Interaction of Phosphorylated FceRIγ Immunoglobulin Receptor Tyrosine Activation Motif-based Peptides with Dual and Single SH2 Domains of p72syk

ASSESSMENT OF BINDING PARAMETERS AND REAL TIME BINDING KINETICS*

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To examine the characteristics of the interaction of the FceRIγ ITAM with the SH2 domains of p72syk, the binding of an 125I-labeled dual phosphorylated FceRIγ ITAM-based peptide to the p72syk SH2 domains was monitored utilizing a novel scintillation proximity assay. The $K_d$ for this interaction, determined from the saturation binding isotherm, was 1.4 nM. This high affinity binding was reflected in the rapid rate of association for the peptide binding to the SH2 domains. Competition studies utilizing a soluble C-terminal SH2 domain knockout and N-terminal SH2 domain knockouts revealed that both domains contribute cooperatively to the high affinity binding. Unlabeled dual phosphorylated peptide competed with the 125I-labeled peptide for binding to the dual p72syk SH2 domains with an IC50 value of 4.8 nM. Monophosphorylated 24-mer FceRIγ ITAM peptides, and phosphotyrosine also competed for binding, but with substantially higher IC50 values. This, and other data discussed, suggest that high affinity binding requires both tyrosine residues to be phosphorylated and that the preferred binding orientation of the ITAM is such that the N-terminal phosphoryshosyrosine occupies the C-terminal SH2 domain and the C-terminal phosphotyrosine occupies the N-terminal SH2 domain.

Src homology 2 (SH2)1 domains are regions of approximately

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1 The abbreviations used are: SH2 domain, src homology 2 domain; E. coli, Escherichia coli; biotin, biotinylated; ITAM, immunoglobulin receptor tyrosine activation motif; bio-dual GST-Syk SH2, glutathione S-transferase dual C-terminal SH2 domains fusion protein expressed with a biotinylation site in E. coli; b-le-cyto dual GST-SykySH2-Syk SH2, chemically biotinylated dual SH2 domains of p72syk; Nα, C-Syk SH2, Arg42 to Ala mutation; tyrosine kinase domain of p72syk, expressed with a biotinylation site in E. coli; pTyr, phosphorylated tyrosine; Yp, tyrosine phosphopeptide, KSYKDGY(v)pTGLSTRNQETY(p)ETLHKEK-NH₂; pTyr, phosphotyrosine peptide, KSYKDGY(v)pTGLSTRNQETY(p)ETLHKEK-NH₂; Yp, phosphorylated tyrosine; KSYKDGY(v)pTGLSTRNQETY(p)ETLHKEK-NH₂; Yp, phosphorylated tyrosine peptide, KSYKDGY(v)pTGLSTRNQETY(p)ETLHKEK-NH₂; Yp, tyrosine phosphopeptide, KSYKDGY(v)pTGLSTRNQETY(p)ETLHKEK-NH₂; Yp, phosphorylated tyrosine peptide, KSYKDGY(v)pTGLSTRNQETY(p)ETLHKEK-NH₂.
The binding of FcRIγ ITAM-based peptides to the dual and single SH2 domains of p72\textsuperscript{Syk}. Previously, binding of ITAM peptides to a variety of SH2 domains has been assessed by a number of different approaches including ELISA-based assays (15), surface plasmon resonance determination (Biacore) (8, 10, 17, 21, 26), and isothermal titration caloriometry (17, 25). The ELISA-based assays are generally formatted with ITAM-based peptides immobilized to ELISA plates and the SH2 domains in solution. Surface plasmon resonance determination involves immobilizing the ITAM peptides on sensor chips. An increase in binding of the SH2 domains to the immobilized peptides and the resulting increase in mass is detected by a change in the refractive index of the sensor chip surface (17). Isothermal titration caloriometry determines the extent of binding based on the entropy of the binding reaction (25). Unfortunately, published studies utilizing these techniques have shown considerable variation in the apparent affinities of binding. This variation is a consequence of experimental limitations associated with the assay parameters (17). For example, as competition-based binding assays require several washing steps which may not achieve competition equilibrium may not be achieved. Finally ELISA-says utilizing both the ELISA and surface plasmon resonance main interactions (8). Furthermore, as competition-based ascribed in the literature for certain phosphotyrosine-SH2 do-

$$\text{GGGTGCCTTCGCCCTG-3}$$

5-3

$$\text{GCCCTTCTTTTTCG-3}$$

5-3

$$\text{Fc}$$

accurately determine the affinities and the binding kinetics of the reactions: 1, 5

$$\text{GCCCTTCTTTTTCG-3}$$

5-3

$$\text{ITAM-based peptides to the dual and single SH2 do-

mains of p72\textsuperscript{Syk}, a novel scintillation proximity (SPA) based assay system was developed which circumvents the experimental limitations described above.**

