPPPIPP174 PRS-2. The residue numbers for each sequence are shown in parentheses. The number of residues between the C-terminal consensus proline and the N-terminal residue of the SH3 domain are shown on the right for both PRS-1 and PRS-2. The asterisks highlight that the BMX PRS-2 sequences represent an incomplete match to the consensus and binding to SH3 domains has not been experimentally confirmed. The alignment was generated using the program MOLMOL (29). B, alignment of the N-terminal portion of the mouse Tec PRR-SH3 proteins used in this study. The N-terminal Gly-Ser are additional residues remaining after thrombin cleavage of the GST fusion partner. PRR-SH3 wt represents the wild type mouse sequence, PRR-SH3A1 represents a single P158A mutation disrupting the PRS-1 consensus. PRR-SH3A2 represents four P168A/P169A/P170A/P171A mutations disrupting the PRS-2 consensus. PRR-SH3A12 represents a combination of both these mutants. The mutated Ala residues are highlighted by white text on black. SH3 represents the N terminus of the protein sample used to generate the three-dimensional structure.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Site-directed Mutagenesis—A 260-bp region encoding Tec SH3 domain, (amino acids 181–245 in mouse Tec) was PCR amplified from a cDNA library clone (a kind gift from Dr. James Ihle, St. Judes) and then cloned into the expression plasmid pGEX-4T-2 (Amersham Bioscience, Inc.). The PRR-SH3 construct, a 300-bp fragment (amino acids 151 to 245), was also PCR amplified and cloned into pGEX-4T-2. Site-directed mutagenesis was conducted on the wild type pGEX-4T-2-PRR-SH3 construct using a QuikChange site-directed mutagenesis kit (Stratagene) essentially as per the manufacturer’s instructions to mutate specific Pro residues to Ala. PRR-SH3A1 contains a Pro-Ala mutation of residue 158 within PRS-1, PRR-SH3A2 contains Pro-Ala mutations of residues 168–171 within PRS-2, and PRR-SH3A12 harbors both sets of mutations (Fig. 1). Correct mutagenesis and cloning was confirmed by DNA sequencing.

Protein Production—Unlabeled and 15N-labeled samples of Tec SH3. Tec PRR-SH3wt, Tec PRR-SH3A1, Tec PRR-SH3A2, and A12 were prepared essentially as described for the Tec SH3 in Pursglove et al. (23). The purity of protein samples was monitored throughout the purification procedure by denaturing polyacrylamide gel electrophoresis. All proteins produced were buffer exchanged into phosphate-buffered saline, pH 6.0, and concentrated to 10 mM phosphate, 0.01% NaN3, pH 6.0. Sample concentrations of 2.0 and 1.25 mM were used in this study for the unlabeled and 15N-labeled samples, respectively. NMR data were recorded on Varian Inovoa 600 and Bruker AMX-500 and DRX-600 spectrometers. The NMR experiments used to obtain the sequence-specific chemical shifts for the Tec SH3 have been described elsewhere (23). PRR-SH3wt and PRR-SH3A2 proteins were buffer exchanged into phosphate-buffered saline, pH 6.0, and concentrated to ~0.5 mM. HSQC experiments were conducted as previously stated for the SH3 domain (23).

Structural Restraints—Distance restraints were derived from a two-dimensional 1H-1H NOESY spectrum (recorded in D2O) and a three-dimensional 1H-15N HSQC-NOESY spectrum, both with mixing times of 200 ms, in an identical fashion to that described for the fourth domain of the common chain in the interleukin-3, interleukin-5, and granulocyte-macrophage colony-stimulating factor receptors (24). Hydrogen bond restraints were imposed for amide groups detectable in 1H-15N HSQC spectra recorded more than 1 h after protein, lyophilized from H2O, was dissolved in D2O. The hydrogen-acceptor distance was restrained between 1.7 and 2.2 Å and the donor-acceptor distance was restrained between 2.7 and 3.2 Å. Values of 4JHN-H and 2JHN-H were measured.
from F1 and F2 cross-peak line widths in a $^{1}H$-$^{15}N$ HSQC-J experiment (25). Torsion angle restraints for $\phi$, $\psi$-angles of $-120 \pm 40^\circ$ were imposed for $\Delta H_{\text{max}} > 8$ Hz and $-60 \pm 30^\circ$ for $\leq 5$ Hz.

