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Immunology

Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance

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ABSTRACT **Tyrosine phosphorylation of a 17-amino acid immunoreceptor tyrosine-based activation motif (ITAM), conserved in each of the signaling subunits of the T-cell antigen receptor (TCR), mediates the recruitment of ZAP-70 and syk protein-tyrosine kinases (PTKs) to the activated receptor. The interaction between the two tandemly arranged Src-homology 2 (SH2) domains of this family of PTKs and each of the phosphotyrosine-containing ITAMs was examined by real-time measurements of kinetic parameters. The association rate and equilibrium binding constants for the ZAP-70 and syk SH2 domains were determined for the CD3ε ITAM. Both PTKs bound with k_a and K_d values of 5×10^6 $M^{-1} \cdot sec^{-1}$ and ≈25 nM, respectively. Bindings to the other TCR ITAMs (ζ1, ζ2, γ, and δ ITAMs) were comparable, although the ζ3 ITAM bound ≈2.5-fold less well. Studies of the affinity of a single functional SH2 domain of ZAP-70 provided evidence for the cooperative nature of binding of the dual SH2 domains. Mutation of either single SH2 domain decreased the K_d by >100-fold. Finally, the critical features of the ITAM for syk binding were found to be similar to those required for ZAP-70 binding. These data provide insight into the mechanism by which the multisubunit TCR interacts with downstream effector molecules.**

Tyrosine phosphorylation of the T-cell antigen receptor (TCR) is an obligate and early event in TCR activation. Phosphorylation of a highly conserved motif consisting of D/EXXYXXLX$_{6-8}$YXXL [termed ITAM (immunoreceptor tyrosine-based activation motif)] provides the structural basis for interaction of a variety of effector molecules with the receptor (reviewed in refs. 1–3). This motif is present in a single copy in each of the CD3γ, -δ, and -ε subunits and in three copies in the TCR ζ subunit (ζ1-membrane proximal, ζ2, and ζ3-membrane distal). Chimeric receptors consisting of the cytoplasmic domains of the CD3ε or -ζ chains, or chimeras containing solely the ζ1 or ζ2 motifs, are capable of mediating TCR signaling events (4–9).

The presence of multiple ITAMs in the TCR has raised the possibility that various subunits may play distinct roles in TCR signaling. Crosslinking of a chimeric receptor containing the cytoplasmic tail of ε (Tac–ε) results in the induction of a pattern of cellular tyrosine phosphoproteins distinct from those seen after crosslinking of a Tac–ζ chimera (7). In addition, while TCR–CD3 complexes devoid of a functional ζ subunit produce interleukin 2 in response to anti-TCR monoclonal antibodies, crosslinking of Thy-1 or Ly-6 required the presence of ζ (10). Recently, SHC and phosphatidylinositol 3-kinase (PI3K) have been described to associate with ζ (11, 12). In the case of PI3K, a hierarchy of association was demonstrated with the ζ1 motif binding with greater affinity than ζ3 or ζ2 (12). Such differences may be important in mediating differential responses by the TCR.

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ZAP-70 and syk are protein-tyrosine kinases (PTKs) that associate with the TCR. These PTKs contain two tandemly arranged Src-homology 2 (SH2) domains (13, 14) and associate with the phosphorylated receptor (15–17). The presence of two functional SH2 domains within ZAP-70 and phosphorylation of both tyrosine residues in the ζ1 ITAM are required for its efficient association with ZAP-70 (9, 18–20). To determine the ability of individual ITAMs to interact with ZAP-70 and syk, we quantitatively analyzed these interactions by plasmon resonance and determined the amino acid within the ITAM required for syk binding.

MATERIALS AND METHODS

Fusion Proteins and Peptides. Glutathione *S*-transferase (GST)–ZAP-70 and GST–syk constructs were produced within the pGEX-KT vector (21) and contained amino acids 1–263 of human ZAP-70 (14) and 1–264 of human syk (22). Peptides, purified to >98% homogeneity and confirmed with mass spectroscopy and ^{31}P NMR, were purchased from QCB (Hopkinton, MA).

