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Interactions of p59^{fyn} and ZAP-70 with T-Cell Receptor Activation Motifs: Defining the Nature of a Signalling Motif

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The tyrosine-based activation motif is a 20- to 25-amino-acid sequence contained in the cytoplasmic domains of many hematopoietic receptors which is sufficient by itself to reconstitute signalling. This motif is characterized by two YXXL/I sequences separated by approximately 10 residues. The molecular basis of signalling by this motif is unknown. Here we demonstrate that the tyrosine-based activation motif is required and sufficient for association with the tyrosine kinases p59^{fyn} and ZAP-70, suggesting that association with these kinases is a general feature of this motif. Focusing on the single activation motif present in epsilon, we analyzed which residues of the motif were critical for binding of p59^{fyn} and ZAP-70. Surprisingly, we found that no single mutation of any residue of epsilon resulted in the loss of p59^{fyn} association. In contrast, single mutations at five residues of the epsilon activating motif abrogated ZAP-70 binding. Both of the tyrosines and the leucine or isoleucine residues that follow them were critical. The spacing between the tyrosines was also important, as deletion of two residues disrupted binding of ZAP-70, although p59^{fyn} binding was not disrupted. Most of the defined features of the tyrosine activation motif are therefore requirements for ZAP-70 binding. Interestingly, the interaction of ZAP-70 with the motif was dependent on the presence of both ZAP-70 SH2 domains and both of the tyrosine residues in the motif, suggesting that ZAP-70 interacts with two phosphotyrosine residues and that the binding of the two SH2 domains is cooperative. In addition, we demonstrate that the interaction between the tyrosine activation motif is direct and requires prior tyrosine phosphorylation of the motif. We propose that the activation of cells by the tyrosine activating motif occurs in four discrete steps: binding of p59^{fyn}, phosphorylation of the motif, binding of ZAP-70, and activation of ZAP-70 kinase activity.

The T-cell antigen receptor is a multiprotein complex composed of at least seven different proteins. The genes encoding two of the chains, α and β (or γ and δ), undergo extensive rearrangement and nucleotide addition in order to generate a repertoire able to recognize a vast array of different antigens. The other five chains are invariable and are known as the CD3 δ, ε, γ, ζ, and η chains. Although the exact manner in which the T-cell receptor generates signals is largely unknown, multiple studies implicate the activation of one or more tyrosine kinases as the first event triggered by the T-cell receptor (19, 31).

Chimeric proteins containing cytoplasmic domains of the CD3 ζ and ε chains have simplified the analysis of early signalling events in T cells. Several groups have demonstrated that cross-linking of these chimeric proteins is sufficient to reconstitute most of the biochemical events that characterize T-cell activation, including tyrosine kinase activation (18, 27, 28, 36, 37, 50). Mutational analysis has shown that the minimal required sequence is an 18- to 25-amino-acid motif that is present in all of the CD3 proteins and the B-cell antigen receptor proteins Igα and Igβ, as well as in some of the components of the Fc receptors (35). The exact function of this motif, known as the antigen receptor homology motif (2) or tyrosine-based activation motif (TAM) (21), is not known.

Because the T-cell receptor does not itself encode a tyrosine kinase, one possible function of the activating motif, which we will refer to as the TAM, is that it binds and/or activates a tyrosine kinase. Three candidate cytoplasmic tyrosine kinases, p56^{lck}, p59^{fyn}, and ZAP-70, have all been implicated in the activation of T cells (51). p56^{lck}, a member of the Src family of tyrosine kinases, associates with the T-cell receptor coreceptors CD4 and CD8 (38, 47) and, in some cells, has been shown to be essential for T-cell receptor activation (20, 44). Although it functions in the T-cell receptor signalling pathway, there is little evidence to support a direct interaction of p56^{lck} with the T-cell receptor. p59^{fyn}, another member of the Src family, is the only kinase that has been shown to be physically associated with the unactivated T-cell receptor complex (39, 41). Reconstitution experiments demonstrate that this association is specific and that p59^{fyn} can associate directly with multiple CD3 subunits (13). Importantly, p59^{fyn} kinase activity is stimulated by engagement of the T-cell receptor (46), and p59^{fyn} can mediate calcium mobilization and phospholipase C phosphorylation when coexpressed with a ζ fusion protein in a nonlymphoid cell (15). Overexpression studies in T-cell lines and transgenic mice confirm an important role for p59^{fyn} in T-cell activation (7, 8). The third kinase, ZAP-70, is the most recent addition to this group. It is a 70-kDa tyrosine kinase, closely related to the Syk tyrosine kinase (45), and is expressed

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exclusively in T lymphocytes and natural killer cells (4, 5). ZAP-70 was originally identified because it binds to the T-cell receptor ζ chain. However, this binding is restricted to "activated" receptor molecules and is presumed to be dependent on prior phosphorylation of ζ, itself an early consequence of T-cell receptor activation. The precise roles of the two tyrosine kinases that interact with the T-cell receptor, $p59^{fyn}$ and ZAP-70, and their relationship to each other and to the T-cell receptor are not known.

In this study, we demonstrate that $p59^{fyn}$ and ZAP-70 both interact with the T-cell receptor TAMs. This suggests that association with ZAP-70 and $p59^{fyn}$ may be a general property of TAMs. In addition, using the single TAM present in CD3 ε, we analyzed which residues of the motif were critical for binding of $p59^{fyn}$ and ZAP-70. Because we had already demonstrated which of the residues of the ε motif are critical for T-cell activation (28), we could therefore correlate the residues critical for $p59^{fyn}$ and ZAP-70 binding with the residues critical for activation. For the binding of $p59^{fyn}$, we found that no single mutation of any residue of ε resulted in the loss of kinase association. In contrast, single mutations of five separate residues of the activation motif abrogated ZAP-70 binding. All of the residues demonstrated to be critical for kinase binding were also essential for T-cell activation. Interestingly, three mutations that abolished T-cell activation did not affect binding of either ZAP-70 or $p59^{fyn}$. This result suggests that simple binding of the two kinases may not be sufficient for cell activation. We also examined the requirements for the interaction of ZAP-70 with the T-cell receptor ζ chain. Binding required phosphorylation of ζ and also required both ZAP-70 SH2 domains, suggesting that ZAP-70 binds to two phosphorylated tyrosine residues. This idea was further supported by demonstrating that the spacing between the tyrosines was critical for ZAP-70 binding to the ε TAM. This supports a model of T-cell activation in which Src family kinases, constitutively bound to either the T-cell receptor or coreceptor molecules, lead to the phosphorylation of T-cell receptor subunits, which in turn results in the binding and activation of a second tyrosine kinase, ZAP-70.

MATERIALS AND METHODS

Cloning of ZAP-70. A ZAP-70-specific DNA probe was isolated by using degenerate oligonucleotides to amplify tyrosine kinase domains from day 16 thymocyte cDNA. Products were ligated into pBluescript (Stratagene) and sequenced. From the similarity of the DNA sequence with the published human ZAP-70 sequence, one clone, 4, was used to screen a UniZAP (Stratagene) cDNA library prepared from day 16 thymocyte RNA. Hybridization-positive plaques were sized by PCR with ZAP-70-specific primers. A single 2.2-kb insert was sequenced by using Sequenase (U.S. Biochemical).

DNA constructs and mutagenesis. The construction of the plasmids encoding G epsilon, GZeta, GZetaA32*, and $p59^{fyn}$ was described previously (13). The G T− construct was created by removal of a *Bam*HI fragment of G epsilon which encodes the epsilon cytoplasmic domain and religation. The resulting sequence of the cytoplasmic domain encodes the amino acids RVGIL and then a stop codon. G epsilon Δ145–165 and G epsilon −2 were generated by religation of the PCR products from inverse PCR by using G epsilon as a template (13). G epsilon P166* and G epsilon Y181* were generated by PCR with a T7 primer and an oligonucleotide which causes a stop codon to replace codon 166 or 181, respectively, and introduces an *Xba*I site. After digestion of the PCR products with *Eco*RI and *Xba*I, the DNA fragments were ligated into pGem3Z+ (Promega). G epsilon mutants 1 through 21 were generated by PCR from SRα plasmids encoding the TTε mutant constructs (28) by using the oligonucleotides 5′-AAGTCTCGGATCCGGAAGGCCAAGGCCAAG-3′ and 5′-GGATCTAGATGTGTCAGAGGTTTTCAC-3′ to create a *Bam*HI-compatible *Bsm*AI site at the start of the mutated epsilon cytoplasmic domains and an *Xba*I site in the vector portion of the TTε constructs. The resulting PCR fragments were digested with *Bsm*AI and *Xba*I and exchanged with the *Bam*HI-*Xba*I fragment of G gamma* (13). G epsilon mutant 6 I-A was generated by religation of the inverse PCR product by using G epsilon as a template and the primers 5′-GCCCGC AAAGGCCAGCGAGATCTGTATTCTGG-3′ and 5′-GGG CTCATAGTCTGGGTTGGG-3′.