**Structure Calculations**—Structures were calculated in X-PLOR (version 3.8.31) (26) using the ARIA method (27) as implemented previously (24). This restrained minimization protocol began with 40 structures calculated as defined in Table I. The 20 structures with the lowest overall energies were retained. Ten ARIA iterations followed during which the assignment parameter ($N_A$) was reduced from 1.0 to 0.75 and the donation tolerance ($V_{\text{don}}$) was reduced from 2.0 to 0 Å. Hydrogen bond restraints were included for the slowly exchanging amide groups when a single acceptor was found with suitable hydrogen bonding geometry in at least 50% of the retained structures. A total of 100 structures were calculated using the final restraint list (generated with $N_A$ of 0.75 and $V_{\text{don}}$ of 0 Å). These final restraints included 1228 NOE derived distance restraints. Of these, 956 were assigned unambiguously (468 intraresidue, 173 sequential, 55 residue and 19 long range restraints), 1566 ambiguous restraints, and 31 $\phi$, $\psi$ angle restraints. The 10 structures with the lowest overall energy were retained. Ten ARIA iterations followed during which the assignment parameter ($N_A$) was reduced from 1.0 to 0.75 and the donation tolerance ($V_{\text{don}}$) was reduced from 2.0 to 0 Å. Hydrogen bond restraints were included for the slowly exchanging amide groups when a single acceptor was found with suitable hydrogen bonding geometry in at least 50% of the retained structures. A total of 100 structures were calculated using the final restraint list (generated with $N_A$ of 0.75 and $V_{\text{don}}$ of 0 Å). These final restraints included 1228 NOE derived distance restraints. Of these, 956 were assigned unambiguously (468 intraresidue, 173 sequential, 55 residue and 19 long range restraints), 1566 ambiguous restraints, and 31 $\phi$, $\psi$ angle restraints. The 10 structures with the lowest overall energy were selected as the final ensemble.

**Structural Analyses**—Hydrogen bonding and secondary structure were analyzed using MOLMOL (29) and Ramachandran properties and torsion angle restraints for (25). Torsion angle restraints for $\phi$, $\psi$-angles have angular order parameters greater than 0.9 (192$^{\text{th}}$, $^{193}$His, $^{194}$Lys). Well defined residues were identified by iterative fitting of the C$^\alpha$ atoms to define the subset with the best defined C$^\alpha$ positions. Following each iteration the C$^\alpha$ atom with the highest RMSD was excluded prior to the next fit. At each iteration the C$^\alpha$ RMSD for the retained subset was divided by the number of residues in the subset. The set of best defined residues was the subset for which this ratio was the minimum.

**Ligand Titration Experiments**—PRS-1p was added stepwise to a 0.75 mM $^{15}$N-labeled SH3 sample to an 8-fold molar excess. $^{1}H$-$^{15}N$ HSQC spectra were recorded for each peptide:ligand ratio. Chemical shift change versus peptide concentration data for peaks that moved significantly upon ligand addition were fitted using nonlinear regression analysis ( Prism3) to the relationship described for peptide binding to the Fyn SH3 domain (31) to obtain equilibrium dissociation constants.

**BiAcore Experiments**—Surface plasmon resonance experiments were performed on a BiAcore 2000 instrument. Purified Tec SH3 domain was amine-coupled to a CM5 chip according to the manufacturer’s directions resulting in the deposition of 1500 or 3000 response units (RU) of Tec SH3 domain. The PRS-SH3 proteins were buffer exchanged into HEPES-buffered saline and concentrations ranging from 1 to 100 $\mu$M were flowed past the SH3 domain at a rate of 10 $\mu$l/min. Report points were taken preinjection, postinjection, and after 50 min of continuous flow. Sensorgram analysis was carried out using the BiAevalua (Amersham Bioscience, Inc.) software. Kinetic studies were conducted at equilibrium to determine the steady-state $K_d$ values. The concentrations used for these experiments, ranged from 1 to 85 $\mu$M for PRR-SH3wt, 1–45 $\mu$M for PRR-SH3A1, 1–25 $\mu$M for PRR-SH3A2, and 1–60 $\mu$M for PRR-SH3A12. These injections were conducted in duplicate and randomly to avoid incremental errors. It was determined that at a flow rate of 10 $\mu$l/min there were no significant changes in response units due to bulk effects. Regeneration of the SH3-coated lanes was not possible by standard methods without destroying the SH3 domain coupled to the chip. However, it was determined that 12 injections were possible over one SH3 coupled lane before a decrease in response was observed.

**Analytical Ultracentrifugation Experiments**—Sedimentation equilibrium experiments to characterize the self-association of Tec PRR-SH3 wild type and mutants were carried out on a Beckman Optima XL-A analytical centrifuge equipped with an An-60Ti rotor. Protein samples were made up in phosphate-buffered saline, pH 7.0, at concentrations corresponding to $c_{\text{protein}}$ = 0.08, 0.27, and 0.8 and $A_{280}$ = 0.8 and 0.27 (for a 1-cm path-length). Data were recorded at both 4 and 20 °C at speeds of 20,000, 30,000, 42,000, 48,000, and 56,000 rpm. Data were collected in six-sector cells as absorbance (248 and 280 nm) versus radius scans (0.001-cm increments). Scans were collected at 4-h intervals and compared to determine when the sample reached equilibrium. Analysis of the data was carried out using NONLIN (32), and the best model and final parameters were determined by examination of the residuals derived from fits to monomer, monomer $\leftrightarrow$ dimer, monomer $\leftrightarrow$ trimer, and monomer $\leftrightarrow$ tetramer models (all ideal species models). The partial specific volumes of each domain were determined from the amino acid sequences (33), and the solvent density was taken to be 1.0066 g ml$^{-1}$ at 20 °C.