Plasmon Resonance Studies. Binding experiments were performed with BIACore (Pharmacia) plasmon resonance measurements according to the manufacturer's recommendations. The basic principles and detection methods have been reviewed (23). In brief, 0.8 ng of an N-terminal biotinylated ligand (i.e., peptide) was immobilized onto avidin-coated (SA5) sensor chips and GST fusion proteins infused at 5 μl/min at 12°C. For equilibrium experiments, a 50-μl injection of the GST fusion protein was used. For competition experiments, increasing amounts of nonbiotinylated peptides were preincubated with the GST fusion proteins and analyzed in a similar fashion. dR/dt was determined for the initial linear portion of association and the degree of inhibition in dR/dt was graphed as a function of inhibiting peptide concentration.

RESULTS

Affinity of the ZAP-70 and syk SH2 Domains for the CD3ε ITAM. To determine the affinities of the ZAP-70 and syk SH2 domains to the CD3ε ITAM, bacterially produced GST fusion proteins containing the two SH2 domains of ZAP-70 or syk were produced (Fig. 1 *A* and *B*) and their affinities for a peptide encoding the CD3ε ITAM (Fig. 1*C*) were measured by surface plasmon resonance at equilibrium. Binding increases the mass on the chip and is measured as an increase in resonance units (Fig. 2*A*). Scatchard analysis of binding at equilibrium revealed a K_d of 21.4 ± 11 nM (mean ± SD) for the ZAP-70 SH2 fusion protein and a K_d of 27.2 ± 7 nM for the syk SH2 fusion protein (Table 1). Association rates (k_a) of ZAP-70 and syk fusion proteins, derived by analyzing ln (dR/dt) as a function of time, were also similar for the two

Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif; GST, glutathione *S*-transferase; PTK, protein-tyrosine kinase; SH2, Src-homology 2; TCR, T-cell antigen receptor.

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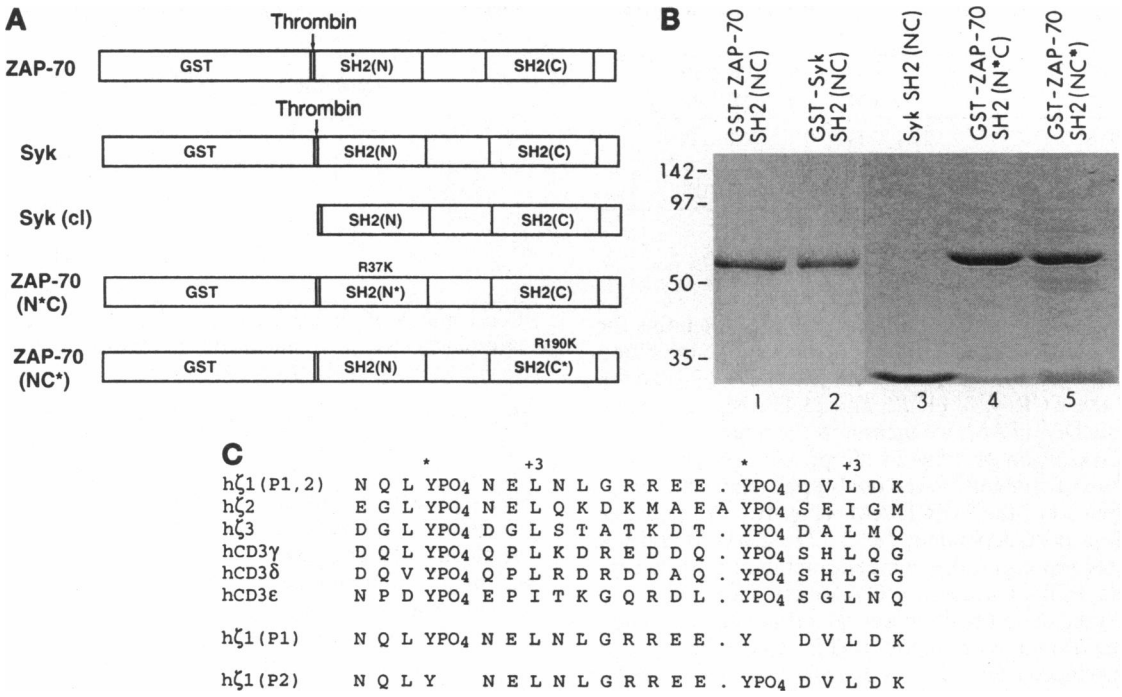

FIG. 1. (*A*) Schematic of fusion proteins of ZAP-70 and syk used in this study. (*B*) GST fusion proteins. Approximately 2 μg of each protein was analyzed by SDS/PAGE and Coomassie blue staining. The SH2(N*C) and SH2(NC*) fusions have ≈15% contamination with a GST degradation fragment (lanes 4 and 5). The remaining proteins were >95% homogeneous. Number on left are kDa. (*C*) ITAM peptide sequences. *, N- and C-terminal tyrosine residues. The two +3 positions are labeled. P1 and P2 refer to singly phosphorylated peptides.