To generate the ZAP-70 plasmid, the phage clone containing the full-length ZAP-70 cDNA was excised and recircularized by following the manufacturer's instructions (Stratagene, La Jolla, Calif.). The *myc*-tagged ZAP-70 construct was generated by using PCR to append an *Xba*I site and the sequences encoding the epitope SMEQKLISEEDLN to the C terminus of ZAP-70. The ZAP-70-*myc* construct was ligated into the *Bam*HI and *Xba*I sites of pGem3Z+ (Promega). Expression of the ZAP-70 SH2 domains alone was achieved by using PCR to generate a DNA fragment that encoded a stop codon instead of the alanine at codon 261 as well as *Bam*HI fragments at both ends by using the oligonucleotides 5′-CGCGGATCCCGCG GCGCACCTG-3′ and 5′-GATCGGATCCTTAGCTGGCG CTGCTGTTGGG-3′. After digestion with *Bam*HI, the fragment was ligated into the plasmid vector pET14a (Novagen) at the *Bam*HI site. The constructs containing deletions of the individual SH2 domains were also generated by PCR. A *myc*-tagged ZAP-70 construct lacking sequences encoding the N-terminal SH2 domain (residues 10 to 162) was generated by PCR with the M13 reverse sequencing primer and the oligonucleotide 5′-GCGCGAATTCTGGATAATGCCAGACCCC GCGGCGCACCTGCCCTGGTATCACAGCAGCCTGAC-3′. After digestion with *Eco*RI and *Xba*I, the DNA fragment was ligated into the *Eco*RI and *Xba*I sites of pGem3Z+ (Promega). A *myc*-tagged ZAP-70 construct lacking sequences encoding the C-terminal SH2 domain (residues 106 to 256) was generated by inverse PCR (13) with the oligonucleotides 5′-AGCAGCGCCAGCAACGC-3′ and 5′-CGGCCGGTTA CACGGCTT-3′ and plasmid pGem-ZAP-70-*myc* as the template.

The accuracy of all of the mutations was verified by dideoxy sequencing with Sequenase (U.S. Biochemical) (40).

DNA transfections, protein expression, and in vitro kinase assays. The vaccinia virus-T7 expression system for expression of vesicular stomatitis virus (VSV) G chimeric proteins, $p59^{fyn}$, and ZAP-70 in HeLa cells was used as described previously (13) except that the digitonin lysis buffer used for the $p59^{fyn}$ and epsilon mapping consisted of 1% digitonin (WAKO), 25 mM Tris (pH 8.0), 25 mM NaF, 150 mM NaCl, and the protease inhibitor aprotinin (500 kallikrein inhibitor units/ml; Sigma).

For the ZAP-70 experiments, HeLa cells were lysed on ice with a buffer containing 1% Triton X-100, 25 mM Tris (pH 7.4), 150 mM NaCl, 25 mM NaF, 100 μM sodium orthovanadate and aprotinin (500 kallikrein inhibitor units/ml). Insoluble material was removed by centrifugation at 4°C for 5 min, and the lysates were precleared for 30 min with preimmune rabbit serum and protein A-Sepharose. Generally, 5×10^5 cells were used and lysed in a volume of 1 ml. For mixing experiments, 250-μl aliquots of precleared lysates were used. Phosphatase treatment was performed by adding 1/10 volume of 10× calf intestinal phosphatase buffer and 10 μl of calf intestinal

phosphatase for 30 min at 37°C. Samples were cooled on ice before being mixed with ZAP-70-containing lysates. The antibodies used and the kinase reaction protocols have been described previously (13). For purification of nonphosphorylated and phosphorylated GZeta and G epsilon, the VSV G-specific monoclonal antibody I1 was incubated with protein A-Sepharose (Sigma), washed, and cross-linked to the support by using dimethylpimelimidate (Pierce). Cell lysates were incubated with antibody-conjugated beads, washed extensively with 0.1% sodium dodecyl sulfate (SDS), and then eluted twice in 100 mM glycine, pH 2.5. The buffer was exchanged and the protein was concentrated with spin columns (Amicon). In some experiments, proteins were treated with 10 mM fluorosulfonylbenzoyladenosine (Sigma) to ensure that no active kinases were copurifying with the protein.

Expression and purification of the bacterially expressed, histidine-tagged ZAP-70 SH2 domain construct were done as described in the manufacturer's instructions (Novagen) with the bacterial strain BL21(DE3) (*lysS*). Biotinylation (Enzotin Diagnostics) of the ZAP-70 SH2 domains construct was performed by following the manufacturer's instructions.

Immunoblotting of whole-cell lysates prepared from duplicate cultures of cells was performed as described previously (13).

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been deposited in GenBank (accession number U04379).

RESULTS

Mapping residues of CD3 ε required for association of p59^{fyn}. We demonstrated previously that multiple T-cell receptor chains could bind p59^{fyn} and that multiple independent binding domains were present in the CD3 ζ cytoplasmic domain (13). Because all of the CD3 proteins (γ, δ, ε, ζ, and η) contain the TAM and because the motif is present in multiple copies in ζ and η, we postulated that p59^{fyn} might be specifically associated with the TAM. To test this hypothesis, we investigated which residues in the TAM of ε were critical for binding. We focused on ε because it contains only a single copy of the motif and because mutagenesis of the ε chain has already demonstrated which residues are critical for T-cell activation (28).

For all of these experiments, we used a chimeric molecule, G epsilon, which contains the extracellular and transmembrane domains of the VSV glycoprotein (VSV G) fused to the cytoplasmic domain of CD3 ε. As reported previously, this allowed us to express ε independently of other T-cell receptor subunits and also allowed us to use a well-characterized monoclonal antibody to VSV G (I1 [26]) for all of our coprecipitation experiments (13). To confirm that the association of p59^{fyn} with ε was mediated specifically by sequences contained within the TAM, we tested the ability of a truncated G epsilon molecule that lacks the TAM to coprecipitate p59^{fyn}. The TAM is contained within the C-terminal 23 residues of the 56-amino-acid CD3 ε cytoplasmic domain. A chimeric molecule that lacks these 23 residues, G epsilon P166* (depicted in Fig. 1A), was generated and then coexpressed in HeLa cells with p59^{fyn} in the vaccinia virus-T7 expression system (12). Association of p59^{fyn} with the G epsilon mutant was assessed by testing G epsilon immunoprecipitates which were prepared from digitonin-containing lysates for associated p59^{fyn} kinase activity. Full-length G epsilon and a construct which contains only the extracellular and transmembrane domains of VSV G, G T−, were used as positive and negative controls, respectively, to confirm the specificity of any association detected (Fig. 1B). As described previously, in vitro kinase reactions of immunoprecipitates of full-length G epsilon result in the phosphorylation of proteins of 70 and 60 kDa which we previously showed represent phosphorylated G epsilon and p59^{fyn}, respectively (13). Immunoprecipitates of G epsilon P166* did not demonstrate any significant p59^{fyn} kinase activity, confirming our hypothesis that p59^{fyn} binds directly to the ε TAM (Fig. 1B). Another mutant, G epsilon Δ145–165, which lacks the 20 residues of the ε cytoplasmic domain just membrane proximal to the TAM, was used to confirm that p59^{fyn} binds specifically to the TAM. As expected, G epsilon Δ145–165, which lacks most of the non-TAM sequences in the ε cytoplasmic domain, still coprecipitated p59^{fyn} kinase activity (Fig. 1B). Immunoblotting of duplicate lysates demonstrated similar levels of p59^{fyn} and VSV G chimeric proteins, confirming that the lack of p59^{fyn} kinase activity in the VSV G immunoprecipitates was not due to lack of expression (Fig. 1C and D). Sequences contained within the TAM are therefore required for the association of p59^{fyn} with CD3 ε. As reported previously, coprecipitation of p59^{fyn} was only observed when digitonin was used; complex formation was sensitive to more stringent detergents such as Triton X-100 (13, 39).

Two additional G epsilon mutants were generated to further define the requirements for p59^{fyn} association with the ε TAM (depicted in Fig. 1A). To test whether the spacing of approximately 10 residues between the tyrosines of the TAM was important for p59^{fyn} binding, G epsilon −2 was created. This mutant contains a deletion of the amino acids glycine at position 176 and glutamine at position 177, giving a spacing of eight residues between the tyrosines of the TAM. This chimeric protein was coexpressed with p59^{fyn} in HeLa cells and tested for associated p59^{fyn} kinase activity by coprecipitation with the VSV G monoclonal antibody. Coprecipitation of p59^{fyn} kinase activity with the G epsilon −2 protein demonstrated that a spacing of 10 residues between the two tyrosines was not required for p59^{fyn} binding (Fig. 1B). Similar results were obtained when an additional G epsilon mutant which contained an internal deletion of six residues within the TAM was tested (data not shown).

To determine whether the carboxy-terminal portion of the TAM was important for p59^{fyn} binding, another G epsilon mutant, G epsilon Y181*, in which the codon encoding the second tyrosine of the TAM was changed to a stop codon, was tested. When this chimeric protein was tested for p59^{fyn} binding, greatly reduced kinase activity was repeatedly detected in VSV G immunoprecipitates (Fig. 1B). The addition of an exogenous substrate to the in vitro kinase reaction mixes confirmed that the total amount of kinase activity was substantially decreased (data not shown). Therefore, these data suggest that although the spacing between the tyrosines of the ε TAM is not critical for p59^{fyn} binding, the sequence from leucine 180 to valine 189 of the TAM is required.

