Substrate Recognition by the Lyn Protein-tyrosine Kinase

NMR STRUCTURE OF THE IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF SIGNALING REGION OF THE B CELL ANTIGEN RECEPTOR*

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The immunoreceptor tyrosine-based activation motif (ITAM)1 plays a central role in transmembrane signal transduction in hematopoietic cells by mediating responses leading to proliferation and differentiation. An initial signaling event following activation of the B cell antigen receptor is phosphorylation of the CD79a (Ig-α) ITAM by Lyn, a Src family protein-tyrosine kinase. To elucidate the structural basis for recognition between the ITAM substrate and activated Lyn kinase, the structure of an ITAM-derived peptide bound to Lyn was determined using exchange-transferred nuclear Overhauser NMR spectroscopy. The bound substrate structure has an irregular helix-like character. Docking based on the NMR data into the active site of the closely related Lck kinase strongly favors ITAM binding in an orientation similar to binding of cyclic AMP-dependent protein kinase rather than that of insulin receptor tyrosine kinase. The model of the complex provides a rationale for conserved ITAM residues, substrate specificity, and suggests that substrate binds only the active conformation of the Src family tyrosine kinase, unlike the ATP cofactor, which can bind the inactive form.

The immunoreceptor tyrosine-based activation motif (ITAM)1 plays a central role in transmembrane signal transduction in hematopoietic cells. Since the recognition of this motif (1), it has been identified in the cytoplasmic domains of numerous antigen and Fc receptors, as well as certain viral proteins (2, 3). Although the ITAM occurs on many receptors, and in multiple copies in some instances, the transduced signals mediated by different ITAM regions can lead to distinct pathways. Only 6 residues of the approximately 26-residue-long sequence [(D/E)2(Y)2(L/I)] are conserved across receptors, suggesting that the functional specificity of different ITAMs may be determined by the 20 nonconserved residues (2). Neither the basis for the evolutionary pressure to conserve these 6 residues nor the structural determinants for specificity of ITAM are completely understood.

We report here the first structure determination of an ITAM region bound as a substrate to a Src family tyrosine kinase. Lyn (4, 5), one of nine members of the Src family, associates with the B cell antigen receptor following activation by antigen binding (6, 7). In an initial event of B cell signaling, Lyn preferentially phosphorylates the first tyrosine of the CD79a ITAM (8–10). Asymmetrical phosphorylation of the two tyrosines of the ITAM has also been demonstrated for phosphorylation by Fyn, another Src family kinase (11), and for in vivo phosphorylation (12). After phosphorylation, the ITAM becomes a membrane docking site for the SH2 domains of the Syk protein-tyrosine kinase to further signaling in the B cell (10, 13, 14). Src family tyrosine kinases are targets for drug discovery in allergic diseases, autoimmunity, transplantation rejection, and cancer (15, 16). As no structure of a bound substrate has been previously determined, and few natural substrates are defined for these kinases, the structure of ITP reported here is potentially a useful template in drug design efforts.

The complex between the Lyn tyrosine kinase and a 12-residue peptide derived from the ITAM region of CD79a, acetylD178ENLYEGLNLDD-NH2, was examined using exchange-transferred nuclear Overhauser effect spectroscopy (et-NOESY). The resulting NMR structure of the ITAM substrate was docked onto the crystallographic structure of an activated Src family kinase (17) for the purpose of gaining insight into ITAM specificity determinants and Src kinase function in signaling. The results suggest that ITAM substrate binds in a cleft between the two lobes of the kinase domain similar to the binding of cyclic AMP-dependent protein kinase peptide inhibitors (18). This mode of binding differs from that of a peptide substrate bound to the insulin receptor kinase (19). The complex model of ITAM-Lck provides a rationale for conservation of some of the conserved residues of the ITAM, the substrate specificity of Lyn, and suggests a possible role of substrate binding to activated Src in stabilization of the activated conformation.