**RESULTS**

**Solution Structure of Tec SH3 domain**—The solution structure of the Tec SH3 domain was determined using NMR spectroscopy with calculations carried out in X-PLOR using the ARIA methodology (27). The raw peak lists from the homonuclear two-dimensional NOESY and three-dimensional $^{1}H$-$^{15}N$ HSQC-NOESY experiments were interrogated to generate an initial restraint list of those NOEs able to be assigned unambiguously based purely on the chemical shift assignments. Ten ARIA iterations were completed then coordinate sets were refined in explicit solvent resulting in 20 final structures, the structural parameters of which are presented in Table I. The final ensemble of 20 structures is shown in Fig. 2. The protein backbone is well defined except for the termini and residues 190–195 in the RT loop, as indicated by the RMSD values plotted against residue number (Fig. 3).

The SH3 domain of Tec kinase, like those found in other proteins, has two triple-stranded anti-parallel $\beta$-sheets arranged in a $\beta$-sandwich, with the two sheets at right angles to each other (Fig. 2). The second strand is shared between the two sheets (designated as B and B’). Strand B contains a $\beta$-bulge due to hydrogen bonding of both HN Leu$^{208}$ and HN Glu$^{209}$ to the carbonyl O of Arg$^{217}$. A single turn of $\beta_1$, corresponding to Ser$^{210}$-$^{212}$ links $\beta$-strands D and E. The first sheet is made up of $\beta$-strands A (Ile$^{188}$-$^{189}$Ala$^{184}$), B (Gly$^{202}$-$^{205}$), and E (Val$^{215}$-$^{218}$) (Fig. 2). The 310 helix forms hydrogen bonds with amino acids from both of the $\beta$-sheets. The RT loop, between strands A and B, contains Tyr$^{187}$ which is conserved in a variety of SH3 domains including Tec family and Src family kinases. Tyr$^{187}$ is autophosphorylated in Tec, which renders these kinases fully active and, in the three-dimensional structure, is on the surface near the binding pocket (Fig. 4). The conformation of the RT loop in Tec SH3 is stabilized by the presence of a hydrogen bond between Phe$^{190}$ and Leu$^{217}$. By comparison, the n-Src loop between $\beta$-strands B and C, spanning amino acids Asn$^{211}$ and Leu$^{213}$, was not found to contain any hydrogen bonds.

**Peptide Binding by the SH3**—A peptide corresponding to PRS-1 (PRS-1p) was titrated into a sample of $^{15}$N-labeled SH3 domain and the change in chemical shift of resonances in the $^{1}H$-$^{15}N$ HSQC spectrum was measured as a function of ligand concentration. These data allowed us to define the interaction surface on the SH3 domain and to estimate the $K_d$ for the interaction to be $2 \times 0.3$ mM (Fig. 4). Twenty-seven backbone amide resonances (Glu$^{191}$, Val$^{194}$, Met$^{196}$, Asp$^{198}$ Phe$^{199}$, Ala$^{201}$, Leu$^{204}$, Tyr$^{206}$, Leu$^{208}$, Glu$^{210}$, Asn$^{211}$, Asp$^{212}$, His$^{214}$, Trp$^{216}$, Gly$^{226}$-$^{228}$, and Ser$^{230}$-$^{235}$) underwent chemical shift changes of more than 0.2 ppm ($^{15}$N) and/or 0.02 ppm ($^{1}H$) in the presence of 6 mM PRS-1p. Mapping these amino acids onto the structure of the SH3 domain identifies a shallow indentation on the surface of the SH3 domain for the peptide-binding site (Fig. 4C). Pro$^{229}$ lies near the center of this surface and is expected to be involved in ligand binding, however, due to its lack of a backbone NH atom it could not be detected in these experiments. Tyr$^{187}$ is located at the edge of the binding site, not integral to it, which is consistent with observations that phosphorylation of the corresponding residue in Btk has an
The SH3 domain is vital to the regulation of the tyrosine kinase activity of Src kinases due to the intramolecular contacts made in the inactive conformation (14–16). Although it appears that regulation of the tyrosine kinase activity of Tec family members is different to that of Src, it is likely that the SH3 domain still plays an important role (18, 21). Deletion of the Tec SH3 domain (35) or mutation of Tyr187 result in a constitutively active form of the kinase (36).

Tec kinase has two main isoforms, with TecIII lacking the C-terminal 22 amino acids (223–245) of the SH3 domain relative to Tec. To understand the structure and function of the Tec SH3 domain, structural statistics were obtained (Table I).