To determine whether any individual residues in the TAM were required for p59^{fyn} binding, a series of G epsilon mutants which had single-amino-acid substitutions at 20 different positions were generated and tested for p59^{fyn} binding (Fig. 2). All of the mutants were coexpressed with p59^{fyn} in HeLa cells in the vaccinia virus-T7 expression system. After several hours, cells were lysed in a buffer containing 1% digitonin, and immunoprecipitates were prepared with a monoclonal antibody to VSV G. Coprecipitating p59^{fyn} was detected by performing an in vitro kinase assay on the immunoprecipitates. Immunoblotting of duplicate cell lysates with antibodies to p59^{fyn} and VSV was performed to confirm that both proteins were equivalently expressed in all of the transfections (data not shown). Of the 20 mutants tested, none demonstrated a

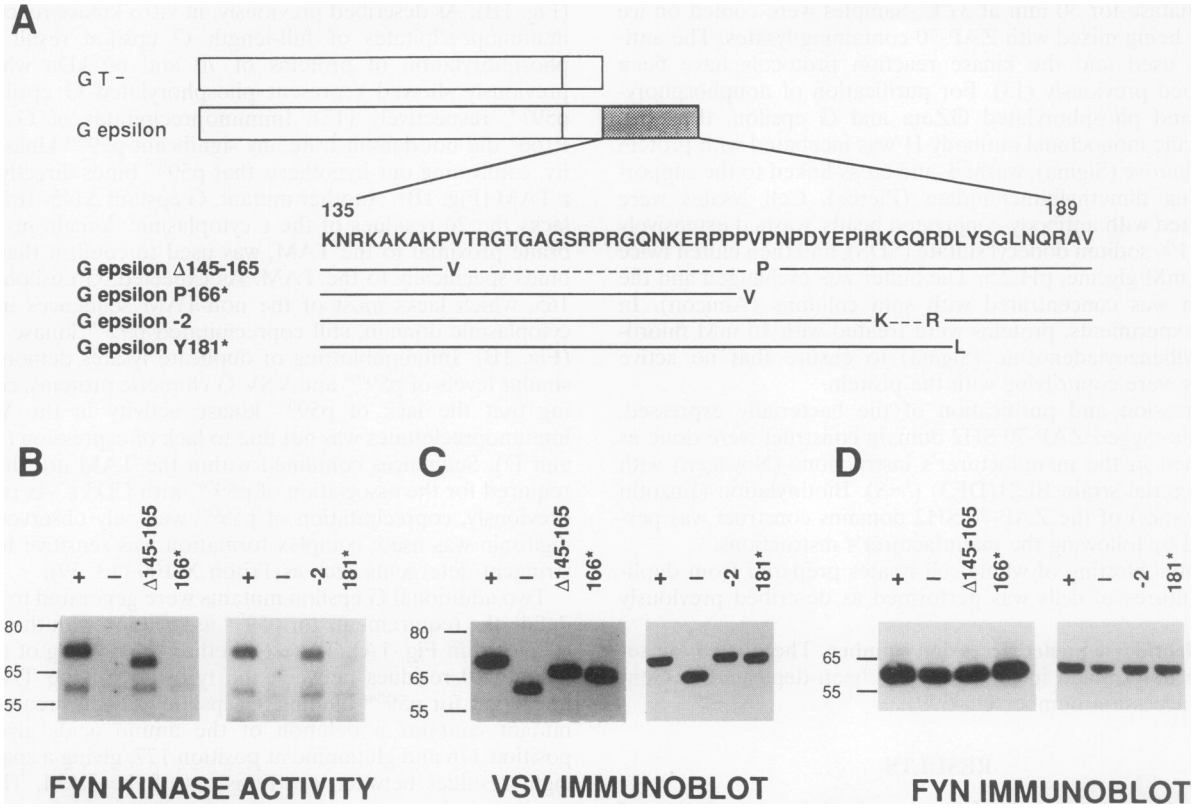

FIG. 1. p59^{fyn} binds to the ε TAM. (A) Schematic depiction of the full-length chimeric protein, G epsilon, G T−, G epsilon Δ145–165, G epsilon P166*, G epsilon −2, and G epsilon Y181*. Open boxes represent VSV G extracellular and transmembrane domains; the shaded box represents the CD3 ε cytoplasmic domain. The one-letter amino acid code is used to indicate the residues of the ε cytoplasmic domain. The internally deleted residues are represented by dashes, whereas solid lines indicate unchanged residues. G T− lacks the complete cytoplasmic domain of ε; G epsilon Δ145–165 contains an internal deletion of most of the non-TAM sequences; G epsilon P166* lacks the complete TAM; G epsilon −2 lacks glycine 176 and glutamine 177 within the TAM; and G epsilon Y181* was truncated by changing the second tyrosine of the TAM to a stop codon. (B) Sequences within the TAM are required for the association with p59^{fyn}. The VSV G chimeric proteins indicated in panel A were coexpressed with p59^{fyn} in HeLa cells with the vaccinia virus-T7 expression system. In vitro kinase assays were performed on immunoprecipitates prepared from digitonin-containing lysates with an antibody to VSV G (I1). Phosphoproteins were analyzed on an 8% polyacrylamide–SDS gel and autoradiographed. (C and D) VSV G and p59^{fyn} immunoblots of lysates from coexpressing cells. Duplicate plates of cells from panel B were lysed in Laemmli sample buffer. Proteins were separated on 8% polyacrylamide–SDS gels, transferred to nitrocellulose, and immunoblotted with rabbit polyclonal antibodies to VSV (C) and p59^{fyn} (D). Immunoblots were developed with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin serum and the enhanced chemiluminescence system (Amersham). Lanes +, positive control (full-length G epsilon); lanes −, negative control (G T−). Sizes are shown in kilodaltons.

consistent diminution of coprecipitating p59^{fyn} kinase activity (summarized in Fig. 2). Although the binding of p59^{fyn} to mutants 3, 14, and 15 was variable, when protein expression levels were optimal, we always detected significant binding of p59^{fyn} to these mutants. Thus, no single residue of ε appears to be critical for p59^{fyn} association. It is possible, however, that some of the ε mutants had reduced affinity for p59^{fyn} but that these differences were not detected because of the high protein levels achieved in our expression system. We suspect that the redundant YXXL/I motif in the TAM is sufficient for p59^{fyn} binding or that p59^{fyn} binding is dependent on structural features of the TAM that are not disrupted by substitution with leucines.

	P	D	Y	E	P	I	R	K	G	Q	R	D	L	Y	S	G	L	N	Q	R
MUTANT #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
SUBSTITUTION	L	L	L	L	L	L	L	L	L	L	L	L	A	L	F	L	L	A	L	L	L
Activation	+	+	−	−	−	−	+	+	+	+	+	−	+	−	−	+	+	−	+	+	+
fyn binding	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

FIG. 2. Sequences and phenotypes of ε mutants. The 21 residues encoding the CD3 ε TAM are depicted in the one-letter amino acid code. Below each residue number is the number of each mutant and the residue which was substituted. The symbols below the substituted residue indicate whether a particular mutation in the context of a Tac/ε chimeric protein could activate cells after expression and cross-linking in BW5147 cells, as reported previously (28), and bind to p59^{fyn}.

Cloning and expression of mouse ZAP-70. Current data suggesting that ZAP-70 may also bind to residues within the TAM (5, 17) led us to evaluate the chimera containing the ζ cytoplasmic tail and the more defined G epsilon chimera for assembly with ZAP-70. We cloned the cDNA for mouse ZAP-70 from an embryonic day 16 thymocyte cDNA library. A 250-bp ZAP-70-specific probe was generated by PCR with degenerate primers recognizing conserved tyrosine kinase residues. A 2.2-kb clone containing the entire ZAP-70 coding sequence was isolated by hybridization with the ZAP-70 probe and sequenced. Comparison of the deduced amino acid sequence demonstrated that the mouse form of ZAP-70 is very

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mouse ZAP-70    ..... ..... ..... ..... ..... .....   60
Human ZAP-70    ..... ..... ..... ..... ..... .....   60
Consensus       MPDPAAHLPF FYGSISRAEA EEHLKLAGMA DGLFLLRQCL RSLGGYVLSL VHDVRFHHFP   60

mouse ZAP-70    ..... ..... ...CQ...Q. ..... .....P.... .....   120
Human ZAP-70    ..... ..... ...VE...R. ..... .....S.... .....   120
Consensus       IERQLNGTYA IAGGKAHCGP AEL..FYS.D PDGLPCNLRK PCNRP.GLEP QPGVFDCLRD   120

mouse ZAP-70    ...C..... .....D.... ..... ..... ..... .....   180
Human ZAP-70    ...R..... .....E.... ..... ..... ..... .....   180
Consensus       AMV.DYVRQT WKLEG.ALEQ AIISQAPQVE KLIATTAHER MPWYHSSLTR EEAERKLYSG   180

mouse ZAP-70    Q..... ..... ..V..... ..... ..... .....   240
Human ZAP-70    A..... ..... ..I..... ..... ..... .....   240
Consensus       .QTDGKFLLR PRKEQGTYAL SL.YGKTVYH YLISQDKAGK YCIPEGTKFD TLWQLVEYLK   240

mouse ZAP-70    ........R. ..V..... A.VA.--... ......F.Q. ...V..... ........LA   298
Human ZAP-70    ........C. ..A..... N.SG.AA... ......L.H. ...I..... ........IT   300
Consensus       LKADGLIY.L KE.CPNSSAS .A..A..PTL PAHPST.T.P QRR.DTLNSD GYTPEPAR..   300

mouse ZAP-70    .ST..... ..... ..... .....E...V ..... .....   358
Human ZAP-70    .P-..... ..... ..... .....D...I ..... .....   359
Consensus       S..DKPRPMP MDTSVYESPY SDPEELKDKK LFLKR.NLL. ADIELGCGNF GSVRQGVYRM   360

mouse ZAP-70    ..... ..... KD..... ..... ..... .....   418
Human ZAP-70    ..... ..... TE..... ..... ..... .....   419
Consensus       RKKQIDVAIK VLKQGTEKAD ..EMMREAQI MHQLDNPYIV RLIGVCQAEA LMLVMEMAGG   420