**MATERIALS AND METHODS**

*Plasmid Expression Vectors—* Human p72\textsuperscript{Syk} cDNA was initially cloned by reverse transcription-PCR from the KU812 human mast cell line. Three oligonucleotide primers were then prepared on an Applied Biosystems DNA synthesizer: A, 5’-TCAGTGCCAGGACACCCCTGGCCTCCTTCTTTTGCG-3’; B, 5’-TCAGGCAATCTCCTTATTTTTGACATGGGAAGAAGAAA-3’; C, 5’-CATGGAATACCTCCTATTTTGACATGGGAAGAAGAAA-3’. Primers A and C were used to amplify a segment of human p72\textsuperscript{Syk} cDNA (47) corresponding to amino acid residues 10–261, using standard PCR methodology (48). The PCR fragment was cleaved with XhoI and BglII and then ligated with the XhoI/BamHI vector fragment of pdW363 (49) to construct pBio-dual syk SH2 (pTC1). C-terminal SH2 domain constructs were constructed by amplifying a portion of a previously constructed dual GST-Syk SH2 fusion vector with primers B and C containing the same SH2 domain amino acid residues as stated above, cleaving the PCR product with XhoI and BglII, and then ligating it with the XhoI/BamHI vector backbone of pdW363 (49). Standard recombinant DNA techniques were employed (48), and the structures of the recombinant plasmids were confirmed by dideoxy nucleotide sequencing (50).

**Mutagenesis of p72\textsuperscript{Syk} N- and C-terminal SH2 Domains—** The arginine residues 42 and 45 of the N terminus and arginine residue 195 of the C terminus SH2 domains of p72\textsuperscript{Syk} were mutated to alanines by two-step PCR mutagenesis. The following PCR primers were used in the reactions: 1, 5’-TCAGTCGCTAGATGCTCCAGGATCTACCCGCATCTGCTCCTTCCTCTTTTGCG-3’; 2, 5’-GCGGCTCTGGCCGCAGAAATTTACTCTGCTCCTTCCTCTTTTGCG-3’; 3, 5’-ATTTGCTGCGCCAGGACCCGCAG-3’; 4, 5’-TCAGTCGCTAGATGCTCCAGGATCTACCCGCATCTGCTCCTTCCTCTTTTGCG-3’; 5, 5’-ATTTGCTGCGCCAGGACCCGCAG-3’; 6, 5’-TCAGTCGCTAGATGCTCCAGGATCTACCCGCATCTGCTCCTTCCTCTTTTGCG-3’; 7, 5’-GCGGCTCTGGCCGCAGAAATTTACTCTGCTCCTTCCTCTTTTGCG-3’; 8, 5’-GCGGCTCTGGCCGCAGAAATTTACTCTGCTCCTTCCTCTTTTGCG-3’; 9, 5’-ATTTGCTGCGCCAGGACCCGCATCTGCTCCTTCCTCTTTTGCG-3’; 10, 5’-GCGGCTCTGGCCGCAGAAATTTACTCTGCTCCTTCCTCTTTTGCG-3’ (underlined bases indicate the alanine mutations).

For the N-terminal Arg-42 mutation, PCR reactions were performed using the dual SH2 domain of p72\textsuperscript{Syk} as the template with primers 1 and 2 (product A) and primers 3 and 5 (product B). Aliquots of products A and B were combined in a second PCR reaction with primers 1 and 4. For the N-terminal Arg-45 mutation, PCR reactions were performed using the dual SH2 domains of p72\textsuperscript{Syk} as the template with primers 6 and 7 (product A) and primers 5 and 8 (product B). Aliquots of products A and B were combined in a second PCR reaction with primers 5 and 6. For the C-terminal mutation, PCR reactions were performed with primers 3 (product C) and primers 5 and 10 (product D) using the p72\textsuperscript{Syk} dual SH2 domains as the template. Aliquots of products C and D were combined in a second PCR reaction with primers 5 and 6. The second PCR products (mutated p72\textsuperscript{Syk} SH2 domain constructs) were purified (Wizard PCR prep, Promega), cleaved with BamHI and EcoRI, purified using low melt agarose followed by Wizard PCR prep, and subcloned into the pGEX-3X vector (Pharmacia). DNA sequences were sequenced to verify inclusion of the desired mutations.

