ZAP-70 Binding Specificity to T Cell Receptor Tyrosine-based Activation Motifs: The Tandem SH2 Domains of ZAP-70 Bind Distinct Tyrosine-based Activation Motifs with Varying Affinity

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Summary

Engagement of the T cell antigen receptor (TCR) results in activation of several tyrosine kinases leading to tyrosine phosphorylation of protein substrates and activation of multiple biochemical pathways. TCR-mediated activation of the src-family kinases, Lck and Fyn, results in tyrosine phosphorylation of the TCRγ chain and CD3 chains. The site of phosphorylation in these chains is the tyrosine-based activation motif (TAM), a 15–16 amino acid module containing two tyrosine residues. Tyrosine-phosphorylated TAMs serve as targets for binding of the zeta-associated protein (ZAP-70) tyrosine kinase via its tandem SH2 domains. This binding correlates with activation of ZAP-70, a critical event in T cell activation. To further define the structural requirements for ZAP-70 interaction with the TCR, we developed a binding assay using immobilized glutathione S-transferase fusion proteins containing the NH2- and/or COOH-terminal SH2 domains of ZAP-70, and soluble synthetic peptides with the sequence of the cytoplasmic region of the TCRγ chain (TCRγcyt) or individual TCRγ and CD3ε TAM motifs. Direct binding studies demonstrated that the tandem ZAP-70 SH2 domains bind phosphorylated, but not nonphosphorylated, TCRγcyt. The NH2-terminal ZAP-70 SH2 domain also binds to TCRγcyt but with 100-fold lower affinity. No binding was observed with the COOH-terminal ZAP-70 SH2 domain. Similar studies demonstrated that the ZAP-70 tandem SH2 domain can bind a TCRγ TAM peptide in which both tyrosine residues are phosphorylated: Little or no binding was observed with peptides phosphorylated at only one tyrosine residue, or a nonphosphorylated peptide. Binding of the tandem SH2 domains to the other two TCRγ TAM peptides and to a CD3ε TAM peptide was also observed. All four doubly tyrosine phosphorylated TAM peptides cross-compete with each other for binding to the tandem SH2 domains of ZAP-70. The affinity of these peptides for the tandem SH2 construct demonstrated a hierarchy of TAM1γ > TAM2γ > TAMε > TAM3γ. The results provide further evidence that the ZAP-70 interaction with the TCR requires prior phosphorylation of both tyrosine residues within a TAM motif. Binding of ZAP-70 to phospho-TAMs is notable for the high level of cooperativity between the two SH2 domains, which individually demonstrate low affinity interaction with the ligand. The cooperativity ensures higher affinity for the doubly phosphorylated ligand. Affinity differences of as much as 30-fold indicates a significant specificity of interaction of ZAP-70 SH2 domains for different phospho-TAMs.

Studies with mutant T cell lines and transfectants expressing chimeric TCR molecules have identified a 15–16 amino acid sequence motif in the cytoplasmic portion of the TCRγ and CD3 chains subunits that is both necessary and sufficient for coupling the receptor to early and late signaling events. The sequence, first noted by Reth (1), and referred to as a tyrosine-based activation motif (TAM), has the general structure YXX(L/I)X(s-z)YXX(L/I), and is sufficient to confer upon a chimeric receptor the potential to induce cell activation after receptor cross-linking (2, 3). TAM motifs are found in three copies in the TCRγ chain and in one copy each in the CD3γ, δ, and ε chains (1, 3–6). From the time
of their discovery, these motifs have been thought to be involved in coupling the TCR to downstream effector molecules. Investigators have suggested that multiple TAMs in the TCR are required for binding multiple copies of a particular molecule in order to amplify the signal given by a rare binding event (7). Others have postulated that each of the different TAM motifs within a single TCR may function as a docking region for distinct effector molecules (3).

T cell activation via the TCR is dependent on multiple protein tyrosine kinases (PTKs) that phosphorylate numerous substrates (8), including the TCR/CD3 subunits that are phosphorylated on their TAM motifs (9–11). These multiple TCR subunits lack intrinsic PTK activity; instead, they interact with and activate, cytoplasmic PTKs that couple the receptor to the signaling apparatus.

Four PTKs have been implicated in this process: the src family members Lck and Fyn, and the syk family members, Syk and zeta-associated protein (ZAP-70) (reviewed in references 7, 12). One function of the Lck and/or Fyn molecules activated directly or indirectly by the TCR is the tyrosine phosphorylation of the TCR TAM motifs. The Syk PTK is found primarily in B cells, mast cells, thymocytes, and, at a low level, in some T cell lines. The ZAP-70 PTK is found in the cytosol of resting T cells and, upon TCR stimulation, is rapidly recruited to the TCR/CD3 complex (13, 14). This sequential PTK activation model has been studied in COS cells using transfected TCR chimeras and PTKs (15). The association of the ZAP-70 kinase with tyrosine phosphorylated TCR subunits after T cell activation has been shown to be mediated by the tandem SH2 domains of this kinase (15–17).