mouse ZAP-70    .......L.. K..... .....A... ..... ..... ..R.....   478
Human ZAP-70    .......V.. R..... .....S... ..... ..... ..A.....   479
Consensus       GPLHKFL.GK .EEIPVSNVA ELLHQV.MGM KYLEEKNFVH RDLAARNVLL VN.HYAKISD   480

mouse ZAP-70    ..... ..... ..... ..... ..... ...F.....   538
Human ZAP-70    ..... ..... ..... ..... ..... ...L.....   539
Consensus       FGLSKALGAD DSYYTARSAG KWPLKWYAPE CINFRKFSSR SDVWSYGVTM WEA.SYGQKP   540

mouse ZAP-70    .......Q.L D..K..... ........M. ..... ..... .....NY...   598
Human ZAP-70    .......E.M A..E..... ........L. ..... ..... .....AC...   599
Consensus       YKKMKGP.V. .FI.QGKRME CPPECPPE.Y ALMSDCWIYK WEDRPDFLTV EQRMR..YYS   600

mouse ZAP-70    P..RA....Q CE.V.....G X                                   619
Human ZAP-70    L..KV....G ST.K.....A -                                   619
Consensus       .AS..EGPP. ..Q.AEAAC.                                     621
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FIG. 3. Sequence comparison of predicted protein products of mouse and human ZAP-70 cDNAs. The predicted protein product of the mouse ZAP-70 cDNA is compared with the predicted protein sequence of human ZAP-70 as reported by Chan et al. in the one-letter amino acid code (5). Dots represent identical residues.

similar to the published human sequence. Mouse ZAP-70 encodes a 618-amino-acid protein which differs from the human sequence at only 41 positions (Fig. 3).

The cDNA was appended with sequences encoding a Myc epitope recognized by monoclonal antibody 9E10 (11). This modified ZAP-70 cDNA was transfected into HeLa cells and expressed with the vaccinia virus-T7 expression system. Immunoprecipitates prepared with the 9E10 antibody were tested for ZAP-70 expression in an in vitro kinase assay. Phosphorylation of a 70-kDa protein was detected in lysates of cells transfected with the ZAP-70 cDNA, while lysates from mock-transfected cells demonstrated no kinase activity (data not shown). The cDNA that we isolated therefore appears to encode murine ZAP-70.

Association of ZAP-70 with cytoplasmic sequences of ζ in vivo and in vitro. We next tested the ability of our ZAP-70 clone to associate with the ζ chain by first testing whether association could occur in vivo. In the vaccinia virus-T7 expression system, ZAP-70 was coexpressed with the GZeta chimera, and GZeta/ZAP-70 complex formation was analyzed by measuring ZAP-70 kinase activity present in anti-VSV G immunoprecipitates (Fig. 4). Anti-VSV G immunoprecipitates from cells coexpressing GZeta and ZAP-70 demonstrated significant kinase activity, with phosphorylation of a 70-kDa protein that is likely ZAP-70 (Fig. 4, lane 2). This association was specific, because a mutated form of GZeta lacking the entire cytoplasmic domain of ζ, GZetaA32* (13), did not coprecipitate any kinase activity (Fig. 4, lane 1).

To directly evaluate the requirements for the association of ζ with ZAP-70, we tested whether GZeta and ZAP-70 could form a stable complex in vitro. We also tested whether phosphorylation of GZeta by the T-cell isoform of $p59^{fyn}$ could enhance complex formation. Cell lysates from cells expressing GZeta alone or from cells coexpressing GZeta and $p59^{fyn}$ were mixed with equal amounts of a cell lysate containing ZAP-70 and then incubated on ice. GZeta immunoprecipitates were then tested for associated ZAP-70 kinase activity. Consistent with our previous results, GZeta was able to coprecipitate ZAP-70 kinase activity when expressed without a Src family kinase (Fig. 5A, lane 3). GZeta/ZAP-70 complexes can therefore be formed in vitro. Interestingly, the amount of coprecipitating ZAP-70 kinase activity was strongly enhanced when

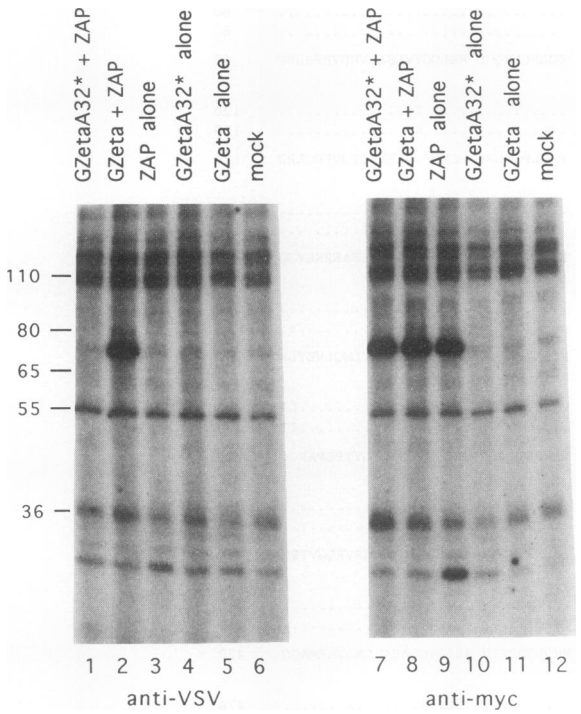

FIG. 4. Specific association of ZAP-70 with cytoplasmic sequences of ζ in vivo. HeLa cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase and transfected with the indicated cDNAs. At 10 to 12 h postinfection, cell lysates were divided, immunoprecipitates were prepared with monoclonal antibodies to VSV G (lanes 1 to 6) and to Myc (lanes 7 to 12), and in vitro kinase assays were performed. Phosphoproteins were analyzed on a 10% polyacrylamide–SDS gel and by autoradiography. Sizes are shown in kilodaltons.

GZeta was coexpressed with $p59^{fyn}$, suggesting that tyrosine phosphorylation of GZeta may enhance complex formation (Fig. 5A, lane 5). Immunoblotting of GZeta immunoprecipitates with polyclonal rabbit antibodies to ZAP-70 confirmed that the association of ZAP-70 protein was increased (Fig. 5A, lanes 10 and 11). VSV immunoblots of GZeta immunoprecipitates confirmed that roughly equivalent amounts of GZeta were present in the immunoprecipitates (Fig. 5A, lanes 6 and 7), and antiphosphotyrosine immunoblotting of GZeta immunoprecipitates confirmed that coexpression of GZeta with $p59^{fyn}$ resulted in greatly enhanced tyrosine phosphorylation of GZeta (Fig. 5A, lanes 8 and 9). Importantly, a small amount of phosphotyrosine could be detected on GZeta molecules expressed without $p59^{fyn}$, suggesting that the ability of GZeta molecules to bind ZAP-70 when expressed without a Src family kinase may be due to tyrosine phosphorylation by endogenous *src* family kinases. These results suggest that tyrosine phosphorylation of GZeta by $p59^{fyn}$ may be required for its binding with ZAP-70. Note that although we used $p59^{fyn}$ to phosphorylate GZeta in vivo in these experiments, this effect was not specific to $p59^{fyn}$. Coexpression of other Src family tyrosine kinases with GZeta, such as $p60^{src}$, $p62^{yes}$, $p58^{fgr}$, and $p56^{lck}$, gave similar results (data not shown).

Association of ZAP-70 with cytoplasmic sequences of ζ requires tyrosine phosphorylation of ζ. Although the previous experiment suggested that tyrosine phosphorylation is required for ζ/ZAP-70 complex formation, the evidence was indirect and complicated by the fact that cell lysates were used instead of purified proteins. Therefore, to directly evaluate the role of ζ tyrosine phosphorylation, we developed an in vitro assay with purified components. If the association with ZAP-70 required tyrosine phosphorylation of GZeta, as we suspected, it was likely to be mediated by the ZAP-70 SH2 domains. To test this hypothesis, a recombinant protein containing the portion of ZAP-70 which includes the two ZAP-70 SH2 domains was generated in *Escherichia coli*. The recombinant protein, which includes a polyhistidine tag at the amino terminus to facilitate purification, has an apparent molecular mass of 30 kDa. The ability of this fusion protein to bind GZeta was tested both in a competition assay (data not shown) and by direct binding to GZeta.

Direct binding of the recombinant ZAP-70 SH2 domains was tested by first biotinylating the protein and then adding it to a mock-transfected cell lysate or cell lysates containing either GZeta and $p59^{fyn}$, G epsilon and $p59^{fyn}$, or G T− and $p59^{fyn}$ (Fig. 5B, lanes 1 to 3). Anti-VSV G immunoprecipitates were prepared, analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose. The presence of the biotinylated ZAP-70 recombinant protein in the VSV G immunoprecipitates was detected by using horseradish peroxidase conjugated to avidin. A biotinylated protein of approximately 30 kDa coprecipitating with GZeta and G epsilon was detected (Fig. 5B, lanes 1 and 2). This result was specific, because the VSV G molecule lacking the cytoplasmic domain (G T−) did not coprecipitate the biotinylated recombinant ZAP-70, nor was the biotinylated ZAP-70 molecule recognized by the antibodies to VSV G (Fig. 5B, lanes 3 and 4). These results demonstrate that the ZAP-70 SH2 domains are sufficient for binding to GZeta and G epsilon.