**Expression of Biotinylated and Non-biotinylated Forms of Syk SH2 Proteins in Escherichia coli—** Cultures of MC1061 cells (51) containing pTC1 or pTC2 were grown overnight from single colonies at 37 °C in LB (48) supplemented with 50 μg tetracycline and 100 μg/ml ampicillin. The cultures were diluted 10-fold in the same medium, grown to OD<sub>600</sub> = 1.0 at 37 °C, and, after reducing the temperature to 30 °C, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Cells were collected by centrifugation and the cell pellet was resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM diithiothreitol, 5% glycerol, 100 μg/ml phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml pepstatin A, and 1% Triton X-100) and sonicated (20 burst of 1 s, pulse duration 1 μs). Lysates were added to the cell suspension and the cells were gently shaken for 1 h at 4 °C. MgCl<sub>2</sub> (final concentration: 20 mM), DNase, and RNase (f inal concentration: 20 μg/ml) were then added and the cells were incubated for an additional 1 h at 4 °C with shaking. The cell debris was removed by centrifugation for 45 min at 10,000 × g and the proteins were purified from the supernatant as described below.

**Purification of Biotinylated and Non-biotinylated Syk SH2 Proteins—** The bio-dual Syk SH2 and bio-dual GST-Syk SH2 proteins were purified by affinity chromatography using Soft-link monomeric avidin resin (Promega) or glutathione-Sepharose (Pharmacia), respectively, in accordance with the instructions provided by the manufacturers. After elution from the resin, the proteins were dialyzed exhaustively against Tris buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl) then concentrated by diafiltration using an Amicon membrane with a 10-kDa molecular mass cut off. Protein concentrations were determined spectrophotometrically (A<sub>280</sub> reading) or by a BCA protein determination kit (Pierce).

Non-biotinylated p72\textsuperscript{Syk} SH2 domain protein was expressed in E. coli as a GST fusion protein, with a Factor Xa cleavage site directly preceding the p72\textsuperscript{Syk} SH2 domains. The fusion protein was purified (>90% pure) by affinity chromatography on a glutathione-Sepharose (Pharmacia) column and a fraction was then digested with Factor Xa to release the SH2 domain fragment. The fragment was purified to homogeneity from the Xa-digest. On SDS gels, this protein appeared as a 29-kDa band. The fusion protein was purified (>90% pure) by affinity chromatography on a glutathione-Sepharose (Pharmacia) column. N-terminal sequencing and mass spectrometry analysis indicated that the protein was >95% pure. For some assays, the purified GST fusion protein was biotinylated chemically using either a 5-fold molar excess of sulfo-succinimidyl-6-(biotinamido)-hexaese (Immunopure-NHS-L-Leucine, Pierce) or a 10-fold molar excess of biotin-XX-NHS (Calbiochem) in the presence of phosphate-buffered saline, pH 7.4. The excess biotin was removed by extensive dialysis against phosphate-buffered saline containing 2 mM dithiothreitol at 4 °C.

**SDS-PAGE and Western Blot—** Samples were removed from the cultures before and after induction with isopropyl-1-thio-β-D-galactopyranoside, and the cells were pelleted at 10,000 × g. The pellets were resuspended in 200 μl of sample buffer (52) and heated at 100 °C for 4 min. The proteins in the samples were resolved by SDS-PAGE on a 12% pre-cast mini-gel (Bio-Rad). After electrophoresis, the gel was either stained with Coomassie Brilliant Blue or the proteins were electroblotted onto a nitrocellulose membrane using a Bio-Rad Trans-Blot SD device at 100 volts for 1 h. Following transfer, the membrane was treated overnight at 4 °C with blocking buffer (25 ml Tris-HCl, pH 7.5, 0.15 x NaCl, 0.2% Tween 20 and 5% BSA), washed three times with blocking buffer (without BSA), then incubated with streptavidin-horse-radish peroxidase conjugate (Boehringer Mannheim; 1/2000 dilution) for 1 h at room temperature. After washing the membrane three times with blocking buffer (without BSA), the streptavidin-reactive proteins were visualized using the ECL detection system (Amersham).
**RESULTS**