PTKs (Table 1). Thus, the binding constants at equilibrium and association rates, and by inference the dissociation rates, for the SH2 domains of ZAP-70 and syk appear equivalent. Removal of the GST portion of the infusion protein did not affect the affinity of the syk SH2 domain for the phosphorylated peptide (Table 1).

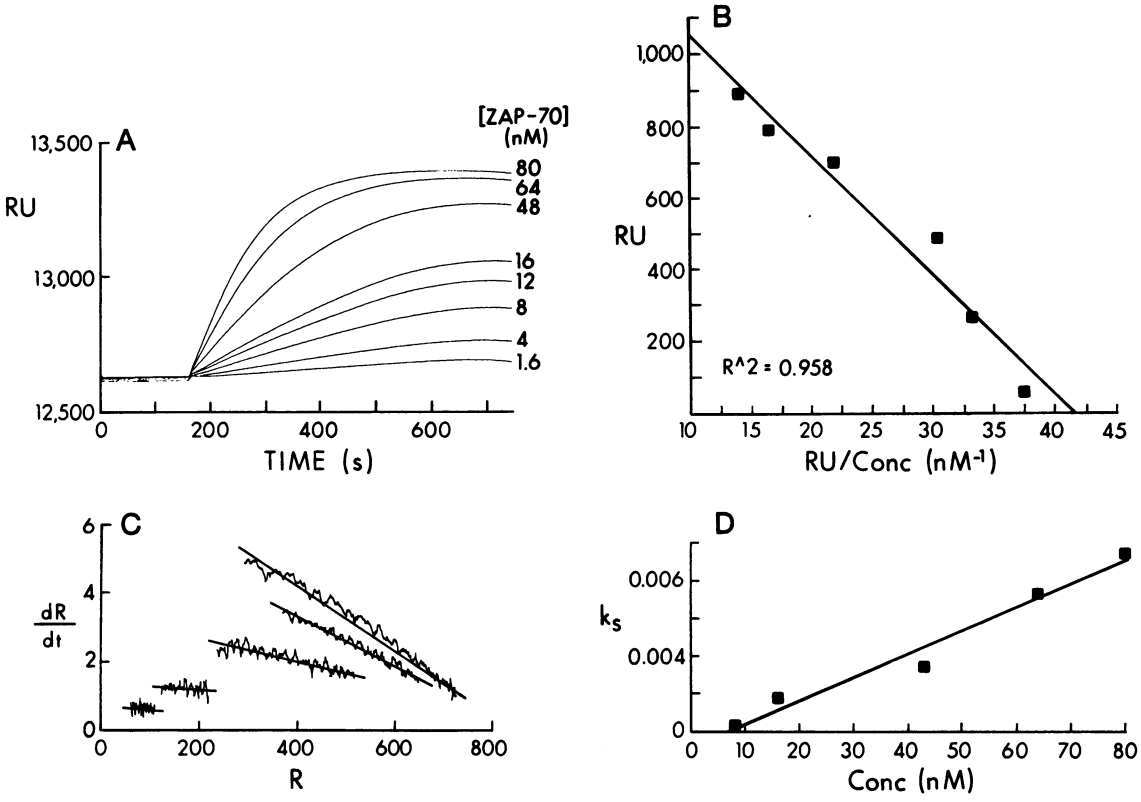

FIG. 2. (*A*) Binding of ZAP-70 SH2(NC) to the CD3ε ITAM. Binding was measured as an increase in resonance units (R.U.). (*B*) Scatchard analysis of ZAP-70 binding to the CD3ε ITAM. Binding (R.U.) at equilibrium was studied as a function of R.U./[ZAP-70] (nM). Slope = K_d. (*C*) dR/dt vs. R. Association phase in *A* was graphed (dR/dt) as a function of binding. Slope = k_s. (*D*) k_s vs. concentration. k_a was determined by k_s/concentration. Slope = k_a; R^2 = 0.98.