A direct role for tyrosine phosphorylation in the interaction between ZAP-70 and ζ was assessed by purifying both nonphosphorylated and phosphorylated GZeta molecules over VSV G immunoaffinity columns and adding a portion of the purified protein preparation to the biotinylated recombinant ZAP-70 SH2 domains (Fig. 5C). Binding of the GZeta molecules was assessed by precipitating the recombinant ZAP-70 molecules with a nickel-containing resin (which binds to the polyhistidine tag) and then immunoblotting with antibodies to VSV. Phosphorylated GZeta coprecipitating with the recombinant ZAP-70 protein was easily detected (Fig. 5C, lane 2). In contrast, little if any nonphosphorylated GZeta binding to ZAP-70 could be detected (Fig. 5C, lane 1). Furthermore, the binding of phosphorylated GZeta to ZAP-70 was sensitive to treatment with alkaline phosphatase, which significantly reduced binding to ZAP-70 (data not shown). VSV immunoblotting of the purified GZeta proteins was used to confirm that equivalent amounts of GZeta (phosphorylated and nonphosphorylated) were incubated with the ZAP-70 SH2 domains (Fig. 5C, lanes 3 and 4, respectively). Phosphotyrosine immunoblotting confirmed the presence of tyrosine phosphorylation in the phosphorylated GZeta preparations (Fig. 5C, lanes 5 and 6). These experiments demonstrate that the interaction between GZeta and ZAP-70 is direct, is mediated by the SH2 domains, and requires prior tyrosine phosphorylation of GZeta.

Association in vitro of ZAP-70 with cytoplasmic sequences of CD3 γ, ε, and η. We then tested whether ZAP-70 could associate with the cytoplasmic domains of other T-cell receptor subunits, such as CD3 γ, ε, and η, by using a set of VSV G chimeras generated previously (13). For these experiments, we measured ZAP-70 binding to chimeric T-cell receptor proteins by mixing an equal amount of a cell lysate containing ZAP-70 with lysates from cells expressing the chimeric proteins. Because tyrosine phosphorylation is required for the association

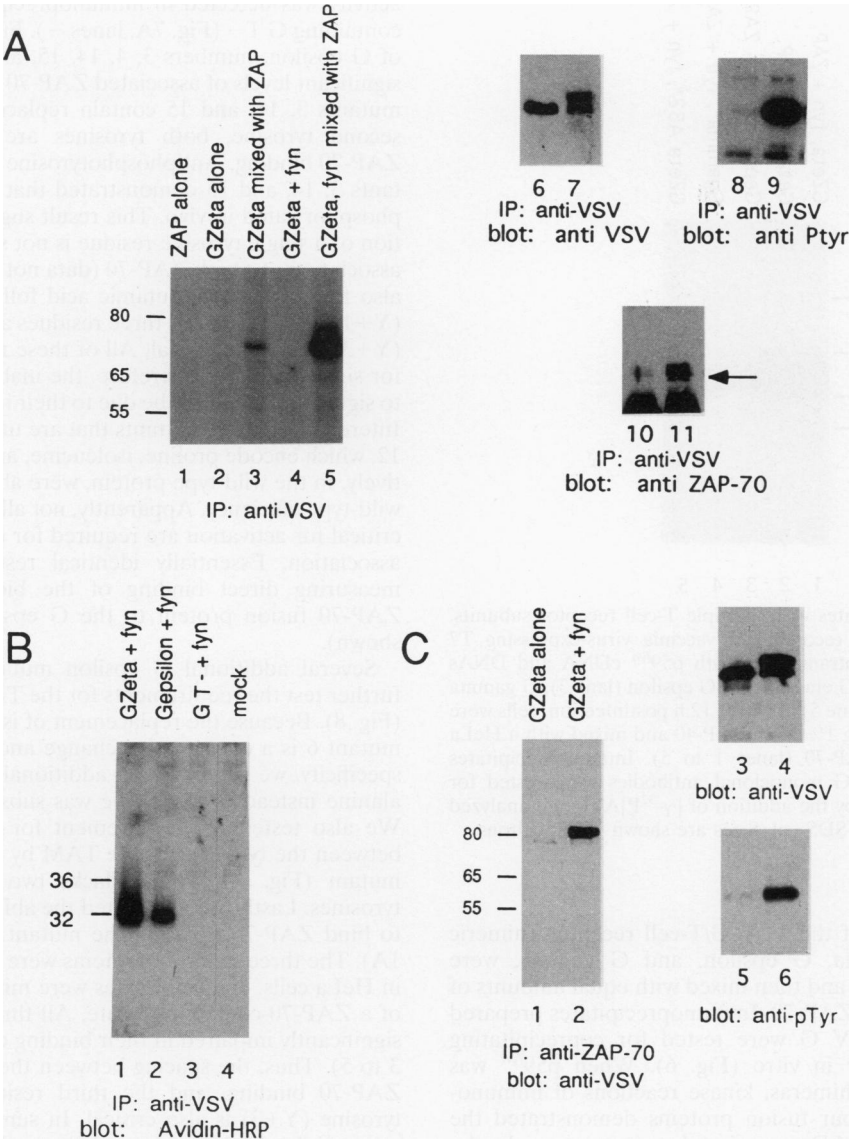

FIG. 5. Association in vitro of ZAP-70 with tyrosine-phosphorylated GZeta. (A) Association in vitro of ZAP-70 and GZeta by mixing cell lysates. Lysates from HeLa cells expressing ZAP-70 (lane 1), GZeta alone (lanes 2, 3, 6, 8, and 10), or GZeta and $p59^{fyn}$ (lanes 4, 5, 7, 9, and 11) were either left untreated (lanes 2 and 4) or mixed with an equal amount of a cell lysate containing ZAP-70 (lanes 3 and 5). Immunoprecipitates (IP) were prepared with antibodies to VSV G and divided. In vitro kinase assays were performed on a portion of each immunoprecipitate and analyzed on a 10% polyacrylamide gel (lanes 1 to 5). A portion of each VSV G immunoprecipitate was also used for immunoblotting (lanes 6 to 11). The immunoprecipitates used in lanes 3 and 5 (GZeta mixed with ZAP-70 and GZeta coexpressed with $p59^{fyn}$ and mixed with ZAP-70, respectively) were immunoblotted with antibodies to VSV (lanes 6 and 7), phosphotyrosine (anti Ptyr, lanes 8 and 9), and ZAP-70 (lanes 10 and 11). (B) Association of recombinant ZAP-70 SH2 domains with phosphorylated GZeta and G epsilon. Recombinant ZAP-70 SH2 domains to which a polyhistidine tag was appended were expressed in bacteria, purified, and biotinylated. Equal amounts of protein were then mixed with a cell lysate containing GZeta coexpressed with $p59^{fyn}$ (lane 1), G epsilon coexpressed with $p59^{fyn}$ (lane 2), G T− coexpressed with $p59^{fyn}$ (lane 3), or a mock-transfected cell lysate (lane 4). Anti-VSV G immunoprecipitates were separated on 10% polyacrylamide–SDS gels, transferred to nitrocellulose, and developed with avidin conjugated to horseradish peroxidase (HRP) to detect associated biotinylated ZAP-70 SH2 domains. (C) Direct interaction of purified ZAP-70 SH2 domains with purified tyrosine-phosphorylated and nonphosphorylated GZeta. GZeta was purified from HeLa cell lysates containing GZeta alone (nonphosphorylated GZeta, lanes 1, 3, and 5) or GZeta coexpressed with $p59^{fyn}$ (phosphorylated GZeta, lanes 2, 4, and 6) by using anti-VSV G monoclonal antibodies. Equal amounts of nonphosphorylated and phosphorylated purified GZeta protein (as determined by immunoblotting, lanes 3 and 4) were incubated with purified recombinant ZAP-70 SH2 domains (described for panel B). ZAP-70 was precipitated with a nickel-containing resin. Coprecipitating GZeta proteins were detected by immunoblotting with antibodies to VSV after separation by SDS–10% PAGE and transfer to nitrocellulose (lanes 1 and 2). Immunoblotting with anti-phosphotyrosine (anti-pTyr) antibodies (lanes 5 and 6) was performed to confirm the tyrosine phosphorylation content of the "phosphorylated GZeta" preparation. Sizes are shown in kilodaltons.

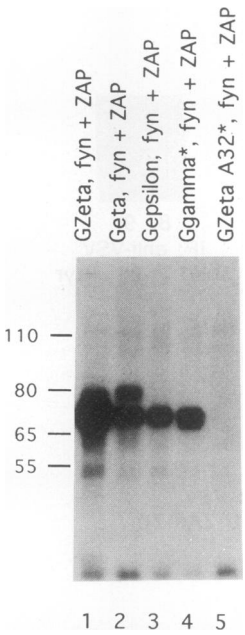

FIG. 6. ZAP-70 associates with multiple T-cell receptor subunits. HeLa cells infected with recombinant vaccinia virus-expressing T7 RNA polymerase were cotransfected with $p59^{fyn}$ cDNA and DNAs encoding GZeta (lane 1), G eta (lane 2), G epsilon (lane 3), G gamma (lane 4), or GZetaA32* (lane 5). At 10 to 12 h postinfection, cells were lysed in a buffer containing 1% Nonidet P-40 and mixed with a HeLa cell lysate containing ZAP-70 (lanes 1 to 5). Immunoprecipitates prepared with anti-VSV G monoclonal antibodies were tested for associated kinase activity by the addition of [γ-^{32}P]ATP and analyzed on a 10% polyacrylamide–SDS gel. Sizes are shown in kilodaltons.

of ζ and ZAP-70, all of the VSV G/T-cell receptor chimeric proteins, GZeta, G eta, G epsilon, and G gamma, were coexpressed with $p59^{fyn}$ and then mixed with equal amounts of a cell lysate containing ZAP-70. Immunoprecipitates prepared with antibodies to VSV G were tested for coprecipitating ZAP-70 kinase activity in vitro (Fig. 6). When $p59^{fyn}$ was coexpressed with the chimeras, kinase reactions of immunoprecipitates from all four fusion proteins demonstrated the phosphorylation of a 70-kDa protein substrate presumed to be ZAP-70. The amount of kinase activity coprecipitated by G gamma, G eta, and G epsilon, however, was lower than that seen with GZeta, perhaps reflecting a higher affinity of ζ cytoplasmic sequences for ZAP-70. These experiments demonstrate that ZAP-70 can bind to T-cell receptor subunits in addition to ζ.