EXPERIMENTAL PROCEDURES

GST-Lyn Expression—A cDNA coding for the catalytic domain of Lyn, kLyn, beginning at the codon for Arg221 and extending through the polyadenylation site, was isolated from a B cell A-zap cDNA expression library by screening with anti-phosphotyrosine antibodies. The Lyn cDNA was subcloned into the XbaI-XhoI site of the pGEX-5X vector (20) to allow expression of the kinase in Escherichia coli as a glutathione-S-transferase (GST) fusion protein. The kLyn fusion protein was isolated from lysates of isopropyl-1-thio-β-D-galactopyranoside-induced cells by chromatography on glutathione-agarose (Sigma). The GST-kLyn fusion product was eluted with two applications of 10 mM glutathione.

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1 The abbreviations used are: ITAM, immunoreceptor tyrosine-based activation motif; ITP, ITAM peptide substrate, residues 178–189; kLyn, catalytic domain of Lyn tyrosine kinase, residues 221–491; NOE, nuclear Overhauser effect; et-NOESY, exchange-transferred nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; SH, Src homology; GST, glutathione S-transferase; cAPK, cyclic AMP-dependent protein kinase; IRK, insulin receptor tyrosine kinase.

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thione in phosphate-buffered saline, dialyzed against phosphate-buffered saline, and concentrated. Protein concentration was estimated from the 280-nm UV absorbance using an extinction coefficient value of 5.3 x 10³ liters cm⁻¹ mol⁻¹.

Peptide Phosphorylation—The peptides were synthesized by the Purdue University Peptide Synthesis Laboratory with solid phase synthesis, and purified by fast protein liquid chromatography using a Pharmacia C₄-C₁₈ column and a solvent system of 0.1% trifluoroacetic acid in H₂O (solution A) and 0.1% trifluoroacetic acid in 95% HPLC-grade acetonitrile, 5% H₂O (solution B). For analysis of the in vitro peptide phosphorylation, the peptides acet-DENLYEGLNLDD-NH₂ and acet-ENLYEGLNLDDSMYEDI-NH₂ (160 µM) were incubated with expressed kLyn-GST fusion protein in reactions containing 5 mM HEPES, pH 8.4, 10 mM MnCl₂, 5 mM p-nitrophenylphosphate, 50 µM [γ³²P]ATP, and 1 mM 2-mercaptoethanol for the times indicated. Reaction components were separated by electrophoresis on an 40% alkaline-polyacrylamide gel as described (21). Phosphopeptides were recovered from the gel and incubated for 2 h with 10 µg of Staphylococcus aureus V8 protease in 50 mM NH₄CO₃, pH 7.8, at 37 °C, followed by an additional 2-h incubation with a freshly added 10 µg of protease. The resulting samples were again separated by 40% alkaline-PAGE. Phosphopeptides were visualized by autoradiography.

Enzyme Assay for Peptide Phosphorylation—Phosphorylation of peptides by kLyn-GST fusion protein and the accompanying production of ADP were monitored by enzymatic coupling to the oxidation of NADH using phosphonopyruvate, pyruvate kinase, and lactate dehydrogenase in a final volume of 1 ml. The substrate sequences recognized by Lyn determined either from a combinatorial peptide library or by phage display are also shown.

NDM Spectroscopy—TOCSY (23), ROESY (24), and et-NOESY using WATERGATE solvent suppression (25) were run at 5 °C in a Shigemi™ tube with a Varian VXR 500 or a Varian Unity Plus 600 spectrometer equipped with a pulsed z-field gradient unit. The mixing time for TOCSY was 70 ms and for ROESY was 300 ms.

Phosphorylation of CD79α ITAM peptides by kLyn. A, phosphorylation of ENLYEGLNLDDSMYEDI (18-mer) and DENLYEGLNLDD (12-mer). Peptides were phosphorylated in vitro with kLyn for the indicated times and then separated by 40% alkaline-PAGE. The migration positions of the corresponding peptides are indicated. B, location of phosphorylation sites on the ITAM 18-mer. Phosphopeptides isolated from the gel illustrated in panel A were digested with S. aureus V8 protease and the reaction products separated by 40% alkaline-PAGE. The migration of the two cleavage products are indicated.