Table 1. Binding of ZAP-70 and syk SH2 domains to the CD3ε ITAM

	K_d, nM*	$k_a \times 10^{-6}$, $M^{-1}\cdot sec^{-1}$
ZAP-70 SH2 (NC)	21.4 ± 11	6.0 ± 3.16
syk SH2 (NC)	27.2 ± 7	5.0 ± 0.21
syk SH2 (NC)-cl	22.6 ± 2.4†	ND‡

*Mean (±SD) of four independent experiments.
†Mean (±SD) of two independent experiments.
‡Not determined.

Relative Affinity of the ZAP-70 and syk SH2 Domains for the TCR γ, δ, ε, ζ1, ζ2, and ζ3 ITAMs. Competitive inhibition was used to determine the relative affinities of ZAP-70 and syk for each of the TCR γ, δ, ζ1, ζ2, and ζ3 ITAMs. Using the immobilized CD3ε ITAM, we measured the binding of either ZAP-70 or syk fusion proteins in the presence of increasing concentrations of nonbiotinylated doubly phosphorylated peptides encoding all of the TCR ITAMs (Fig. 1*C*). All peptides competed in a dose-dependent fashion for ZAP-70 and syk binding. Three representative competition binding assays representing the TCR ζ1, ζ3, and ε ITAMs are shown in Fig. 3 *A–C*. The degree of competition was quantitated as a function of the degree of inhibition on *dR/dt* (Fig. 3*D*). While ζ1 and ε peptides produced a 50% decrease in *dR/dt* at 57 and 56 nM, respectively, the ζ3 peptide required 192 nM to obtain a similar decrease (Fig. 3 *A–C* and Table 2). Peptides ζ2, δ, and γ were similar to ζ1 in their avidities for ZAP-70 binding (Table 2). Thus, only ζ3 showed any significant difference in its ability to bind ZAP-70.

Comparable findings were also seen with the syk SH2 domains. Again, ζ3 demonstrated a 2- to 3-fold lower avidity to bind syk; the remaining ITAMs were otherwise comparable in their binding in syk (Table 2). In addition, consistent with the K_d values measured for ZAP-70 and syk with the immobilized CD3ε peptide, the ability of the nonbiotinylated CD3ε peptide to compete for ZAP-70 and syk binding was comparable.

Affinity of a Single Functional ZAP-70 SH2 Domain for the N- or C-Terminal ITAM Tyrosine. Since ZAP-70 contains two SH2 domains, we determined (*i*) the affinity of a single functional SH2 domain to the phosphorylated ζ1 ITAM and (*ii*) whether either of the SH2 domains [designated SH2(N) and SH2(C)] demonstrated any specificity toward the N- and C-terminal tyrosine residues within the ζ1 ITAM. To analyze the binding of a single SH2 domain, the reciprocal SH2 domain was made nonfunctional by substituting a lysine for a conserved arginine residue that resides in the Tyr-PO_4 binding pocket of the SH2 domain. Thus, SH2(N) and SH2(C) domains were each mutated by altering Arg-37 or Arg-190, respectively, to lysine and are designated ZAP-70 SH2(N*C) and ZAP-70 SH2(NC*), respectively (Fig. 1*A*). Equilibrium measurements and Scatchard analysis of the ZAP-70 SH2(N*C) and ZAP-70 SH2(NC*) proteins are summarized in Table 3. The affinity of the SH2(N) domain for the doubly phosphorylated ζ1 ITAM was comparable to that of SH2(C). More important, however, the affinity of a single functional SH2 domain was ≈100-fold less than the dual functional SH2 constructs (Tables 1 and 3).

To determine whether the SH2(N) or SH2(C) domain of ZAP-70 demonstrated any specificity toward either of the two tyrosine residues within the ζ1 ITAM, the K_d and k_a values for the binding of ZAP-70 SH2(N*C) and ZAP-70 SH2(NC*) to peptides that were singly phosphorylated on either the N- or the C-terminal tyrosine (designated P1 and P2, respectively; Fig. 1*C*) were determined and are summarized in Table 3. The

Table 2. Inhibition of binding by competing peptides to the SH2 domains of ZAP-70 and syk

Activation motif	Concentration, nM*	
	ZAP-70	syk
ζ1	56.7	96.4
ζ2	58.8	68.3
ζ3	129.0	159.0
ε	55.6	65.0
γ	56.2	60.0
δ	41.9	49.5

*Concentration of peptide required to obtain a 50% decrease in *dR/dt* as demonstrated in Fig. 3. Experiment shown here is representative of three independent experiments.