Mapping of residues in CD3 epsilon critical for association with ZAP-70. The ability of the G epsilon chimera to bind ZAP-70 allowed us to scan the CD3 ε TAM for residues critical for the binding of ZAP-70. Each of the 21 G epsilon mutants described in Fig. 2 was coexpressed with $p59^{fyn}$ in HeLa cells. Cell lysates were then mixed with equal portions of a lysate from HeLa cells expressing ZAP-70. After a brief incubation, anti-VSV G immunoprecipitates were prepared and tested for associated kinase activity in an in vitro kinase assay (Fig. 7) or by direct binding to the biotinylated recombinant ZAP-70 SH2 domains (data not shown). The full-length G epsilon chimera and the construct which contains only the transmembrane and extracellular domains of VSV G, G T−, were used as controls. As described above, ZAP-70 kinase activity was easily detected in immunoprecipitates of the unmutated G epsilon chimera when coexpressed with $p59^{fyn}$ (Fig. 7A, lanes +). No kinase activity was detected in immunoprecipitates from cell lysates containing G T− (Fig. 7A, lanes −). Five of the mutated forms of G epsilon, numbers 3, 4, 14, 15, and 18, demonstrated no significant levels of associated ZAP-70 kinase activity. Because mutants 3, 14, and 15 contain replacements of the first and second tyrosine, both tyrosines are therefore critical for ZAP-70 binding. Antiphosphotyrosine immunoblotting of mutants 3, 14, and 15 demonstrated that they were still tyrosine phosphorylated in vivo. This result suggests that phosphorylation of a single tyrosine residue is not sufficient to mediate the association of ε with ZAP-70 (data not shown). In addition, we also found that the glutamic acid following the first tyrosine (Y+1) and the leucine three residues after the second tyrosine (Y+3) were also critical. All of these residues are also critical for signalling (28). Therefore, the inability of these ε mutants to signal in T cells may be due to their inability to bind ZAP-70. Interestingly, three mutants that are unable to signal, 5, 6, and 12, which encode proline, isoleucine, and aspartic acid, respectively, in the wild-type protein, were able to bind ZAP-70 with wild-type efficiency. Apparently, not all of the residues that are critical for activation are required for either ZAP-70 or $p59^{fyn}$ association. Essentially identical results were obtained by measuring direct binding of the biotinylated recombinant ZAP-70 fusion protein to the G epsilon mutants (data not shown).

Several additional G epsilon mutants were generated to further test the requirements for the TAM for ZAP-70 binding (Fig. 8). Because the replacement of isoleucine with leucine in mutant 6 is a conservative change and unlikely to affect SH2 specificity, we generated an additional mutant, 6I-A, in which alanine instead of isoleucine was substituted at this position. We also tested the requirement for the 10-residue spacing between the tyrosines in the TAM by using the G epsilon −2 mutant (Fig. 1A), which lacks two residues between the tyrosines. Lastly, we also tested the ability of a truncated TAM to bind ZAP-70 by using the mutant G epsilon Y181* (Fig. 1A). The three mutated proteins were coexpressed with $p59^{fyn}$ in HeLa cells, and cell lysates were mixed with equal amounts of a ZAP-70-containing lysate. All three of the mutants were significantly impaired in their binding of ZAP-70 (Fig. 8, lanes 3 to 5). Thus, the spacing between the tyrosines is critical for ZAP-70 binding, and the third residue following the first tyrosine (Y+3) is also critical. In summary, these data demonstrate that both tyrosines, their spacing in the TAM, and the isoleucines or leucines at the Y+3 positions are all critical for ZAP-70 binding. In addition, the spacing of the critical residues (the Y+1 and Y+3 residues following the first tyrosine and the Y+3 residue following the second tyrosine) is consistent with what is known about SH2 recognition and supports our data demonstrating that this is an SH2-specific interaction. All of the conserved features of the TAM are therefore requirements for ZAP-70 SH2 domain binding.

Association of ZAP-70 with ζ requires two SH2 domains. As demonstrated above, complex formation between ZAP-70 and tyrosine-phosphorylated T-cell receptor chimeric proteins was mediated by at least one of the two ZAP-70 SH2 domains. To determine which of the ZAP-70 SH2 domains was binding to ζ, two mutant ZAP-70 molecules were generated, lacking either the N-terminal SH2 domain (ZAPΔSH2N) or the C-terminal SH2 domain (ZAPΔSH2C) (Fig. 9A). Both of these molecules were also tagged with the Myc epitope recognized by monoclonal antibody 9E10 (11). To ensure that the deletion of either SH2 domain did not disrupt ZAP-70 kinase activity, both mutants were expressed in HeLa cells with the T7-vaccinia virus system, and in vitro kinase reactions were

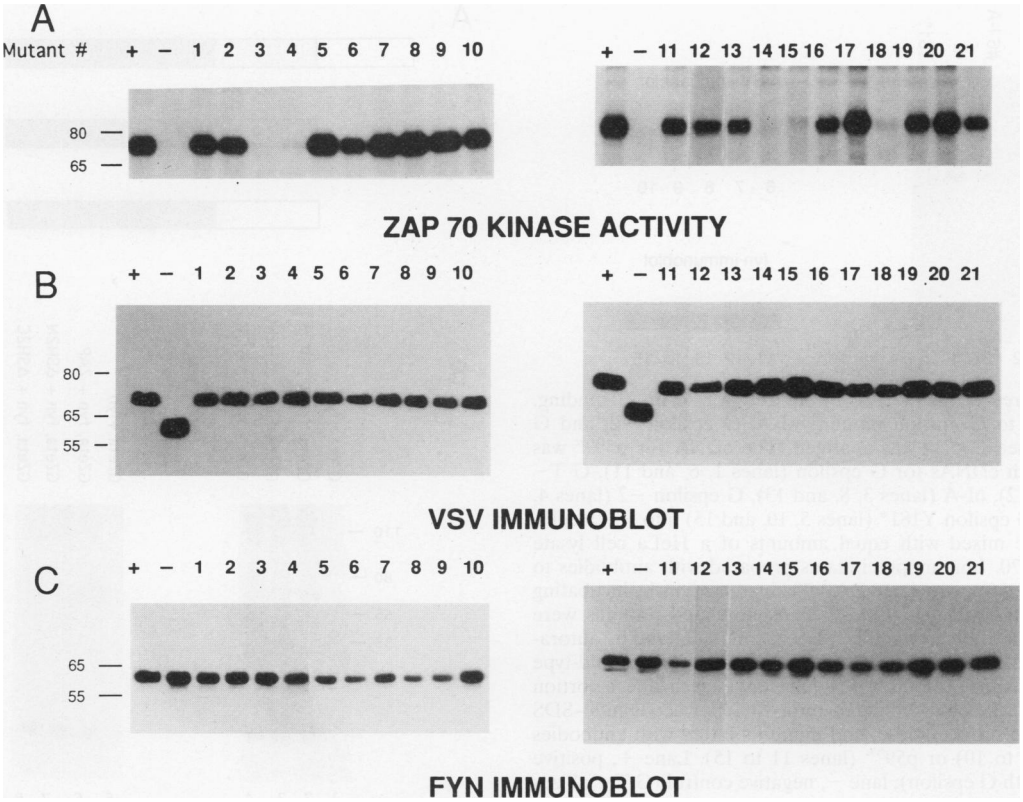

FIG. 7. Multiple residues of ε are required for ZAP-70 binding. (A) Coimmunoprecipitation assays of ZAP-70 associated with ε mutants. HeLa cells infected with recombinant vaccinia virus expressing T7 RNA polymerase were cotransfected with $p59^{fyn}$ cDNA and DNAs encoding each of the ε mutants described in the legend to Fig. 2 and designated by number (1 to 21). After 10 to 12 h, each cell lysate was mixed with an equal aliquot of a HeLa cell lysate containing ZAP-70, and immunoprecipitates were prepared with antibodies to VSV G. Associated ZAP-70 kinase activity was detected by incubating immunoprecipitates with [γ-^{32}P]ATP and analyzed on a 10% polyacrylamide–SDS gel by autoradiography. (B) VSV immunoblot of lysates prepared for kinase assay. Duplicate cultures of cells, prepared as described for panel A, were lysed directly in sample buffer, separated on 10% polyacrylamide–SDS gels, transferred to nitrocellulose, and immunoblotted with antibodies to VSV. (C) $p59^{fyn}$ immunoblot of lysates prepared for kinase assay. Duplicate cultures of cells, prepared as described for panel A, were lysed directly in sample buffer, separated on 10% polyacrylamide–SDS gels, transferred to nitrocellulose, and immunoblotted with antibodies to $p59^{fyn}$. Lanes +, positive control (full-length G epsilon); lanes −, negative control (G T−). Sizes are shown in kilodaltons.

performed on anti-Myc immunoprecipitates from the cell lysates. Phosphorylated proteins with an apparent mobility of 55 kDa, which is consistent with the predicted molecular masses of the two mutated proteins, were observed (Fig. 9B, ΔSH2N and ΔSH2C). Thus, the removal of either SH2 domain of ZAP-70 did not disrupt its kinase activity. To test the ability of the ZAP-70 mutants to bind GZeta, cell lysates containing either the full-length ZAP-70 or the deleted ZAP-70 molecules were mixed with a lysate from cells coexpressing GZeta and $p59^{fyn}$. Kinase activity was detected only in VSV G immunoprecipitates prepared with the full-length ZAP-70 molecule (Fig. 9B, lane GZeta, fyn + ZAP). Neither of the two deleted ZAP-70 molecules could be detected in GZeta immunoprecipitates (Fig. 9B, lanes GZeta, fyn + SH2N and GZeta, fyn + SH2C). This result suggests that both ZAP-70 SH2 domains are required for association with phosphorylated TAMs. This hypothesis is supported by the findings described above demonstrating that both tyrosines in the ε cytoplasmic domain are required for ZAP-70 binding. In summary, our data strongly suggest that the tandem SH2 domains of ZAP-70 recognize the TAMs via their precisely spaced, dually phosphorylated tyrosines as well as via specific interactions at the Y+1 and Y+3 positions.