**Table I: Structural statistics for Tec SH3 domain**

| Statistic                                      | Value                          |
|-----------------------------------------------|--------------------------------|
| RMSD from experimental distance restraints    |                                |
| 956 Unambiguous (Å)                           | 0.022 ± 0.002                  |
| 272 Ambiguous (Å)                             | 0.021 ± 0.005                  |
| 17 Hydrogen bond (Å)                          | 0.023 ± 0.007                  |
| 31 Dihedral angle (°)                         | 0.48 ± 0.3                     |
| RMSD versus average structure (Å)             |                                |
| Backbone (N, Cα, C')                          | 3.21 ± 0.90, 0.68 ± 0.13, 0.48 ± 0.06 |
| All heavy atoms                               | 3.67 ± 0.90, 1.26 ± 0.15, 1.07 ± 0.12 |
| Deviation from idealized geometry             |                                |
| Bond (Å)                                      | 0.004 ± 0.0002                 |
| Angle (°)                                     | 0.59 ± 0.04                    |
| Improper (°)                                  | 0.52 ± 0.03                    |
| Non-bonded energies in CSDX/OPLS force field (kcal · mol⁻¹) |                                |
| vdW                                           | −257.7 ± 18.7                  |
| Elec                                          | −1959.1 ± 107.6                |
| Residues in allowed ϕ/ψ regions of the Ramachandran plot (%) |                                |
| Most favored region                           | 66.7 (81.9)                    |
| Additionally allowed region                   | 27.6 (17.8)                    |
| Generously allowed region                     | 27.6 (17.8)                    |
| Disallowed regions                            | 2.4 (0.1)                      |

**Fig. 2.** NMR solution structure of the mouse Tec SH3 domain. A, stereo view of the Cα trace of the final 20 structures. Residues in β-sheet one composed of β-strands A, B, and E are shown in red, whereas residues in β-sheet two, composed of β-strands B', C, and D are shown in blue. The 3₁₀ helix is shown in magenta. The N and C termini and every tenth residue are labeled. B, stereo view showing β-strands A, B, B', C, D, and E as arrows and the 3₁₀ helix. Every tenth residue and the nSrc and RT loops are labeled. Coloring is as described in part A. The side chain of tyrosine 187 is shown in yellow.

**Fig. 3.** Secondary structure, NOE restraints, and coordinate variability. A, the sequence position of the β-strands (arrows) and 3₁₀ helix (rectangle) are shown relative to the length of the protein used in the structure determination. B, residue by residue breakdown of the unambiguous NOE restraints for the Tec SH3 domain. Intraresidue NOE restraints are shown in black, sequential restraints are shown in white, medium range restraints are shown in red, and long range restraints are shown in green. C, Cα atom RMSDs from the mean coordinates calculated following superposition of the well defined backbone N, Cα, and C' atoms.
tive to TecIV. This region includes the $\beta_2$-$\beta_3$-strands and forms part of the proline-rich ligand surface. While the biological role of TecIII is not clear, the effect of SH3 truncation is expected to have a profound impact on the structure of the enzyme and its interactions with signaling partners and cellular localization.

**PRR-SH3 Mutagenesis, Protein Production, and Preliminary Analysis**—To determine which, if any, of the SH3-binding sites in the Tec PRR can interact with the Tec SH3 domain, we generated a series of Tec PRR and SH3 domain containing proteins that harbor mutations in the PRR. Based on the results of mutating Itk Pro192 to Ala (18), mutation of Tec Pro158 to Ala (PRR-SH3$^{1}$) should inhibit formation of a polyproline type-II helix and thus prevent PRS-1 from binding (Fig. 1). The region of protein corresponding to PRS-2 of Tec (165KRRPPP174) contains four adjacent prolines (168–171) which were mutated to Ala (PRR-SH3$^{2}$) to ensure that all possible consensus sequences were abolished. Finally, both sites were mutated in the same protein to act as a control (PRR-SH3$^{12}$).

Treatment of purified recombinant GST fusion proteins with thrombin resulted in complete digestion within 2 h, suggesting the PRR region forms a flexible tail and provides easy access for the protease. This is in stark contrast to the GST-SH3 construct, which requires much longer incubation times to achieve comparable cleavage. Size exclusion chromatography produced suboptimal separation between the GST fusion partner and the wild type PRR-SH3 protein suggesting wild type PRR-SH3 protein may have been aggregating (data not shown). Initial homonuclear NMR experiments conducted on this protein resulted in a broad peak shape and a lack of $^1$H-$^{15}$N HSQC peaks, also indicative of protein oligomerization (data not shown). In contrast, preliminary NMR data collected from a $^1$H-$^{15}$N HSQC experiment on PRR-SH3$^{12}$ suggested that this protein was predominately monomeric, based on peak shape and number (data not shown).

**Surface Plasmon Resonance Analysis of Tec PRR-SH3 Proteins**—The preliminary NMR data highlighted the possibility that the SH3 consensus binding sites within the PRR have different specificities and thus may perform different functions. These proteins were analyzed using surface plasmon resonance (BIAcore) to gain a better understanding of the specificity and strength of the interactions occurring between the SH3 domain and the different components of the Tec kinase PRR.