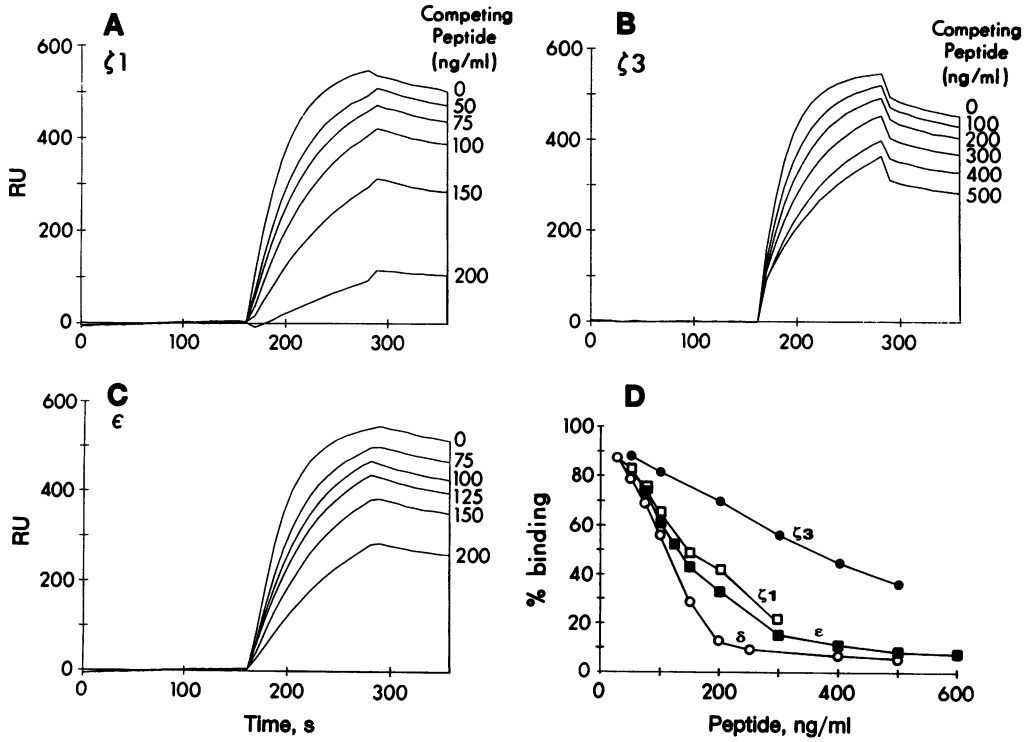

FIG. 3. (*A–C*) Competition of ζ1 (*A*), ζ3 (*B*), and ε (*C*) nonbiotinylated peptides for ZAP-70 binding. Binding of ZAP-70 SH2(NC) at 10 μg/ml to an immobilized CD3ε ITAM peptide was inhibited by increasing concentrations of nonbiotinylated ζ1, ζ3, and ε ITAM peptides. Concentrations of competing peptides are denoted on the right *y* axis. (*D*) Inhibition of *dR/dt* by ζ1, ζ3, δ, and ε peptides. Degree of inhibition of the initial linear portion of association (*dR/dt*) in *A–C* was graphed as a function of peptide concentration.

Table 3. Binding of ZAP-70 SH2 point mutations to singly phosphorylated ζ1 ITAMs

	P1,2		P1		P2	
	K_d, μM	$k_a \times 10^{-3}$, $M^{-1}\cdot sec^{-1}$	K_d, μM	$k_a \times 10^{-3}$, $M^{-1}\cdot sec^{-1}$	K_d, μM	$k_a \times 10^{-3}$, $M^{-1}\cdot sec^{-1}$
N*C	3.5 ± 0.28	8.9 ± 8.7	4.3 ± 1.6	4.9 ± 0.82	4.0 ± 0.93	5.2 ± 0.7
NC*	1.8 ± 1.4	12.4 ± 6.1	2.2 ± 1.0	10.0 ± 4.3	2.0 ± 1.1	11.0 ± 2.0

P1,2, doubly phosphorylated ζ1 ITAM peptide; P1, singly phosphorylated ζ1 ITAM peptide on the N-terminal tyrosine residue; P2, singly phosphorylated ζ1 ITAM peptide on the C-terminal tyrosine residue. Data represent means ± SD of three independent experiments.