In the present study we analyze the biochemical and structural requirements for ZAP-70 interaction with individual TAM motifs. Using glutathione S-transferase (GST) fusion proteins containing the NH2- and/or COOH-terminal SH2 domains of ZAP-70, we study binding requirements using nonphosphorylated or tyrosine phosphorylated synthetic peptides that correspond in sequence to the TCRγ cytoplasmic region (TCRγC) or to the CD3ε and to each of the three TCRγ TAM motifs. We find that the tandem SH2 domains bind the doubly tyrosine phosphorylated CD3ε TAM and the three doubly tyrosine phosphorylated TAMs from the TCRγ chain. Competition binding studies reveal that the affinities of these binding interactions differ.

Materials and Methods

Peptides and GST Fusion Proteins. The cytoplasmic domain of the TCRγ (TCRγC; residues 52–164) has been chemically synthesized as described (18). Individual phosphorylated TAM motif-containing peptides (Table 1), were prepared by Michael Berne (Tufts University, Boston, MA). A second set of nonphosphorylated 21-mer TAMγ (HDGLYQGLSTATKDTYDALHM) and 22-mer TAMε (QEGLYNELQDKMAEAYEIGM) peptides were purchased from Peptide Technologies Corp. (Gaithersburg, MD). Peptide concentrations were verified by amino acid analysis. Briefly, peptides were hydrolyzed in 6 M HCl at 150°C for 60 min. The hydrolyzed samples were derivatized with phenylisothiouronium and the resulting derivatized amino acids were quantitated on a PICOTAG amino acid analysis system (model 840; Waters, Medford, MA).

TCRγγ and individual TAM peptides were phosphorylated in vitro with recombinant Lck essentially as described (18). To ensure highest possible stoichiometry of phosphorylated peptide, an excess of ATP was used over available tyrosine residues in each reaction. γ[32P]ATP (ICN Radiochemicals, Irvine, CA) was added to kinase reactions to a known specific activity in order to (a) allow quantitation of reaction efficiency; and (b) permit calculation of peptide concentration after repurification via Cerenkov counting. Peptides were repurified by reverse phase-HPLC on a Waters 600E system to remove unreacted ATP and Lck. TCRγTAMγ was repurified on a C4 column (2.1 × 150 mm; Vydac Hesperia, CA) eluting bound peptide with a short acetonitrile gradient. Individual TAM peptides were repurified on a Vydac C8 column (4.6 × 250 mm), eluting bound peptides with a shallow acetonitrile gradient to achieve separation of the singly and doubly phosphorylated forms. A Sciex TOBY single quadrupole mass spectrometer was used to identify the nonphosphorylated, singly phosphorylated and doubly phosphorylated TAM-containing HPLC peaks. GST-ZAP-70 fusion proteins were prepared as described (16).

Binding Assay. 2–4 μg of GST or GST fusion proteins comprising the SH2 domains from ZAP-70 were immobilized on glutathione agarose beads. These beads were washed and incubated with the indicated concentrations of 32P-labeled peptides with or without competing “cold” peptides in 0.5 ml buffer A (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM Na3VO4, 5 mM EDTA, 10 μg/ml each leupeptin and aprotinin, and 25 μM p-nitrophenyl p'-guanidinobenzoate). After 1 h of incubation at 4°C on a rotator, the beads were extensively washed in buffer A, and bound radioactivity was determined by SDS-PAGE (on 15% gels) and autoradiography, or by scintillation counting.

Results and Discussion

Specific Binding of Tyrosine Phosphorylated TCRγγ to the ZAP-70 Tandem SH2 Domains. Experiments with immobilized fusion proteins containing the ZAP-70 tandem SH2 domains demonstrated that they can precipitate the TCRγγ and CD3ε chains from lysates of activated Jurkat T cells (16). The following studies were performed to confirm that the interaction of ZAP-70 SH2 domains and TCR represents a direct interaction. Moreover, we wished to compare the relative affinities of individual and tandem SH2 domains for binding to the TCRγ chain. We established a sensitive binding assay using

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**Table 1. Amino Acid Sequences of TAM Peptides**

| TAMγ1 | LYNELNKGREEYDVLD | TAMε | DYPFIRKQGRLYSGLN |
| TAMγ2 | LYNELQDKMAEAYEIG | TAMγ3 | LYQGLSTATKDTYDALH |
ZAP(SH2)2 Domains Exhibit Specific Binding to the Doubly
Tyrosine Phosphorylated TAM3 Peptide. The TCRζ possess
sees three TAM sequences, and on the average, only 2.7–2.9
out of a possible 6 tyrosine residues are phosphorylated after
an in vitro tyrosine phosphorylation with recombinant Lck.
To better define the requirements for ZAP-70/TCRζ inter-
action, we performed binding studies with the tandem SH2