Docking of ITP—ITP was manually docked onto the known structure of activated Lck tyrosine kinase (Protein Data Bank code 3lck (17) using the graphics program QUANTA, followed by distance-restrained molecular dynamics and energy minimization with CHARMM (31). The manual docking of ITP onto kLck was guided by known structures of the complex of cyclic AMP-dependent protein kinase (cAPK) with a peptide inhibitor and ATP (Protein Data Bank code 1atp) (18), and the insulin receptor tyrosine kinase (IRK) complex with a peptide substrate and AMP-PNP (Protein Data Bank code 1i3r) (19). The three kinase structures were superpositioned by a least-squares fit of the main chain atoms in the catalytic segment and in the core secondary structure of
the C-lobe subdomain. An NMR ITP structure was then aligned with either the cAPK or IRK peptide by superposition of the main chain atoms of the acceptor residues. These two template structures gave distinct initial positions for ITP on the kLck surface. The superposition was followed by manual, graphical manipulation of ITP to remove obvious steric conflicts.

Each of the 20 best ITP NMR structures were aligned with the coordinates for both of the two manually docked models by superposition of the ITP main chain atoms of residues 1–7. Each model was subjected to 450 steps of restrained energy minimization, followed by 5-ps molecular dynamics at an initial temperature of 500 K and cooled to 300 K over a 5-ps period, and then optimized by 150 steps of Powell energy minimization. NMR distance restraints were applied in all molecular mechanics and dynamics with a force constant of 100 kcal/mol/Å and a soft-square-well potential. The backbone atoms of kLck were harmonically constrained to their crystallographic coordinates with a force constant of 100 kcal/mol/Å², while residues with no atoms in a 10-Å radius of any ITP atom were fixed in space.

RESULTS AND DISCUSSION

ITAM Phosphorylation by Lyn in Vitro—To explore the substrate specificity of Lyn for the phosphorylation of the two ITAM tyrosine residues of CD79a, we prepared peptides of sequence acet-D178ENLYEGLNLDD-NH₂ (ITP) and acet-E179NLYEGLNLDDCSMYEDI-NH₂ to use as substrates for in vitro phosphotransferase assays. These peptides correspond in sequence to the CD79a ITAM (Scheme 1) and contain either one or both of the conserved ITAM tyrosines. Peptides were incubated in the presence of [γ-32P]ATP with the catalytic domain of Lyn (kLyn), and the phosphopeptides separated from the reaction components by electrophoresis. kLyn was capable of catalyzing the phosphorylation of both peptides (Fig. 1). Each phosphopeptide was then recovered from the gel and digested with S. aureus V8 protease under conditions in which proteolysis is restricted to the COOH termini of glutamate residues. This separates the 18-residue peptide into two distinct fragments, each of which contains one of the ITAM tyrosines (Fig. 1). Only a single phosphopeptide was generated from the 18-residue peptide, indicating that only a single tyrosine had been phosphorylated to a detectable extent. The migration of this phosphopeptide on the alkaline gel was identical to that of the phosphopeptide generated from the 12-residue peptide, which contains only the amino-terminal ITAM tyrosine. This result confirms previous results indicating that Src family kinases exhibit a strong preference for phosphorylation of the first of the two ITAM tyrosines.

ITP Structure Determination—The structure was determined of ITP bound in the active site of Lyn. ITP contains the amino-terminal ITAM tyrosine of CD79a (Scheme 1). The form of Lyn was a GST fusion protein containing the Lyn catalytic domain, kLyn. kLyn was expressed in its activated, phosphorylated form as determined by Western blotting with anti-phosphotyrosine antibodies. ITP is phosphorylated by kLyn, with $K_m$ equal to 13 μM as measured by a coupled enzymatic assay for phosphorylation (data not shown).