**Binding of dual GST-Syk SH2 Fusion Proteins to Dual Phosphorylated FcRI-γ ITAM-based Peptide**

Hydroxyamine (pH 3.0, 100 mM Tris-iodination buffer) was washed with 10 ml of Tris-iodination buffer to remove unreacted (Amersham). 50 mg of beads were resuspended in 5 ml of Tris buffer according to the manufacturer’s protocol. Tris-iodination buffer (100 mM Tris-HCl, pH 7.5, 0.4 M NaCl) was added to each well, the incubation continued for 1 h, then the plate was rinsed again. A 1:3000 dilution of goat anti-rabbit horseradish peroxidase-conjugated antibody (Zymed) was then added to each well and the plate was then incubated for an additional hour. After a final rinse, the plate was developed using TMB (Pierce) (1:1) for 5 min at room temperature then the reaction terminated by the addition of 1 N phosphoric acid. The binding was quantified by the ODmax determination in a 96-well plate reader (Molecular Devices).
biotinylated molecules.

Binding of the 125I-Dual Phosphorylated FceRI ITAM-based Peptide to Bio-dual Syk SH2 Proteins—The ability of the bio-dual p72\textsuperscript{syk} SH2 domain protein to bind the Yp\textsuperscript{64-75} peptide was initially confirmed utilizing the ELISA based format (data not shown). To establish parameters for subsequent SPA binding studies, streptavidin-coated SPA beads were incubated with increasing concentrations of bio-dual Syk SH2 domain protein. After rinsing, the beads were incubated to equilibrium (2 h) with increasing concentrations of 125I-Yp\textsuperscript{64-75}. As shown in Fig. 2, the binding of the 125I-dual phosphorylated FceRI ITAM-based peptide to the dual p72\textsuperscript{syk} SH2 protein was specific and saturable, especially for the lower concentrations of the bio-dual Syk SH2 domain protein. Utilizing a protein concentration where there was saturation of binding (3 \(\mu\)g/ml), the binding isotherm revealed that the \(K_d\) for this binding was 1.4 nM (Fig. 3). Scatchard analysis and best fit comparison utilizing "Radlig" software indicated that there was only one binding site (Fig. 3).

Binding of the 125I-Dual Phosphorylated FceRI ITAM-based Peptide to Enzymatically and Chemically Biotinylated Dual GST-p72\textsuperscript{syk} SH2 Domain Fusion Proteins—Previous studies utilizing other binding assays, such as surface plasmon resonance (Biacore), indicate that GST fusion proteins can dimerize in solution resulting in inaccurate determination of the \(K_d\) values (17). As it may not always be possible to utilize the biotinylation vector to express SH2 domain proteins, \(e.g.\) where the proteins need to be expressed in insect cells or the protein yield necessitates a higher binding capacity to solid phase for purification purposes, it may be necessary to express the SH2 domain proteins with a GST tag to purify the proteins on a glutathione column. The ability of bio-dual GST-Syk SH2 protein to bind to the 125I-Yp\textsuperscript{64-75} peptide was, therefore, compared to the ability of bio-dual Syk SH2 to bind to the 125I-Yp\textsuperscript{64-75} peptide. From the equilibrium binding studies (Fig. 3, Fig. 4A), it can be seen that the \(K_d\) for the binding of the 125I-Yp\textsuperscript{64-75} to the GST fusion protein (1.3 nM) was identical to that observed for the non-GST fusion protein (1.4 nM). A Scatchard plot of the data and best fit comparison utilizing

Radlig software revealed only one binding site. Taken together, these data indicate that there is no difference in the binding characteristics of GST and non-GST SH2 domain fusion proteins utilizing the SPA format.

To determine whether chemically biotinylating the p72\textsuperscript{syk} dual SH2 domain protein interfered with its ability to recognize the 125I-Yp\textsuperscript{64-75} peptide, dual GST-Syk SH2 domain protein
that had no biotinylation leader sequence was expressed in *E. coli*, purified, then chemically biotinylated utilizing two different systems (see “Materials and Methods”). The bio-dual GST-Syk SH2 domain protein and le-bio-dual GST-Syk SH2 domains proteins were incubated overnight with SPA beads at the concentrations indicated in the figure. The beads were rinsed and distributed into the individual wells of a 96-well microtiter plate. The ITAM peptide (3 nM) was then added to the wells and binding was allowed to proceed for 120 min at room temperature. The extent of binding was then determined as before.