Tec SH3 domain was covalently linked to a Biacore chip to act as an immobilized receptor for PRR-containing proteins. Accumulation of mass, represented by increasing RU values, was therefore an indicator of intermolecular interactions between immobilized SH3 domains and PRR proteins. Sensorgrams for the various PRR proteins are shown in Fig. 5 and the observed equilibrium dissociation constants are summarized in Table II. Wild type PRR-SH3 bound to the immobilized SH3 domain surface with an equilibrium dissociation constant of 68 $\mu$M, consistent with the self-aggregation observed in the preliminary NMR studies. Disruption of the PRR-1 (PRR-SH3$^{1}$) had no significant effect on the ability of the protein to bind intermolecularly to the SH3 domain surface ($K_d$, 64 $\mu$M), indicating that PRR-1 is not critical for this interaction. Disruption of PRR-2, however, had a profound effect on the ability to bind...
In this paper we describe inter- and intramolecular interactions between the SH3 domain and SH3-binding sequences present in the PRR of Tec kinase. The three-dimensional structure of the SH3 domain was determined and NMR chemical shift perturbation experiments demonstrated that a ligand representing Tec PRR-1 binds to a shallow pocket on the surface of the SH3. Using surface plasmon resonance and analytical ultracentrifugation we have shown that PRR-1 can bind intramolecularly to the SH3 domain, while PRR-2, which has a higher affinity for the SH3 domain, is unable to bind intramolecularly and instead outcompetes PRS-1, resulting in dimer formation (Fig. 7).

The presence of PRR sequences is conserved within the Tec family of enzymes. Itk PRR-SH3 contains only PRS-1 and therefore displays intramolecular SH3 binding (18). Btk and Tec have both PRS-1 and PRS-2, and the Btk PRR-SH3 region was recently shown to form dimers with a dissociation constant of 60 μM, similar to that shown here for the Tec PRR-SH3 (22). These results suggest that Tck, which lacks PRS-1 but retains a PRS-2 sequence, would be unable to form intramolecular PRR/SH3 interactions, but would be capable of intramolecular binding. The situation in Bmx is harder to predict. It has a PRR-1 sequence but no PRS-2, suggesting a situation more like Itk, however, the divergence in the sequence of the SH3 domain itself may mean that the Bmx SH3 domain has no affinity for its own PRR. It will be interesting to see whether this SH3-like region folds into the canonical β-barrel shape and binds PRR ligands in the same manner as the other Tec family members.

Recently, it has been demonstrated that a construct containing the SH3 and SH2 domains of Itk dimerizes via a novel mechanism whereby a surface on the SH2 domain, composed largely of loops from different sections of the sequence, binds to the SH3 domain in the place of more conventional proline helix ligands (37). The Kd of this SH32 monomer-dimer equilibrium was estimated to be 25 μM, which is about a factor of two tighter than the PRR-mediated dimerization of Btk and the PRS-2-mediated monomer-dimer equilibrium in Tec and it was suggested that the SH32 self-association could provide a novel mode of kinase regulation (37). Fifteen of the 33 residues of the Itk SH2 domain implicated in the dimer interface are conserved in Tec including six of nine interacting residues within the BG loop. However, given that the nonconserved residues are markedly different in character, it is difficult to predict from sequences alone whether such a mechanism may also apply to the Tec protein.

The key challenge will now be to translate these observations to the regulatory mechanisms acting in the intact enzyme. The SH3 domain of Tec plays both positive and negative roles in enzyme regulation. Deletion of the SH3 domain of Tec results in enzyme activation (35) consistent with a negative regulatory role for the SH3 domain, while truncating mutations in the Btk SH3 domain have been associated with the agammaglobulinemia disease phenotype, indicating a positive role for SH3 function (10). Activation of Tec family kinases occurs through translocation to the membrane and engagement of the pleckstrin homology domain by phosphatidylinositol lipids. Conforma-

DISCUSSION

In this paper we describe inter- and intramolecular interactions between the SH3 domain and SH3-binding sequences present in the PRR of Tec kinase. The three-dimensional structure of the SH3 domain was determined and NMR chemical shift perturbation experiments demonstrated that a ligand representing Tec PRR-1 binds to a shallow pocket on the surface of the SH3. Using surface plasmon resonance and analytical ultracentrifugation we have shown that PRR-1 can bind intramolecularly to the SH3 domain, while PRR-2, which has a higher affinity for the SH3 domain, is unable to bind intramolecularly and instead outcompetes PRS-1, resulting in dimer formation (Fig. 7).

The presence of PRR sequences is conserved within the Tec family of enzymes. Itk PRR-SH3 contains only PRS-1 and therefore displays intramolecular SH3 binding (18). Btk and Tec have both PRS-1 and PRS-2, and the Btk PRR-SH3 region was recently shown to form dimers with a dissociation constant of 60 μM, similar to that shown here for the Tec PRR-SH3 (22). These results suggest that Tck, which lacks PRS-1 but retains a PRS-2 sequence, would be unable to form intramolecular PRR/SH3 interactions, but would be capable of intramolecular binding. The situation in Bmx is harder to predict. It has a PRR-1 sequence but no PRS-2, suggesting a situation more like Itk, however, the divergence in the sequence of the SH3 domain itself may mean that the Bmx SH3 domain has no affinity for its own PRR. It will be interesting to see whether this SH3-like region folds into the canonical β-barrel shape and binds PRR ligands in the same manner as the other Tec family members.