NMR spectra of free ITP and ITP in the presence of kLyn are shown in Fig. 2 (A and B, respectively). The broadening of the 1H resonances from ITP upon titration with kLyn is due to the...
averging of the linewidths from the bound and free states of ITP when exchange is fast with respect to chemical shift differences. Moreover, assuming diffusion-limited binding, the dissociation rate is estimated to be $10^3 \text{ s}^{-1}$. This rate is significantly faster than cross-relaxation in the complex, so that exchange-transferred NOE interactions are observed and distances may be readily estimated from et-NOE intensities (32).

That ITP binds specifically to kLyn was tested by competition with lavendustin, a high affinity, active site inhibitor of Src family tyrosine kinases (33, 34). Addition of lavendustin to a sample containing ITP and kLyn (Fig. 2C) results in reversal of the broadened ITP resonances to the narrower linewidths characteristic of the free state of ITP. This reversal by competition with lavendustin indicates that ITP binds specifically to kLyn at the high concentrations required for the NMR experiment.

Examination of the amide region of the et-NOESY spectrum shown in Fig. 2D finds a significant number of well resolved cross-peaks resulting from intramolecular NOE interactions of ITP bound to kLyn. A NOESY spectrum of ITP measured with identical parameters either in the absence of protein, or in the presence of glutathione S-transferase, showed only a small number of intraresidue NOEs between protons separated by a single dihedral angle. These control experiments confirm that the NOE interactions measured in the presence of kLyn reflect the structure of ITP bound to kLyn.

Exchange-transferred NOESY cross-peaks, categorized as strong, medium, or weak intensities, provided 107 structurally useful distance restraints for ITP, with 53 of the distance restraints derived from NOE interactions involving amide protons. Many of the NOE interactions involving Asp11 and Asp12 could not be assigned unambiguously, and therefore were not interpreted for distance restraints in the structure determination. ITAM residues 1 and 3–10 have one to five midrange NOE interactions to a non-neighboring residue. The occurrence of midrange NOE interactions over most of the peptide indicates that a reliable model of the bound structure may be obtained using the et-NOESY method of structure determination (35). The energy averaged over the 20 best in vacuo ITP structures generated by simulated annealing and restrained molecular dynamics was $-131.0 \pm 12 \text{ kcal/mol}$, and the average NOE energy was $1.4 \pm 0.24 \text{ kcal/mol}$. Interproton distances in the 20 best structures did not violate the NOE restraint distances by more than 0.2 Å, and the average number per structure of NOE violations greater than 0.1 Å was 0.6.

**NMR Structure of Bound ITP**—A representative structure of bound ITP is shown in Fig. 3A. The kinase substrate peptide binds in an irregular helix-like conformation with most of the side chains oriented in one direction. The polypeptide backbone in this enzyme-substrate complex is not extended, unlike the peptide structure of phosphotyrosine-containing peptides recognized by SH2 and phosphotyrosine binding domains.

The structure of ITP bound to kLyn for residues 1–7 and 8–12 is well determined by the NMR data, but the main chain conformation varies across Gly7 despite an NOE distance restraint between Gly7 and Asn8. Ten models from the set of 20 best in vacuo NMR structures are superimposed in Fig. 3B. The precision of the NMR structures is high when the main chain atoms of either residues 1–7 or residues 8–12 are superimposed, while the precision is lower when full-length ITP is superimposed (Table I). The average root mean square difference from the average structure when residues 1–7 are superimposed was 0.80 and 0.89 Å for all non-hydrogen atoms and main chain atoms, respectively, and for residues 8–12 was 1.76 and 0.71 Å, respectively. When full-length ITP is compared, the values were 2.60 and 1.95 Å, respectively. All structural models satisfy the Gly7-Asn8 NOE restraint. As such, the single midrange restraint on Gly7 is not sufficient for defining the backbone conformation of this residue with high precision. Since we find no experimental evidence, such as differential linewidths (36), to support actual conformational disorder in the complex, the heterogeneity in the NMR models likely reflects a limitation of the NMR data to define the bound state structure, rather than a property of that binding.