SH2(N) domain appeared to have a slightly higher affinity and association rate when compared to SH2(C) in this context. However, both SH2 domains did not demonstrate any specificity to either YXXL motifs within the ITAM in the system analyzed here.

Mapping of the CD3ε ITAM for syk Binding. We have previously mapped the residues within the CD3ε chain required for ZAP-70 association (20). All of the conserved features of the ITAM were demonstrated to be requirements for ZAP-70 binding (Fig. 4*A*). A similar analysis was performed to determine whether conserved features of the ITAM were also required for binding of the syk SH2 domains. While the wild-type G–ε protein was able to bind GST–syk, no significant binding was detected to the construct lacking the ε cytoplasmic domain (Fig. 4*B*, lanes 1 and 2). Moreover, mutation of tyrosine residues to leucines as well as the isoleucine/leucine in the +3 position abrogated the ability of GST–syk to bind G–ε (Fig. 4*B*, lanes 3, 6, 7, and 10). The spacing between the two tyrosines was also important because a mutant lacking two residues between the two tyrosines was unable to bind GST–syk (data not shown). Similar to ZAP-70, the +1 position following the first tyrosine that was required for ZAP-70 binding was also contributory to syk binding. Thus, consistent with the plasmon resonance data, both tyrosine residues are important for binding of ZAP-70 and syk.

DISCUSSION

The presence of multiple ITAMs within a single TCR complex has raised the intriguing possibility for differential signaling

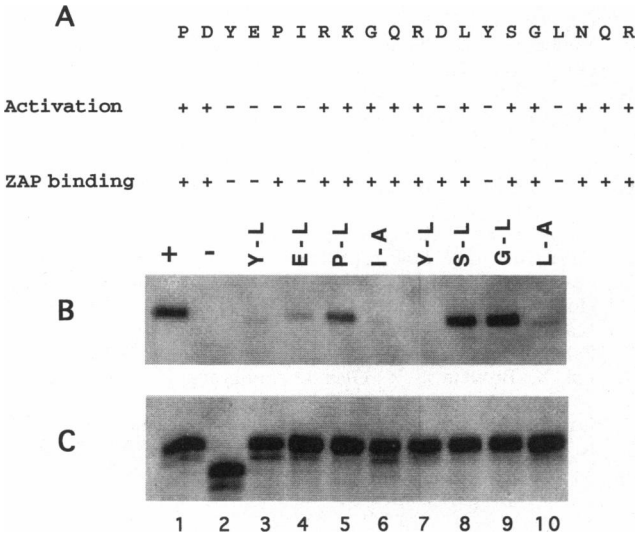

FIG. 4. Requirements of the CD3ε ITAM for syk binding. For comparison, *A* summarizes the requirements for ZAP-70 binding (20). A panel of vesicular stomatitis virus (VSV)–ε chimeras containing point mutations within the ITAM was phosphorylated by coinfection with $p59^{fyn}$ in HeLa cells. Cell lysates were incubated with a GST–syk(SH2) fusion protein immobilized on glutathione-Sepharose. Binding of the VSV–ε chimera mutants was analyzed by Western blot analysis with an anti-VSV antibody (*B*). Comparable levels of expression of each of the VSV–ε chimeras (*C*) and fyn (data not shown) were confirmed by Western blot analysis.

roles for the CD3 and ζ-chain subunits. Since both ZAP-70 and syk associate with the phosphorylated TCR, we addressed the possibility that these two PTKs may interact differentially with the various ITAMs present within the TCR. Consistent with previous results, we demonstrate that the SH2 domains of ZAP-70 can directly bind the doubly phosphorylated ITAM (9, 18–20). However, we extend these findings and demonstrate here that the binding and association rates of a fusion protein containing both SH2 domains of ZAP-70 to the ζ1, ζ2, γ, δ, and ε ITAMs are comparable. Interestingly, the binding of ZAP-70 to the ζ3 ITAM was somewhat less (≈2.5-fold) compared to ITAM peptides encoding other chains. These data are consistent with studies in which crosslinking of chimeric receptors containing ε, ζ1, or ζ2 is able to mediate calcium mobilization, induction of tyrosine phosphoproteins, and interleukin 2 production (4–9). While the ζ3 motif binds somewhat less efficiently to ZAP-70, the ability of each of the three ζ motifs to mediate TCR activation does not appear to be functionally distinct. Expressions of a ζ chain devoid of any ITAMs, a ζ chain containing only the ζ1 or ζ3 ITAMs, or η (a differentially spliced form of ζ containing only the ζ1 and ζ2 ITAMs) in $\zeta^{-/-}$-deficient mice were all able to reconstitute thymic development and mediate TCR activation (24). Thus, while expression of ζ is required for efficient surface expression of the multisubunit TCR, none of the ζ ITAMs are required nor do they contribute a unique signal to TCR signaling or thymic development. Interestingly, an additive effect of the number of ITAMs was observed in the ability to reconstitute T-cell maturation, suggesting an amplification effect of the multiple ITAMs. Also consistent with the amplification hypothesis, a chimeric receptor containing three copies of the ζ1 motif induces ≈3-fold more NFAT-regulated activity when compared to a chimeric receptor containing only a single ζ1 motif (8). In addition, the Tac–ε chimera containing one ITAM requires 10- to 100-fold greater antibody concentration to elicit interleukin 2 secretion compared to the Tac–ζ chimera, which has three ITAMs (7). Together, these data suggest that a major function of having at least 10 ITAMs within a receptor complex is likely to be amplification of the TCR activation signal and thereby increase the sensitivity of the TCR to ligand stimulation.