Figure 1. Specific binding of tyrosine phosphorylated TCRζcyt to GST-
ZAP-70 fusion protein requires both the NH2- and the COOH-terminal
SH2 domains. A synthetic peptide corresponding to the TCRζ cytoplas-
ic domain (TCRζcyt) was 32P phosphorylated on tyrosine residues using
recombinant Lck and γ-[32P]ATP. The radiolabeled peptide, at the indi-
cated amounts, was incubated with 3 μg of GST-ZAP-70 fusion proteins
containing the tandem SH2 domains (A), the COOH-terminal (B) or
NH2-terminal (C) SH2 domains, or GST alone (D), immobilized to
glutathione agarose beads. After 1 h of incubation on a rotator at 4°C,
the beads were washed and the bound radiolabeled peptides were eluted
and subjected to SDS-PAGE under reducing conditions. An autoradiog-
raphy is shown.

Figure 2. Specific interaction between GST-ZAP-70 and TAMζ3 re-
quires the ZAP-70 tandem SH2 domains and phosphorylation of the two
tyrosine residues within the TAM. (A) 2 μg GST-ZAP(SH2)2 (O), GST-
ZAP(SH2)c (A), or GST alone (D), immobilized to glutathione agarose beads, were incubated with the indicated concentrations of γ-[32P]-labeled (pTyr)2-TAMζ3 for 1 h on a rotator at 4°C. The beads were washed and bound radioactivity was measured. (B) 2 μg of
immobilized GST-ZAP(SH2)2 were incubated with 4 nM of [32P]-labeled (pTyr)2-TAMζ3 or TAMζ3 in the presence or absence of cold (pTyr)2-TAMζ3 ( ), (pTyr)N-
TAMζ3 ( ), (pTyr)C-TAMζ3 ( ), or TAMζ3 ( ). Percentage of radiola-
beled peptide bound was determined as in A.
fusion protein and a TAM-3 peptide. This TAM peptide was synthesized, purified by HPLC, and tyrosine phosphorylated with γ-[32P]ATP and recombinant Lck. HPLC was performed on the peptide after phosphorylation. Fractions containing peptides were analyzed by mass spectrometry to identify the doubly tyrosine phosphorylated peptide and the peak containing the mix of the two singly phosphorylated species. Direct binding studies using GST-ZAP(SH2)2 and the doubly phosphorylated peptide indicated that (pTyr)2-TAM-3 binding was saturable. GST-ZAP(SH2)2 bound this phosphopeptide at ~200–250-fold higher affinity than the GST-ZAP(SH2) C (Fig. 2 A). No binding was observed with the GST-ZAP(SH2) C or the GST control protein.

The binding of labeled (pTyr)2-TAM-3 to ZAP(SH2)2 could be competed with increasing concentrations of unlabeled (pTyr)2-TAM-3. The concentration giving half-maximal inhibition was 3–4 μM. Peptides with only one, (pTyr)c-TAM-3 or (pTyr)n-TAM-3, or neither of the two tyrosines phosphorylated failed to compete (Fig. 2 B). A mixture of (pTyr)c-TAM-3 and (pTyr)n-TAM-3, or of the two individual hemi-TAMs, LpYQGLST and TpYDALHM, each representing one half of a tyrosine phosphorylated TAM-3, also failed to compete with 32P-(pTyr)2-TAM-3 binding to GST-ZAP(SH2)2 (not shown).

Comparison of TAM-1, TAM-2, TAM-3, and TAMe Binding Affinity. Cytoplasmic domains of TCR subunits contain six distinct TAM motifs, four of which, TAM-1, TAM-2, TAM-3, and TAMe, are likely to be duplicated in each TCR. Potentially, all of these TAM motifs may serve as docking sites for ZAP-70. However, the amino acid sequence variability in TAMs, both between the paired tyrosine residues, and between the tyrosine and isoleucine or leucine residues, provides the potential for specificity of TAM–ligand interactions. To test whether we could detect differences in TAM binding specificity, we compared the binding of four doubly phosphorylated TAM peptides to GST-ZAP(SH2)2. Direct binding studies with 32P-labeled, doubly phosphorylated peptides demonstrated saturable binding of the four TAM peptides.