**Model for the ITAM Substrate-Kinase Complex**—Structural information on kLyn is not obtained by the et-NOE method because the linewidths of the 56-kDa kLyn-GST protein are too broad to measure accurately. To gain insight into the mechanism of recognition and enzymatic activation of Src family tyrosine kinases, the ITAM substrate-kinase complex was modeled using a known structure of a phosphorylated Src family tyrosine kinase domain, that of Lck (17). This kinase domain of Lck (kLck) is ideal for modeling the kLyn-ITP complex since Lyn and Lck have a 75% sequence identity in the catalytic tyrosine kinase domain, that of Lck (17). This kinase domain of Lck (kLck) is ideal for modeling the kLyn-ITP complex since Lyn and Lck have a 75% sequence identity in the catalytic tyrosine kinase domain, that of Lck (17). The kinase substrate peptide is shown to be reliable with this high sequence identity (37–40). Moreover, the crystallographic structure of Lck is the activated

### Table I

Average pairwise root mean square difference of main chain atoms $N$, $Ca$, and $C$ for the 20 best ITP structures

| Residues | Main chain | Heavy atom |
|----------|------------|------------|
| 1–12     | 1.95       | 2.60       |
| 1–7      | 0.69       | 0.80       |
| 8–12     | 0.71       | 1.76       |
form of the kinase domain in which Tyr^{394} (Tyr^{416} in Src numbering),\(^2\) is phosphorylated, analogous to the form of kLyn used in this NMR study.

All protein kinases have common structural features despite low sequence similarity (41). The kinase domain comprises two subdomains: a smaller amino-terminal lobe (top in Fig. 4), and a larger, COOH-terminal lobe (bottom in Fig. 4). The activation segment lies at the interface of these subdomains, and is differentially color-coded in Fig. 4. In the structure of kLck (blue), Tyr^{394/416} is phosphorylated, and the activation segment (gray) was well ordered in the initial crystallographic determinations. By contrast, in the earlier crystallographic structures of Hck or Src kinase (42–44) in the down-regulated form, where Tyr^{416} is not phosphorylated, the activation segment is disordered, although recent results (45, 46) define the conformation for this segment shown in Fig. 4 (yellow activation segment and green kinase protein).

Structures are known for three kinases with a bound peptide ligand. The initial positioning of ITP was guided by two of these two kinase-peptide complexes and the shape of the kinase surface visualized with the program GRASP (47). In the complex of cAPK, the peptide inhibitor binds partially in the cleft between the N- and C-lobes of this Ser/Thr kinase (18, 48) (Fig. 5A, left), similar to peptide binding of phosphorylase kinase (49). The peptide substrate in the tris-phosphorylated IRK complex binds in a different orientation (Fig. 5A, right), and contacts the C-lobe adjacent to the cleft with the acceptor tyrosine hydrogen-bonded to active site residues (19). Electron density is observed for only 6 of the 18 residues of the IRK peptide substrate. Thus, ITP was modeled in two orientations on the surface of kLck, one in the interlobe cleft based on the position of the cAPK peptide inhibitor (Fig. 5B, red), and a second one oriented mostly contacting the C-lobe in a fashion analogous to the IRK peptide substrate (Fig. 5B, green) (see “Experimental Procedures”). For the cleft model, ITP lies on the opposite side of the activation segment compared with the C-lobe model. Each of the 20 et-NOE structures defined in the absence of the protein was positioned in either orientation, and subjected to distance-restrained molecular dynamics and energy minimization to give 20 conformationally relaxed structures in each orientation.

The results from modeling ITP in the two orientations differed significantly. The 20 structures of ITP docked in the interlobe cleft are in good agreement with the NMR data, and the complexes have good structural properties (Table II). These cleft models satisfy the NMR restraints better than the isolated ITP structures before docking; the average E_{NOE} for the 20 docked ITP structures is 0.7 ± 0.2 kcal/mol, compared with 1.4 ± 0.2 kcal/mol, respectively. In contrast, docking of ITP to the C-lobe resulted in an average E_{NOE} of 4.1 ± 3.3 kcal/mol, in poorer agreement with the NMR data. The influence on the NOE energy for the complex is the result of the intermolecular energy of interaction. There are no added restraint energies for docking. During refinement by restrained molecular dynamics, the cleft-bound models converge to similar conformations (Fig. 5C, red) and have several common intermolecular interactions, while the C-lobe models are substantially less precise (Fig. 5C, green). ITP modeled in the cleft has good chemical and structural complementarity with the kinase; the average number of intermolecular hydrogen bonds is 10 ± 3, in contrast to 7 ± 2 for the C-lobe docked models. Steric complementarity is illustrated in Fig. 5D by color coding the surface for regions of close