Although these data demonstrate that chemical biotinylation of the Syk SH2 domains using the le-reagent did not affect the ability of the SH2 domains to recognize the ITAM peptides, this was likely dependent on the particular SH2 domain in question and the particular biotinylation protocol used. Furthermore, preliminary studies from our laboratory suggest that binding of phosphotyrosine peptides to other SH2 domains may be affected by chemical biotinylation (data not shown). Thus, for development of other SPA-based SH2-domain binding assays, the method of choice for biotinylating the protein would be to use the *E. coli* biotinylation vector. If chemical biotinylation was the only option, it would be necessary to determine whether the biotinylation procedure was interfering with phosphotyrosine recognition.

**Kinetics of Binding of 125I-Dual Phosphorylated FceRI ITAM-based Peptide to Dual p72^syk^ SH2 Domains**—The high affinity binding of the dual phosphorylated FceRI ITAM-based peptide to the dual p72^syk^ SH2 domains was reflected in the fast kinetics of association (Fig. 5A) of the peptide with the Syk protein. The t½ was less than 1 min and was independent of the protein concentration over the range of protein examined (1–30 μg/ml). The dissociation rate was determined by displacing the 125I-Yp^64^Yp^75^ peptide bound to the bio-dual Syk SH2 domain protein at equilibrium with >100-fold excess unlabeled Yp^64^Yp^75^. This produced a rapid rate of dissociation which again was independent of concentration (Fig. 5B). The K_d for binding was determined.
the binding of the doubly phosphorylated FcRI ITAM-based peptide to the dual p72syk SH2 domains was calculated utilizing the relative rates of association (4.17 × 10^{6} M^{-1} s^{-1}) and dissociation (0.98 × 10^{-3} s^{-1}) by “Radlig Kinetic” software. The association rate constant was determined utilizing the data from 4 different concentrations of peptide used in the “on rate” studies. The dissociation constant determined from the 1 and 3 nm peptide concentrations and the association rate data was determined to be 2.34 nm. This value is consistent with the K_d determined from the saturation binding isotherm, indicating that the observed rates of association and dissociation reflected real time kinetics.

**FIG. 6.** The competition binding of the soluble dual GST-Syk SH2 domain protein (●), the soluble dual Syk SH2 domain protein (○), the soluble N^{45},C^{-}-Syk SH2 protein (Δ), the soluble N^{45},C^{-}-Syk SH2 protein (•), and the soluble N^{45},C^{-}-Syk SH2 protein (○) to the 125I-dual tyrosine-phosphorylated FcRIγ ITAM-based peptide. The data represent the means ± S.E. of n = 3. The SYK SH2 domain protein (3 μg/ml) was bound to the SPA beads as described in the legend to Fig. 2 and the soluble SH2 domains were preincubated with the 125I-dual tyrosine-phosphorylated FcRIγ ITAM-based peptide for 30 min prior to addition to the SPA bead-bound bio-dual SYK SH2 domain protein. The binding was then allowed to proceed for 120 min at room temperature.

**DISCUSSION**

Cellular activation following aggregation of receptors belonging to the immunoglobulin receptor superfamily such as the...
TCR, B cell receptor, and the FcRI, and the FcRII, are dependent upon the recruitment of specific tyrosine kinases into the receptor-signal complex (24, 27–34, 43–46). In the case of FcRI-mediated mast cell activation, association of p70 the cytokolic domain of the FcRII subunit is essential for activation of downstream signaling and for subsequent mediator release (33, 34). The recruitment of p72 the consequence of the phosphorylation of the dual tyrosine residues within the ITAM motif of the FcRII cytokolic domain (33, 34) and the resulting high affinity interaction between the phosphorylated ITAM and the dual SH2 domains of p72. In this paper, we have described studies aimed at investigating the nature of this high affinity interaction. This has been accomplished by examining the binding parameters of the interaction between the dual and single SH2 domains of p72 and FcRII ITAM-based peptides.

To examine the properties of the binding of phosphorylated FcRII ITAM-based peptides to dual and single SH2 domains of p72, a novel SPA-based binding assay was developed. This assay has allowed us to circumvent the limitations encountered utilizing other approaches described in the introduction. For example, when the p72 SH2 domain proteins are immobilized on the SPA beads and the binding ITAM-based peptides are in solution, avidity problems associated with GST fusion proteins are minimized (17). To illustrate this, our studies revealed that there was no difference in the $K_d$ values of binding of the FcRII ITAM-based peptide to the E. coli-expressed biotinylated GST fusion Syk SH2 dual domain protein and the E. coli-expressed biotinylated non-GST fusion protein in the SPA-based binding assay. Second, in the competition studies conducted in the SPA assay, both the binding peptides and competing peptides are in the same phase. Thus, a true competition equilibrium should be achieved. Finally, as the SPA assay does not require washing steps or binding of secondary antibodies, the SPA-based assay has proved to be more rapid and reproducible compared to the ELISA assay.