The binding of labeled, doubly tyrosine phosphorylated TAM peptides to GST-ZAP(SH2)2 was used, this time for determination of the relative affinity of each TAM peptide

![Image](378.jpg)

Figure 3. The CD3ε and each of the three TCRζ TAM motif-containing peptides have different binding affinities for the GST-ZAP(SH2)2 fusion protein. 2 μg of immobilized GST-ZAP(SH2)2 were incubated with 32P-labeled (4 nM) derived from the first (A), second (B), or third (C) TCRζ, or the CD3ε (D), TAM motifs, in the presence of various concentrations of the indicated 'cold' peptides (pTyr)c-TAM-1 (O), (pTyr)c-TAM-2 (□), (pTyr)c-TAM-3 (▲), (pTyr)n-TAM-1 (●), (pTyr)n-TAM-2 (▲), (pTyr)n-TAM-3 (●). After 1 h of incubation on a rotator at 4°C, the beads were washed and bound radioactivity was measured by scintillation counting.
for the tandem SH2 domains (Fig. 3). Binding of each of the four $^{32}$P-labeled TAM peptides was subjected to competition analysis with these same four peptides. Regardless of which peptide was labeled, the hierarchy of competition was the same, TAM$_{1}$$>$TAM$_{2}$$>$TAM$_{3}$$>$TAM$_{4}$. The relative apparent affinities of these peptides for GST-ZAP(SH2)$_{2}$, as determined by competition (IC$_{50}$), varied between 0.1 and 3 $\mu$M. A similar order of hierarchy was observed in a direct binding study using the four $^{32}$P-labeled TAM peptides (not shown).

The TAM motif defines a module with potential for interactions mediated by different mechanisms. Residues between the tyrosines can determine binding of src-family tyrosine kinases as demonstrated in the B cell system (19). Likewise, the constitutive interaction of the Fyn PTK with the CD3e TAM does not localize to the tyrosine residues (17). Tyrosine phosphorylation of a single tyrosine residue within a TAM potentially creates an additional binding site. The resulting sequence, pYXXI/L, is recognized by SH2 domains of src-family kinases (20). Enhanced binding of these kinases after receptor phosphorylation may occur via this mechanism (21). Likewise, linker molecules such as SHC, which in turn bind Grb2 and Sos, may bind to tyrosine phosphorylated TAM sequences in this fashion (22).

Published work from several laboratories (15-17, 23) and these current data demonstrate a third type of TAM-mediated interaction. Individually, the COOH-SH2 domain demonstrates no binding and the NH$_{2}$-SH2 domain binds only with low affinity to the phospho-TAMs. These new data demonstrate that the presence of the two SH2 domains, in tandem, raises the affinity for phosphorylated TAMs by at least three orders of magnitude. Thus, this highly cooperative interaction of the tandem SH2 domains of ZAP-70 ensure a high level of specificity of interaction with a doubly tyrosine phosphorylated TAM. The activation of this kinase, which accompanies binding, is thus tightly regulated at the level of assembly with interaction of the Syk kinase with phosphorylated TAMs in B and mast cells.

Our previous work (16) with the tandem SH2 domains of ZAP-70 demonstrated that GST-ZAP(SH2)$_{2}$ could be used to precipitate tyrosine phosphorylated TCR subunits, as well as associated endogenous ZAP-70 kinase. These data indicate that the ZAP-70 SH2 domains can bind multiple TAMs. The current work confirms this result by demonstration of direct binding of GST-ZAP(SH2)$_{2}$ to four of the six TCR TAMs. This observation provides further evidence for the suggestion that the presence of multiple TAMs offers the potential for binding multiple copies of a particular effector molecule, thus resulting in signal amplification (7).

However, data have also been provided that support the possibility that individual TCR subunits and their TAMs are differentially coupled. In this regard, stimulation of T cells expressing receptor chimeras with either TCR$_{p}$ or CD3e cytoplasmic domains results in different patterns of substrate tyrosine phosphorylation (3). Two specific examples of TAM binding preference are the linker protein SHC, which binds phospho-TAM$_{2}$, and not phospho-TAM$_{3}$ (22), and phosphatidylinositol (PI) 3-kinase, which associates with TAM$_{4}$ (25). The most striking results of this present study are the affinity differences of the four phospho-TAMs for the tandem ZAP SH2 domains. Regardless of which of the four phosphorylated TAMs was used for the binding studies, the hierarchy of binding demonstrated by competition was TAM$_{1}$$>$TAM$_{2}$$>$TAM$_{3}$$>$TAM$_{4}$. The apparent affinities differed by as much as 30-fold. These observations provide further evidence of the capacity for TAM-binding specificity. The marked sequence differences between TAMs outside of the conserved tyrosine and leucine/isoleucine residues have a significant effect on binding. The functional consequences of these affinity differences remain to be determined by careful mutagenesis studies.

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Note added in proof: The term Tyrosine-based Activation Motif (TAM) should be replaced with the term Immunoreceptor Tyrosine-based Activation Motif (ITAM). This nomenclature has recently been adopted by a group of investigators in the field. A letter to this effect has been submitted for publication.
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