\(^2\) Amino acid numbers shown in parentheses are c-Src numbering.
intermolecular contact. For ITP binding in the cleft, the blue contact surface (Fig. 5D, left) is continuous around the peptide, while the magenta surface in the case of ITP binding on the C-lobe (Fig. 5D, right) is interrupted by patches of uncolored area where the ITP and kinase residues are not in close contact. The average accessible surface area (1.4-Å probe radius) of kLck contacted by ITP is 1455 ± 180 Å² for the cleft model, and 1000 ± 230 Å² for the C-lobe model. Importantly, after refinement with ITP docked in the cleft, the acceptor tyrosine, Tyr⁵, remains in the active site, while refinement of complexes with ITP oriented on the C-lobe results in Tyr⁵ moving away from the active site and having little intermolecular contact.

Taken together, the results summarized in Table II and Fig. 5 strongly favor the cleft model for binding of ITP. The good agreement with the NMR data and the soundness of the structural features suggest that Src family kinases bind the ITAM substrate in the cleft region between the N- and C-lobes, similar to peptide binding in cAPK and unlike that in IRK, despite the closer protein structural similarity between IRK and Lck. The reason for the different orientations in substrate binding observed in the crystallographic structures for cAPK and IRK is not clear. It is of interest to note in this regard that, in the IRK complex, the peptide substrate occupies the site where the unphosphorylated activation segment is located in the down-regulated form of IRK. That is, the IRK peptide residues Asp⁹ (the P-1 position) and Tyr¹⁰ (the P site) closely mimic the IRK activation segment (11, 51) as well as the activation segment in the unphosphorylated kinase. It is also the case for the tria-phosphorylated IRK complex that cleft residues of IRK at the end of helix C (1038, 1039, and 1042) and on the activation segment (1166, 1167, and 1168) are in close contact with neighboring molecules in the crystal, which could interfere with cleft binding by a ligand by blocking access to this site. Although the different orientations of substrate binding have been considered to distinguish Ser/Thr kinases from Tyr kinases (50), the cleft model proposed here would support a general substrate binding mode that is conserved among protein kinases.

Hereafter, we consider only the cleft model of ITP bound to kLck, given the results summarized in Table II. We describe in the remainder of this section the intermolecular interactions (Table III), which occurred with high frequency in the 20 docked and conformationally relaxed structures, and illustrate these interactions using one of the models (Fig. 6).

**Catalytic Interactions**—Residues of ITP are well positioned for catalysis in the model of ITP-kLck. Fig. 6B illustrates some of the hydrogen bond interactions between ITP and the kinase active site residues. The acceptor tyrosine, Tyr⁵, interacts with Asp³⁶⁴(386) and Arg³⁶⁶(388) while Asp¹ interacts with Arg³⁶⁶(388) and Asp³⁶⁴, conserved among all known protein kinases, is thought to play the role of catalytic base during phosphoryl transfer (10). An analogous interaction was observed in the IRK kinase-peptide complex between Asp¹¹³² and the acceptor tyrosine of that peptide (19). Further, in the model of the ITP-kLck complex, Tyr⁵ is well oriented with the respect to what is known about binding the ATP cofactor. The docking and molecular dynamics refinement of ITP were done in the absence of ATP. When ATP from the cAPK structure (48) was positioned in the active site of the ITP-kLck model structure according to a least-squares superposition of the C-lobe of the kinase domain, Tyr⁵ hydroxyl was found (Fig. 6C) to be oriented in a near-optimal position for nucleophilic attack of the nucleotide γ-phosphate group. Thus, the docking of ITP to kLck resulted in an excellent model for the catalytically active complex.