DISCUSSION

Recent extraordinary progress in elucidating the signalling pathways utilized by receptor tyrosine kinases such as the epidermal and platelet-derived growth factor, insulin, and colony-stimulating factor 1 receptors provides a picture of how the tyrosine phosphorylation of critical substrates, most strikingly the cytoplasmic tails of the receptors themselves, results in activation cascades via two molecular mechanisms. First is the regulation of enzymes by tyrosine phosphorylation, such as phospholipase C-γ (32) and $p60^{c\text{-}src}$ (3, 22, 33). Allosteric regulation of enzymes by phosphorylation, as first elucidated for the serine/threonine kinase pathways, has provided a paradigm for tyrosine phosphorylation. The second novel mechanism underlying the biochemical effects of tyrosine phosphorylation is the assembly of specific macromolecular complexes via the recognition of short phosphotyrosine peptide sequences by widely distributed SH2 domains, found in a large number of proteins (23, 30, 42). The consequences of SH2-phosphotyrosine interactions include the relocalization of proteins, the allosteric activation of SH2 domain-containing molecules, and the assembly of larger activating complexes via SH2-containing adaptor proteins. Although the biochemical

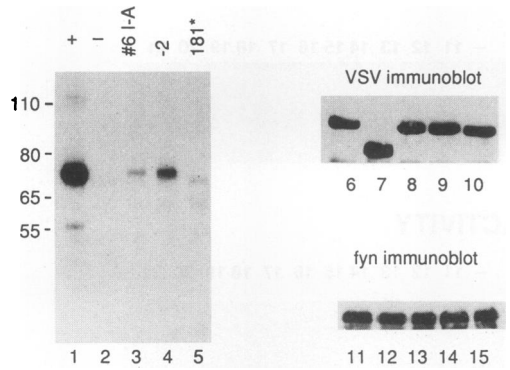

FIG. 8. Features of the ε TAM required for ZAP-70 binding. ZAP-70 binding to G epsilon mutants 6I-A, G epsilon −2, and G epsilon Y181* (see Fig. 1) was analyzed. The cDNA for $p59^{fyn}$ was cotransfected with cDNAs for G epsilon (lanes 1, 6, and 11), G T− (lanes 2, 7, and 12), 6I-A (lanes 3, 8, and 13), G epsilon −2 (lanes 4, 9, and 14), and G epsilon Y181* (lanes 5, 10, and 15) into HeLa cells. Cell lysates were mixed with equal amounts of a HeLa cell lysate containing ZAP-70. Immunoprecipitates prepared with antibodies to VSV G were tested for associated ZAP-70 kinase activity by incubating immunoprecipitates with [γ-^{32}P]ATP. Phosphorylated proteins were separated on a 10% polyacrylamide–SDS gel and analyzed by autoradiography. To confirm that expression levels of $p59^{fyn}$ and the wild-type and mutated G epsilon proteins were equivalent in each lane, a portion of each cell lysate was also analyzed on a 10% polyacrylamide–SDS gel, transferred to nitrocellulose, and immunoblotted with antibodies to VSV (lanes 6 to 10) or $p59^{fyn}$ (lanes 11 to 15). Lane +, positive control (full-length G epsilon); lane −, negative control (G T−). Sizes are shown in kilodaltons.

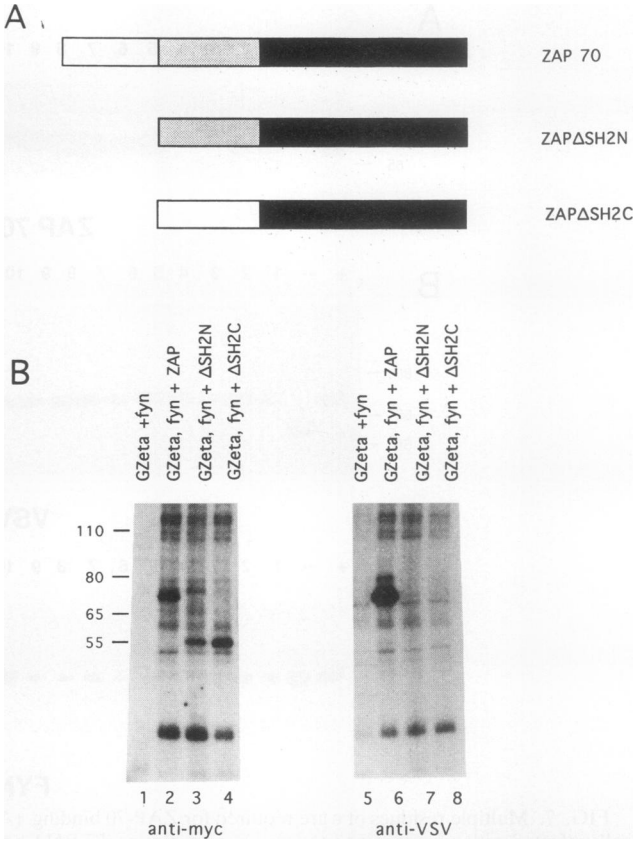

FIG. 9. Requirement for two SH2 domains for association of ZAP-70 with ζ in vitro. (A) Diagram of the full-length and deleted ZAP-70 constructs. The amino- and carboxy-terminal SH2 domains are depicted as open and stippled boxes, respectively. The kinase domain is depicted as a black box. The Myc epitope tag (recognized by the 9E10 antibody) is appended to all of these constructs at the carboxy terminus. (B) In vitro kinase reactions of ZAP-70 and GZeta immunoprecipitates. Lysates from HeLa cells expressing GZeta and $p59^{fyn}$ were divided into four equal portions and mixed with HeLa cell lysates containing either full-length ZAP-70 (GZeta, fyn + ZAP), the amino-terminal SH2 deletion mutant (GZeta, fyn + SH2N), or the carboxy-terminal SH2 deletion mutant (GZeta, fyn + SH2C) or left untreated. The mixed lysates were divided into two. Immunoprecipitates were prepared with antibodies against Myc and VSV G. In vitro kinase assays were performed on the immunoprecipitates to detect the presence of ZAP-70. Phosphoproteins were separated on SDS-polyacrylamide gels and analyzed by autoradiography. Sizes are shown in kilodaltons.

events that accompany activation of immune recognition receptors, such as the T-cell receptor, share many features with the receptor tyrosine kinases (2, 21, 51, 52), because the immune recognition receptor complexes have no intrinsic kinase activity, the question of which tyrosine kinases are involved in these systems and how they are activated has been a subject of great interest.

Over the past 3 years, two classes of tyrosine kinases have been demonstrated to associate directly or indirectly with the T-cell receptor. First it was shown that the Src family kinase $p59^{fyn}$ could be both coprecipitated with and covalently cross-linked to the T-cell receptor (39, 41). An additional Src family tyrosine kinase, $p56^{lck}$, is tightly but noncovalently bound to the coreceptor molecules CD4 and CD8, which coassemble with the T-cell receptor and its ligand, thus providing an additional or alternative Src family kinase for T-cell activation via the T-cell receptor (9, 14, 38, 47). For Jurkat T cells and the murine T-cell line CTLL-2, genetic evidence has directly implicated $p56^{lck}$ as essential for T-cell receptor-induced activation (20, 44). Either of these kinases could thus play a role analogous to that of the receptor tyrosine kinases and stimulate phosphorylation of the receptor, an event that indeed accompanies T-cell activation and is seen most prominently as multiple tyrosine phosphorylations of the T-cell receptor ζ chain (1). As with the receptor tyrosine kinases, the tyrosine phosphorylation of subunits of the T-cell receptor and other immune recognition receptors could provide an activation-dependent set of binding sites for SH2-containing signalling proteins. The first candidate for the assembly of a new protein onto the activated receptor was provided by Chan et al. with the description of ZAP-70 (4, 5). More recently, the SH2-containing protein Shc has been shown to bind to the tyrosine-phosphorylated T-cell antigen receptor in vitro (34).