Recently, it has been demonstrated that a construct containing the SH3 and SH2 domains of Itk dimerizes via a novel mechanism whereby a surface on the SH2 domain, composed largely of loops from different sections of the sequence, binds to the SH3 domain in the place of more conventional proline helix ligands (37). The Kd of this SH32 monomer-dimer equilibrium was estimated to be 25 μM, which is about a factor of two tighter than the PRR-mediated dimerization of Btk and the PRS-2-mediated monomer-dimer equilibrium in Tec and it was suggested that the SH32 self-association could provide a novel mode of kinase regulation (37). Fifteen of the 33 residues of the Itk SH2 domain implicated in the dimer interface are conserved in Tec including six of nine interacting residues within the BG loop. However, given that the nonconserved residues are markedly different in character, it is difficult to predict from sequences alone whether such a mechanism may also apply to the Tec protein.

The key challenge will now be to translate these observations to the regulatory mechanisms acting in the intact enzyme. The SH3 domain of Tec plays both positive and negative roles in enzyme regulation. Deletion of the SH3 domain of Tec results in enzyme activation (35) consistent with a negative regulatory role for the SH3 domain, while truncating mutations in the Btk SH3 domain have been associated with the agammaglobulinemia disease phenotype, indicating a positive role for SH3 function (10). Activation of Tec family kinases occurs through translocation to the membrane and engagement of the pleckstrin homology domain by phosphatidylinositol lipids. Conforma-

FIG. 5. BIACore analysis of wild type and mutant PRR-SH3 proteins. Sensorgrams showing duplicate injections of increasing concentrations: A, 1, 5, 10, 20, 50, and 85 μM of wild type PRR-SH3. B, 1, 5, 10, 15, 50, and 45 μM PRR-SH3Δ1. C, 1, 5, 10, 15, 20, and 25 μM PRR-SH3Δ2; and D, 1, 5, 10, 15, 30, and 60 μM PRR-SH3Δ12 interacting with a Tec SH3 biosensor surface (A and C, 1500 RU; B and D, 3000 RU) at a flow rate of 10 μl/min in HBS buffer.

the SH3 domain surface (Kd 2200 μM) indicating that this site is critical for intermolecular interactions with SH3 domains. Disruption of both PRR sites, however, results in weak binding to the immobilized SH3 domain (Kd 500 μM), suggesting that this protein undergoes a conformational change relative to the single mutations and is able to interact with the immobilized SH3 domain in a non-PRR directed manner.

These data, taken together with the PRS-1p binding experiments, indicate that both PRS-1 and PRS-2 have moderate affinities for the Tec SH3 domain, but that PRS-2 is sterically inhibited from binding in an intramolecular fashion to the SH3 domain. PRS-2 in Btk is the same distance from the SH3 domain as PRS-2 in Tec (Fig. 1). Therefore, it is very likely that PRS-2 in Btk will be unable to bind to its SH3 in an intramolecular fashion.

Analytical Ultracentrifugation Experiments—Analytical ultracentrifugation was used to further investigate the oligomerization properties of wild-type and mutant PRR-SH3 protein samples. Data recorded for these proteins at a range of sample concentrations and centrifugal forces were found to best-fit a monomer-dimer-tetramer model (Fig. 6), allowing calculation of equilibrium dissociation constants for these reactions (Table II). These values were used to calculate the fraction of each species present at a range of protein concentrations (Fig. 6), providing a graphical comparison of the aggregation properties of the wild-type and mutant proteins. PRR-SH3wt was determined to have a Kd of 125 (91–200) μM for the monomer ↔ dimer equilibrium and 0.83 (0.62–1.25) × 10^{-12} M^3 for the monomer ↔ tetramer equilibrium. PRR-SH3Δ1 has the highest proportion of tetramer at low protein concentrations, and also displays the lowest Kd (50 (33–71) μM) for the monomer to dimer transition. PRR-SH3Δ2 and PRR-SH3Δ12 form tetramer to a lesser extent, compared with PRR-SH3 wild type (Fig. 6) and, at all the concentrations tested (up to 0.1 mM), PRR-SH3Δ2 and PRR-SH3Δ12 samples were predominantly mono-
tional change induced by phosphorylation of the kinase domain by a Src family member, or some other mechanism, could then result in a partially activated enzyme with both the SH3 and PRR becoming available for binding partners. Indeed PRS-2 in Tec, Btk, and Txk (and presumably PRS-1 in Itk and Bmx) may participate in the enzyme dimerization required for cross-phosphorylation of the regulatory tyrosine (Tyr187 in Tec) as suggested by Hansson et al. (22) leading to full activation.