**ITAM Recognition**—All ITP amino acids corresponding to fingerprint residues defining the ITAM (Glu⁵, Tyr⁷, and Leu⁸) or to residues conserved in the CD79a ITAM region (Leu⁴, Glu⁶, and Gly⁷) (11, 51) interact either through hydrogen bonding or by hydrophobic contacts with kinase residues (Table III) that are also highly conserved among Src kinases (52). That these intermolecular interactions may be important recognition determinants is supported by the binding preferences of Lyn identified by peptide libraries and phage display studies (53, 54). The combined substrate sequences indicate preferences for the position P-3, P-1, P+1, and P+3 (Scheme 1). Some of these interactions are shown in Fig. 6. We note in particular Leu⁴ (P+3), which is buried at the interface of the N- and C-lobes (Fig. 6, A and D) by hydrophobic contact with kinase residues Leu⁴(397), Gly(421), Ala(409-421), and Phe(402-424). Leu⁴ methyl resonances are shifted by 0.05 ppm upon binding, and the burial of Leu⁴ deep in a hydrophobic region of the cleft is consistent with this chemical shift perturbation. CD79a ITAM residue Leu⁴ (P-1) has hydrophobic contacts with Tyr⁵ and Phe(256-278), a highly conserved kinase residue, while Glu⁶ (P+1) hydrogen-bonds with Glu(255) (Cys(277)). The lack of conservation among the Src family kinases at this position could provide a basis for Lyn and Lck binding selectivity. We note that there is extensive intermolecular interactions between ITP and the phosphorylated activation segment. Such interaction suggests that phosphorylation of the activation segment
plays an active role in substrate binding as opposed to only eliminating a steric barrier to binding.

**ITAM Substrate Specificity**—The model of the ITP complex gives insight into the substrate specificity of Lyn for the CD79a ITAM. The ITAM includes two Tyr residues (Scheme 1). The first Tyr residue of the ITAM is the major site phosphorylated by Lyn upon activation of the signaling pathway in immune cells, while the second Tyr is less extensively phosphorylated (8–10). In *vitro*, Lyn demonstrates a strong preference for phosphorylation of the NH$_2$-terminal Tyr, P$^+3$ and P$^3$, are part of the ITAM fingerprint. A, transparent molecular surface of kLck showing the enzyme residues in contact with ITP and the steric complementarity between the substrate and enzyme. The acceptor Tyr is colored blue, Leu residues are colored yellow, and polar residues are colored red. B, view of the active site kLck residues Asp$_{364(386)}$ and Arg$_{366(388)}$ with hydrogen bonds to ITP Tyr$^a$ and Asp$^b$. ITP Glu$_3$ (P$^3$) hydrogen bonds to kLck residues Lys$_{405(427)}$ and Asn$_{446(468)}$. C, ATP (not included in the modeling of ITP to kLck) is overlaid on the ITP-kLck model showing the reasonable position for catalysis. D, ITP residues Gly$_7$ (P$^+2$) and Leu$_8$ (P$^+3$) bind in the cleft. Gly$_7$ adapts a main chain conformation energetically favorable only for glycine. The activation segment is colored dark blue, and ITP is colored red in B–D.

The lack of an effect on $K_m$ suggests that the conformational flexibility of Gly$_7$ is not critical for binding, at least within the context of the peptide model.