Concomitant with the identification of potentially important tyrosine kinases in T-cell activation was the identification of relatively small regions of the cytoplasmic tails of several invariant chains of the T-cell receptor complex that were necessary and sufficient to induce T-cell activation when cross-linked (18, 27, 28, 36, 37, 50). Common to all of these peptide regions was an 18- to 25-amino-acid motif known as the TAM (21) or antigen receptor homology motif (2) that is present in many hematopoietic receptors, including the T-cell receptor, the B-cell antigen receptor, and the Fc receptors, and was first recognized by Reth (35). Because chimeric proteins that contain this motif are sufficient to activate hematopoietic cells, this motif defines a minimal signalling element that is likely to be a common feature of hematopoietic signalling systems. Point mutagenesis of the CD3 ε motif (28) demon-

strated that there are a number of essential features of a functional TAM, including the requirement for two tyrosines with an optimum spacing of 10 intervening residues. Another essential feature of the motif is the presence of isoleucine or leucine residues three positions beyond each tyrosine. The molecular basis of signalling by this motif, however, is not known. Here we demonstrate that the T-cell receptor chains that contain the TAM can bind to two cytoplasmic tyrosine kinases, $p59^{fyn}$ and ZAP-70. Mapping of critical residues for binding demonstrated that important features of the TAM are required for ZAP-70 binding and essentially define the requirements for ZAP-70 binding. Therefore, we believe that a major function of the TAM is to bind both of these kinases and that this can explain some or all of the signalling properties of the motif.

We previously postulated that $p59^{fyn}$ binds in a specific manner to the TAM (13). This was based on the fact that $p59^{fyn}$ can associate with multiple T-cell receptor subunits, can bind redundantly to the cytoplasmic domain of ζ, and can also interact with the B-cell antigen receptor (6). This result was specific for $p59^{fyn}$, as we could not detect association of $p56^{lck}$ or $p60^{src}$ with the TAM. However, a direct interaction of $p59^{fyn}$ with the TAM had not been shown, nor was it clear which structural features of the TAM were required for $p59^{fyn}$ binding. Here we have shown that $p59^{fyn}$ interacts specifically with the signalling motif of the CD3 ε chain, as deletion of most of the non-TAM sequences in the cytoplasmic domain had no effect on $p59^{fyn}$ binding, whereas removal of the TAM abrogated $p59^{fyn}$ binding. In addition, most of the residues between the tyrosines are not required for $p59^{fyn}$ association. However, a G epsilon mutant in which the TAM was truncated to the residue N-terminal to the second tyrosine demonstrated that these residues are required for $p59^{fyn}$ binding.

We were somewhat surprised that single-amino-acid substitutions in the TAM had no effect on $p59^{fyn}$ binding. One possible explanation is that some of the mutations generated only subtle changes in affinity that were masked by the very high levels of expression that we attain with the vaccinia virus-T7 expression system. Another explanation is that a redundant motif in the receptor, possibly the YXXL/I sequence, is responsible for $p59^{fyn}$ binding. Lastly, our inability to determine which residues in the TAM are critical for $p59^{fyn}$ binding may have been related to the use of leucine in the scanning procedure. We suspect that the specific association of $p59^{fyn}$ with the TAM may be based on the secondary structure of the TAM and may rely less on the recognition of specific amino acid side chains. We previously showed that the first 10 residues of $p59^{fyn}$ are required for the association of $p59^{fyn}$ with T-cell receptor subunits. Recently, we have shown that residues within this domain are required for myristylation and palmitoylation of $p59^{fyn}$ and have also shown that these fatty acid modifications are required for the association of $p59^{fyn}$ with the T-cell receptor (13a). The role of these fatty acids in the association suggests that the interactions between these two proteins are not completely dependent on the contact of specific amino acid residues on each protein. Rather, it is possible that the acylated amino terminus of $p59^{fyn}$ interacts with some structural feature of the TAM (e.g., an α-helix).

Cloning of the mouse ZAP-70 cDNA allowed us to examine the interactions of ZAP-70 with mouse T-cell receptor subunits in detail. Previously, Chan et al. demonstrated that human ZAP-70 associates only with activated molecules of the human ζ chain of the T-cell receptor in vivo (4, 5). Transfection studies in COS cells showing that expression of a Src family tyrosine kinase such as $p59^{fyn}$ or $p56^{lck}$ was required for ζ/ZAP-70 association suggested that phosphorylation of ζ and/or ZAP-70 was a prerequisite for association. Mapping studies demonstrate that ZAP-70 association with ζ requires the membrane-proximal 46 residues, which contain the first of the three ζ TAMs (18). Here we have extended these previous studies and show now that ZAP-70 can associate with other T-cell receptor chains as well (recently shown by Wange et al. [49]) and demonstrate that these interactions can be reconstituted in vitro. Our ability to reconstitute these interactions in vitro allowed us to demonstrate directly that the TAMs must be tyrosine phosphorylated in order to support high-affinity interactions with ZAP-70. Although there is still no direct evidence that ZAP-70 is required for T-cell activation, its ability to bind to multiple T-cell receptor TAMs suggests that ZAP-70 is involved in the signalling transduced by individual T-cell receptor subunits. Whether ZAP-70 can associate with TAMs from other cells is not known, but the ability of FcE receptor chimeric proteins to signal in T cells (24, 37) suggests that ZAP-70 can associate with TAMs from other signalling molecules.

The ability of ε to bind ZAP-70 allowed us to determine which of the residues in the motif were critical for ZAP-70 binding. Not surprisingly, both tyrosines were required for the association. Because the association of ZAP-70 with ζ requires prior tyrosine phosphorylation, it is likely that at least one of these tyrosines was phosphorylated. A requirement for two phosphorylated tyrosines, however, was implied by our experiments and those of others showing that both of the SH2 domains of ZAP-70 are required for binding (49). We suspect that the two tandem SH2 domains of ZAP-70 bind specifically to dually phosphorylated substrates and that this binding is cooperative. This idea is supported by a recent report demonstrating the inability of single ZAP-70 SH2 domains to bind any specific peptides from a degenerate library of phosphotyrosine-containing peptides (43). High-affinity binding of ZAP-70 to a phosphotyrosine-containing substrate apparently requires both of the SH2 domains. It should also be noted that the positions of the other critical residues are very consistent with what is known about SH2-phosphotyrosine interactions. The crystal structures of the $p60^{src}$ and $p56^{lck}$ SH2 domains bound to a tyrosine-phosphorylated peptide demonstrate that the phosphorylated tyrosine and the Y+3 amino acids are critical and fit into well-defined pockets (10, 48). Consistent with this model, binding of ZAP-70 was blocked by mutation of either tyrosine or by substitution of either of the Y+3 residues with alanine. The additional requirement for the Y+1 residue after the first tyrosine suggests that each of the tyrosines interacts with a distinct SH2 domain; the SH2 domain that interacts with the first tyrosine has a Y+1 and Y+3 specificity, whereas the SH2 domain that interacts with the second tyrosine has only a Y+3 specificity. It is also possible that the substitution of some amino acids with leucine was a conservative substitution at certain positions.

The data presented here suggest a model of T-cell activation in which Src family tyrosine kinases act to phosphorylate receptor TAMs. This may involve the prebound $p59^{fyn}$ kinase and/or the CD4 or CD8 coreceptor-associated $p56^{lck}$ kinase. Perhaps in the cell, $p59^{fyn}$ and $p56^{lck}$ preferentially phosphorylate different sites on the T-cell receptor complex and thus act in concert or work together to amplify initial signals transduced by the receptor. Because it is prebound to the T-cell receptor and because tyrosine phosphorylation occurs almost immediately after engagement of the T-cell receptor, we favor the involvement of $p59^{fyn}$ as the initiating kinase. Interestingly, the YXXL/I sequences found in the TAM are also high-affinity binding sites for the Src family SH2 domains (43). Receptor phosphorylation could therefore serve to activate Src family

kinases by competing with the regulatory tyrosine for binding to the Src family SH2 domains (derepression [29]) and also serve to recruit Src family tyrosine kinases to the receptor. A requirement that both tyrosines on the TAM be phosphorylated before ZAP-70 binding suggests that initial phosphorylation events are specific and regulated and will involve multiple steps. We are working to define these steps in more detail by using in vitro systems. Only after both tyrosines of a motif are phosphorylated is a second kinase, ZAP-70, bound. Once ZAP-70 is bound to the receptor, it may be brought into proximity with important substrates or possibly phosphorylate itself, further recruiting SH2-containing molecules. This model is supported by recent work by Kolanus and coworkers (25), which suggests that $p56^{lck}$ and $p59^{fyn}$ precede and potentiate ZAP-70 signalling and also suggests that ZAP-70 activation is sufficient to stimulate some T-cell receptor activation events.

Although these data suggest that signalling by TAMs is mediated by $p59^{fyn}$ and ZAP-70, the situation in vivo is probably much more complex. The ability of both $p59^{fyn}$ and ZAP-70 to bind to mutated CD3 ε chains that are incapable of signalling (Fig. 2 and 7) (28) suggests, for example, that the binding of $p59^{fyn}$ and ZAP-70 is insufficient for T-cell activation and that other important signalling proteins may be bound. In support of this, the B-cell antigen receptor TAMs have been shown to bind phosphatidylinositol 3-kinase as well as another Src family tyrosine kinase, $p56^{lyn}$ (6). Although the commonality of TAM associations with $p59^{fyn}$ and ZAP-70 suggests that multiple T-cell receptor subunits transduce redundant signals, this seems unlikely given recent reports that the T-cell receptor consists of at least two autonomous signalling modules and that ε signalling can be shown to be distinct from αβ T-cell receptor signalling in some cells (16, 50). It seems more likely, therefore, that the biochemical pathways transduced by individual TAMs will be distinct. Further understanding of the pathways induced by these motifs will require a better understanding of the mechanisms by which these motifs are phosphorylated and further identification of TAM-specific binding proteins.

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The first two authors contributed equally to this study.

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