Utilizing the SPA assay, the binding of the dual phosphorylated peptide, corresponding to amino acids 59–82 of the FcRII ITAM, to the p72 SH2 domains was observed to be of high affinity with a $K_d$ of 1.4 nM. Scatchard analysis and analysis of the rates of association of the dual phosphorylated peptide with the dual Syk SH2 domain protein revealed that there was only one binding site, suggesting that there is a preferred orientation for the binding of the dual phosphorylated ITAM peptide to the p72 SH2 domains. p72 has a 57% sequence homology with the TCR chain-associated tyrosine kinase p70 and it has been suggested that the nature of the SH2 domain-ITAM interactions may be very similar for both p72 and p70 (28). The recently published x-ray crystal structure of p70 has revealed that there is a close interaction between the N- and C-terminal SH2 domains (28). Within the interface between the N- and C-terminal SH2 domains, there is a shared contribution to phosphoryrosine recognition. If the N-terminal SH2 domain is expressed in isolation, the structure is not sufficiently complete to recognize phosphoryrosine (28). Thus, the data suggest that the C-terminal of p70, alone, is not capable of binding phosphoryrosine. The data presented in our study suggest that, to a certain extent, this may be similar for the binding of the single SH2 domain of p72. In this context, the relative abilities for the FcRII ITAM peptide to bind to the dual p72 SH2 domains, an N-terminal SH2 domain knockout, and to a C-terminal SH2 domain knockout in a competition assay, revealed that both domains are necessary to confer high affinity binding. Data presented, also suggest that the binding of the ITAM peptide to the C-terminal SH2 domain is of higher affinity than to the N-terminal SH2 domain of p72. The above findings are supported by the observation that the C-terminal SH2 of p72 alone, but not the N-terminal SH2 domain of p72 alone, can recognize tyrosine phosphorylated proteins from RBL cell lysates (34). Competition data, with the N- C-Syk SH2 knockout, however, suggest that other arginines in the C-terminal SH2 domain, apart from Arg, may contribute to the high affinity binding but to a lesser extent.

Our studies revealed that both tyrosines of the FcRII ITAM peptide are required to be phosphorylated to allow the high affinity interaction with the dual SH2 domains of p72. In this respect, the ability of the monophosphorylated peptides to compete for binding was reduced by 3 to 4 orders of magnitude in comparison with the doubly phosphorylated peptide. In addition, the N-terminal monophosphorylated FcRII ITAM peptide was more efficient than the C-terminal monophosphorylated FcRII ITAM peptide in competing with the dual phosphorylated FcRII ITAM peptide for binding to the dual p72 SH2 domains. The p70 x-ray crystallographic study suggests that the ITAM chain of the ITAM peptides is bound in a pocket formed by both domains, so that the TCR chain ITAM N-terminal phosphotyrosine is in the C-terminus SH2 domain pocket (28). Our data therefore suggests that the preferred orientation of the binding for the FcRII ITAM to the dual SH2 domains of p72 would be similar to the p70 model. In this model, the N-terminal phosphotyrosine occupies the C-terminal SH2 domain pocket and the ITAM C-terminal phosphopeptide occupies the N-terminal SH2 domain pocket. Confirmation of this orientation awaits x-ray crystallographic structural studies.

Finally, the marked differences in the relative affinities of the singly and doubly phosphorylated FcRII ITAM peptides to bind to the SH2 domains of p72 may have important physiological implications. Both the FcRII and FcRII ITAMs are targets for FcRI-associated tyrosine phosphatases (58, 59). When these phosphatases are activated, the resulting dephosphorylation of either tyrosine residue within these ITAMs would shift the binding equilibrium resulting in dissociation of p70 and p72 from the FcRII and FcRII subunits, respectively. Our preliminary studies have demonstrated that tyrosine phosphatases in RBL 2H3 cell lysates are indeed capable of dissociating the FcRII ITAM peptide from the p72 ITAM in the SPA assay (60). The relatively rapid off-rates observed in our study would mean that dephosphorylation of even one of the ITAM tyrosines would result in the termination of ongoing cell activation process.

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