**Kinase Activation**—By binding in the cleft between the N- and C-lobes of the kinase domain, ITP could serve to stabilize the most catalytically active form of the enzyme. Activation of kinases appears to depend in part on the orientation of the N- and C-lobes of the kinase domain, as concluded by comparison of numerous crystallographic structures for various forms and ligation states of these enzymes (19, 45, 46, 55). If the inactive form of Hck or Src is compared with the active form of Lck, the lobe-lobe orientation of the active form being more open (Fig. 4). A least-squares fit of the N-lobe after superposition of the C-lobe requires a 13° rotation. The internal structure of each lobe remains roughly constant between active and inactive forms of Src family kinases (0.8- and 1.0-A main chain root mean square difference after superposition of either the N-lobe or C-lobe, respectively). Thus, the relative lobe movement is largely a rigid body motion. One distinction between the lobe structures is displacement of two helices: $\alpha$-C in the N-lobe (42, 43) and $\alpha$-G in the C-lobe. It is of interest to note that the modeled ITAM substrate contacts both of these helices (described below). Further, ATP binding does not appear to depend on the lobe-lobe orientation since down-regulated apo-Hck and Src complexed with an ATP analogue have similar lobe-lobe orientation. Although the exact consequence of the lobe orientations on catalysis is not fully understood, the differences observed in the crystallographic structures of various kinase forms strongly suggest that the lobe-lobe displacement is tightly coupled to activation.

The extensive interactions between the cleft-bound ITAM substrate and both lobes of the kinase domain would serve to stabilize the orientation of the two lobes most appropriate for enzymatic catalysis. Specifically, the ITAM substrate contacts the N-lobe at the nucleotide binding loop, the $\beta$3-$\alpha$C loop, and the amino terminus of $\alpha$-C. In regard to the C-lobe, extensive
contact by substrate is made with the activation segment, and the amino terminus of α-G helix. The model suggests that ITAM substrate would not bind the down-regulated kinase because the narrower width of the cleft between the two lobes, and the altered positions of α-C and α-G would not optimally accommodate the substrate. A second factor to block binding of the ITAM substrate to the inactive form of the Src family kinase is the substantial steric conflict that arises between the ITAM substrate and the activation segment of the unphosphorylated state. The conflict, illustrated in Fig. 7A by overlay of the C-lobes of inactive Hck and activated kLck, occurs between ITP and the activation segment of Hck, residues 409–411 and 422–425, near the region where the electron density is lost due to disorder in this structure. Rotation of the view in Fig. 7A and visualization of the molecular surface in Fig. 7B clearly illustrate the penetration by ITP residues of the space occupied by the Hck activation segment (colored white in Fig. 7B). This steric clash is even more extensive in the recently determined Src and Hck down-regulated structures with a fully defined activation segment.

Conclusions—As the first structure of a Src family kinase substrate bound to an active form of the enzyme, the et-NOE structure of the ITAM peptide from the B cell antigen receptor is engaged in intermolecular interactions, and the ITAM contact by substrate is associated with stabilizing the active form of the kinase, in contrast to the ATP cofactor, which binds to the orientation of the N- and C-lobes in the catalytic domain. In the cleft-binding mode, all conserved CD79a ITAM residues interact with the tyrosine residue (Table III) are engaged in intermolecular interactions, and the ITAM substrate contacts protein regions that are highly conserved among protein kinases. ITAM binding in the cleft implies that substrate recognition is associated with stabilizing the active form of the kinase, in contrast to the ATP cofactor, which bind to the inactive form of Src family kinase. The ITAM contact region spans both lobes by interactions with the phosphorylated activation segment, the nucleotide binding loop, and the α-helices C and G, all kinase regions implicated in activation or catalysis. Thus, the model of the kinase-substrate complex presented here strongly suggests that substrate recognizes the activated form of the Src family kinase with respect not only to the conformation of the activation segment, but also to the orientation of the N- and C-lobes in the catalytic domain. Recognition of a certain interlobe orientation optimal for enzymatic catalysis has implications on controlling enzymatic activity through domain-domain interactions of the catalytic domain with the SH2 or SH3 domains (45, 56). Another noteworthy outcome of the modeled complex is the predicted basis for the observed preference of Lyn to phosphorylate the amino-terminal Tyr residue in the CD79a ITAM over the carboxy-terminal Tyr. One important factor for recognition appears to be hydrogen-bonding interactions of Glu at the position P–3 from the acceptor site. Gly at the P+2 position binds with a main chain conformation that is energetically favorable only for glycine, but this feature was not essential for recognition within the context of a peptide substrate.

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