The presence of syk in thymocytes and its ability to associate with the activated TCR has suggested a potential role for syk in T-cell development. Our findings here demonstrate that syk binds to all of the ITAMs with affinities comparable to ZAP-70. Moreover, there appeared to be no significant difference in the abilities of ZAP-70 and syk to bind any of the motifs.

While most other PTKs have only a single SH2 domain, ZAP-70 has two tandemly arranged SH2 domains (13, 14), which are both required for the efficient binding to a doubly phosphorylated ζ1 peptide or the phosphorylated ζ subunit (9, 18–20). Our results here provide the quantitative mechanism for this observation. While the fusion protein containing two functional SH2 domains exhibited K_d values of ≈20 nM, the single functional SH2 domains demonstrated K_d values that were at least 100-fold greater. The data provide proof that binding of two tandemly arranged SH2 domains is cooperative rather than additive. Under the conditions tested here, both

SH2 domains bound the N- or C-terminal tyrosine residues within the ITAM with approximately the same K_d and k_a values. However, it is possible that additional steric constraints regulated by contiguous sequences not represented by the peptides or the other ITAMs and/or the catalytic domain of ZAP-70/syk may provide additional specificity to each of the SH2 domains as well as each of the tyrosine residues within the ITAM.

Finally, we examined the fine specificity of the ITAM sequences for syk SH2 binding. Similar to ZAP-70, both tyrosine and +3 residues are required for syk binding. The +1 position following the N-terminal tyrosine was also important for both ZAP-70 and syk binding. This suggests that a difference between the two halves of the ITAM may exist that cannot be measured by the isolated singly phosphorylated peptides used here in these plasmon resonance experiments. This difference may dictate the orientation of the two SH2 domains to the two halves of the ITAM and is the subject of additional investigation.

Interestingly, the binding specificities of the SH2 domains of the src and syk PTKs are similar (25, 26). Both demonstrate a preference for binding to the ITAM motifs. The src family of PTKs associates with the resting T- and B-cell receptors and appears to have a low affinity for the unphosphorylated ITAM (27–32), but this interaction appears to increase in affinity with ITAM phosphorylation via an SH2-TyrPO_4-mediated mechanism (33). Binding of a phosphorylated YEEI peptide to the SH2 domain of src has also been demonstrated to increase src catalytic activity (34). Thus, binding of lck or fyn to a singly phosphorylated ITAM may likewise increase its catalytic activity and promote phosphorylation of the second tyrosine within the ITAM or additional tyrosines within the oligomeric antigen receptor. Phosphorylation of both tyrosines within the ITAM would favor the binding of the syk family of PTKs since the affinity of the syk and ZAP-70 SH2(NC) domains is greater than that of the lck or fyn single SH2 domain (J.-y.B. and A.C.C., unpublished data). Thus, in addition to recruitment of various effector molecules to the activated receptor, this model implies additional protective and activating roles for the SH2 domains of the src family of PTKs.

Note Added in Proof. By using a similar ZAP-70 fusion protein immobilized on glutathione-sepharose and ^{32}P-labeled ITAM peptides, Isakov *et al.* (35) have observed a 30-fold difference in the affinity of the $\zeta 1$ and $\zeta 3$ ITAM sequences for ZAP-70.

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