A key piece of the puzzle recently discovered by Kang et al. (38) is that phosphorylation of Btk serine 180 by the \( \beta \) isoform of protein kinase C (PKC\( \beta \)) is a potent negative regulator of Btk activity. Localization to the plasma membrane as well as phosphorylation at Tyr\( ^{521} \) (by Lyn) and Tyr\( ^{223} \) (Btk autophosphorylation) was reduced upon coexpression of Btk with increasing levels of PKC\( \beta \). A similar effect on Tec was also observed in their system. Interestingly this regulatory serine is conserved in other Tec family members (except for Bmx) and is adjacent to the PRR (4–6 residues N-terminal to PRS-1). The mechanism for this negative regulation has yet to be determined, but introduction of a negatively charged phosphate group near PRS-1 is likely to block interactions with SH3 domain containing binding partners. Mano et al. (39) showed that the SH3

### Table II

| PRR-SH3 | \( K_d \) monomer--dimer (μM) | \( K_d \) monomer--tetramer (μM) |
|---------|-------------------------------|-------------------------------|
| wt      | 68 (10.7)                     | 0.83 (0.58–1.25) \( \times 10^{-12} \) M³ |
| \( \Delta 1 \) | 64 (372)                     | 0.16 (0.11–0.24) \( \times 10^{-12} \) M³ |
| \( \Delta 2 \) | 2200 (4.7)                    | 40 (32–48) \( \times 10^{-12} \) M³ |
| \( \Delta 12 \) | 500 (57.4)                    | 66 (55–77) \( \times 10^{-12} \) M³ |

\( ^a \) Values in brackets are \( \chi^2 \).

\( ^b \) Values in brackets are 95% confidence limits.

### Figure 6

**A** Sedimentation equilibrium analysis of wild type and mutant PRR-SH3 proteins. **A**, residuals, expressed as absorbance versus \( r^2/2 \) for sedimentation equilibrium data of PRR-SH3 fitted to an ideal monomer-dimer-tetramer model. The residuals for three data sets corresponding to different loading concentrations are shown in different shades of gray. The fit to this model was better than to all other models tried. Similar results were obtained for the three PRR-SH3 mutants. **B**, plots of fraction of \( (\bullet) \) monomer, \( (\times) \) dimer and \( (\bigcirc) \) tetramer against total protein concentration. These plots were generated using the equilibrium constants calculated from sedimentation equilibrium data (Table II).
Intramolecular complexes are indicated by respectively. The relative propensity to form intramolecular versus cyan open circle The Tec PRR-SH3 protein is PRS-2 in monomer-dimer equilibria. PRR-SH3 sequences lacking PRS-1 or PRS-2 are denoted by three short black lines and interactions between the PRS regions and the SH3 domains arrows. The wild type structure is denoted by red blue regions. The wild type containing the PRS-1 ( ) and PRS-2 ( ) domains of Lyn can bind to the PRR of Tec, suggesting that one role for phosphorylation at Ser180 (152 in Tec) may be to inhibit enzymes. Serine-specific phosphatases in the regulation of Tec family enzymes. Such a mechanism also suggests a role for both phosphotyrosine and phosphoserine-specific phosphatases in the regulation of Tec family enzymes.

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REFERENCES

1. Sommers, C. L., Huang, K., Shores, E. W., Grinberg, A., Charlick, D. A., Knaz, C. A., and Love, P. E. (1995) Oncogene 11, 245–251
2. Tamagnone, L., Lahtinen, I., Mustonen, T., Virtaneva, K., Francis, F., Muscatelli, F., Altaldo, R., Smith, C. I., Larsson, C., and Altaldo, K. (1994) Oncogene 9, 3683–3688
3. Sileician, J. D., Morrow, T. A., and Desiderio, S. V. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11194–11198
4. Sideras, P., Muller, S., Shiels, H., Jin, H., Khan, W. N., Nilsson, L., Parkinson, E., Thomas, J. D., Branden, L., Larsson, I., Paul, W. E., Alt, F. W., V Vieira, D., Smith, C. I. and Xanthopoulos, K. G. (1994) J. Immunol. 153, 5697–5617
5. Hyvonen, M., and Saraste, M. (1997) EMBO J. 16, 3396–3404
6. Vihinen, M., Nilsson, L., and Smith, C. I. (1994) Biochem. Biophys. Res. Commun. 205, 1270–1277
7. Rosh, M. D. (1999) Biochem. Biophys. Acta 1451, 1–16
8. Kawakami, Y., Yao, L., Han, W., and Kawakami, T. (1996) Immunol. Lett. 54, 113–117
9. Merkel, A. L., Atmosukarto, I. I., Stevens, K., Rathjen, P. D., and Booker, G. W. (1999) Cytogenet. Cell Genet. 84, 132–139
10. Zhu, Q., Zhang, M., Rawlings, D. J., Vihinen, M., Hagemann, T., Saffran, D. C., Kwan, S. P., Nilsson, L., Smith, C. I., and Witte, O. N. (1994) J. Exp. Med. 180, 461–470
11. Mano, H., Mano, K., Tang, B., Koehler, M., Yi, T., Gilbert, D. J., Jenkins, N. A., Copeland, N. G., and Ilele, J. N. (1993) Oncogene 8, 417–424
12. Matsuda, T., Takahashi-Tezakura, M., Fakuda, T., Okuyama, Y., Fujitani, Y., Tsukada, S., Mano, H., Hirai, H., Witte, O. N., and Hirano, T. (1995) Blood 85, 627–633
13. Tang, B., Mano, H., Yi, T., and Ilele, J. N. (1994) Mol. Cell. Biol. 14, 8432–8437
14. Xu, W., Harrison, S. C., and Eck, M. J. (1997) Nature 385, 595–602
15. Sichert, F., Moarefi, I., and Kurzyn, J. (1997) Nature 385, 602–609
16. Williams, J. C., Weijland, A., Goulon, S., Thompson, A., Courtneidge, S. A., Superti-Furga, G., and Wierenga, R. K. (1997) J. Mol. Biol. 274, 757–775
17. Williams, J. C., Wierenga, R., and Saraste, M. (1998) Trends Biochem. Sci. 23, 179–184
18. Andreotti, A. H., Bunnell, S. C., Feng, S., Berg, L. J., and Schreiber, S. L. (1997) Nature 385, 93–97
19. Cheng, G., Yu, Z. S., and Baltimore, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8152–8155
20. Alexandropoulos, K., Cheng, G., and Baltimore, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3110–3114
21. Pesh, M. V., Tseng, S. R., Lian, C. Y., Chen, S. H., and Cheng, J. W. (1997) Proteins 29, 545–552
22. Hansson, H., Okoh, M. P., Smith, C. I. E., Vihinen, M., and Hardt, T. (2001) FEBS Lett. 489, 67–70
23. Purignot, S. E., Mulhern, T. D., Hinds, M. G., Norton, R. S., and Booker, G. W. (1998) J. Biomol. NMR 12, 461–462
24. Mulhern, T. D., Lopez, A. F., and D Mulhern, T. D., Lopez, A. F., Pfandera, R. J., Guan, C., Vandevelde, L., Vadis, M. A., Booker, G. W., and Bagley, C. J. (2000) J. Mol. Biol. 297, 989–1001
25. Wishart, D. S., and Wang, Y. (1998) J. Biomol. NMR 11, 329–336
26. Brunger, A. T. (1993) X-PLOR, A System for X-ray Crystallography and NMR, Yale University Press, New Haven, CT
27. Nilges, M., O'Donoghue, S. I., and Oschkinat, H. (1997) J. Mol. Biol. 269, 408–422
28. Linge, J. P., and Nilges, M. (1999) J. Biomol. NMR 13, 51–59
29. Koradi, R., Billeter, M., and Wuthrich, K. (1996) J. Mol. Graph. 14, 51–55
30. Mulhern, T. D., Shu, G. L., Norton, C. J., Day, A. J., and Campbell, I. D. (1997) Structure 5, 1313–1323
31. Morton, C. J., Pugh, D. J., Brown, E. L., Kahmann, J. D., Renzoni, D. A., and Campbell, I. D. (1996) Structure 4, 705–714
32. Johnson, M. L., Correia, J. J., Yphantis, D. A., and Halvorson, H. R. (1981) Biophys. J. 36, 575–588
33. Perkins, S. J. (1986) Eur. J. Biochem. 157, 169–180
34. Morrogh, L. M., Hinzkelwood, S., Costello, P., Cory, G. O. C., and Kinnon, C. (1999) Eur. J. Immunol. 29, 2269–2279
35. Yamashita, Y., Miyazato, A., Ohya, K., Ikeda, U., Shimada, K., Miura, Y., Ozawa, K., and Mano, H. (1996) Jpn. J. Cancer Res. 87, 1106–1110
36. Park, H., Wahl, M. I., Abar, D. E. H., Turck, C. W., Rawlings, D. J., Yam, C., Scharenberg, A. M., Kinet, J. P., and Witte, O. N. (1996) Immunity 4, 515–525
37. Brazin, K. N., Fulton, D. B., and Andreotti, A. H. (2000) J. Mol. Biol. 302, 607–623
38. Kang, S. W., Wahl, M. I., Chu, J., Kitaura, J., Kawakami, Y., Kato, R. M., Tabuchi, R., Tarakhovsky, A., Kawakami, T., Turck, C. W., Wittie, O. N., and Rawlings, D. J. (2001) EMBO J. 20, 5692–5702
39. Mano, H., Sato, K., Yazaki, Y., and Hirai, H. (1994) Oncogene 9, 3205–3211

Fig. 7. Schematic representation of the role of PRS-1 and PRS-2 in monomer-dimer equilibria. The Tec PRR-SH3 protein is represented as an open circle, with the ligand binding surface indicated in cyan and projecting from the domain is the N-terminal extension containing the PRS-1 (blue) and PRS-2 (red) regions. The wild type structure is denoted by wt and contains both PRS-1 and PRS-2. Mutant PRR-SH3 sequences lacking PRS-1 or PRS-2 are denoted by ΔPRS-1 and ΔPRS-2, respectively. The relative propensity to form intramolecular versus intermolecular complexes are indicated by thickness and direction of the arrows and interactions between the PRS regions and the SH3 domains are indicated by three short black lines.
The Solution Structure and Intramolecular Associations of the Tec Kinase Src Homology 3 Domain

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