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Binding of ZAP-70 to Phosphorylated T-Cell Receptor ζ and η Enhances Its Autophosphorylation and Generates Specific Binding Sites for SH2 Domain-Containing Proteins

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ZAP-70 is a protein tyrosine kinase thought to play a critical role in T-cell receptor (TCR) signal transduction. During T-cell activation, ZAP-70 binds to a conserved signalling motif known as the immune receptor tyrosine activating motif (ITAM) and becomes tyrosine phosphorylated. To determine whether binding of ZAP-70 to the phosphorylated ITAM was able to activate its kinase activity, we measured the kinase activity of ZAP-70 both when it was bound and when it was unbound to phosphorylated TCR subunits. The ability of ZAP-70 to phosphorylate itself, but not exogenous substrates, was enhanced when it was bound to the tyrosine-phosphorylated TCR ζ and η chains or to a construct that contained duplicated ε ITAMs. No enhanced ZAP-70 autophosphorylation was noted when it was bound to tyrosine-phosphorylated CD3 γ or ε. In addition, autophosphorylation of ZAP-70 when bound to ζ or η resulted in the generation of multiple distinct ZAP-70 phosphorylated tyrosine residues which had the capacity to bind the SH2 domains of fyn, lck, GAP, and abl. As the effect was noted only when ZAP-70 was bound to TCR subunits containing multiple ITAMs, we propose that one of the roles of the tandem ITAMs is to facilitate the autophosphorylation of ZAP-70. Tyrosine-phosphorylated ZAP-70 then mediates downstream signalling by recruiting SH2 domain-containing signalling proteins.

Activation of the T-cell antigen receptor results in the initiation of a complex biochemical cascade that involves the activation of multiple protein kinases as well as the influx of calcium and stimulation of phosphoinositide metabolism. The initiating signal is thought to be the activation of a tyrosine kinase (22, 31) and is mediated by a short, conserved sequence motif present in many hematopoietic cell receptors like the T-cell and B-cell antigen receptors (19, 20, 27, 28, 37, 38, 51). This motif, known as the immune receptor tyrosine activating motif (ITAM [5, 39]), the antigen receptor homology (4) motif, or the antigen receptor activation motif (52), consists of two YXXL/I sequences spaced by 5 to 7 variable residues (denoted by X). The ITAM is found in all of the conserved chains of the T-cell receptor (TCR), i.e., the CD3 ε, γ, δ, ζ, and η chains. The CD3 ε, γ, and δ chains each have a single ITAM, while the ζ and η chains have three and two ITAMs, respectively. The function of the multiple ITAMs of ζ and η is not known, but there is evidence to suggest that multiple ITAMs help to amplify signalling by the TCR (19, 20, 27, 28, 37, 38).

Recent work suggests that the ITAM functions by interacting with and activating several tyrosine kinases. The motif is thought to function first by binding src family kinases (16, 34). Receptor aggregation then allows associated and/or other src family tyrosine kinases to phosphorylate both tyrosines within the motif. The doubly phosphorylated ITAM is then competent to bind another tyrosine kinase, ZAP-70 (8), by engaging each of its phosphorylated tyrosines to each of ZAP-70's two SH2 domains (16, 21, 49). What occurs after ZAP-70 binds to the ITAM is not known. However, as all of the conserved features of the ITAM are also required features for ZAP-70 binding (16), it seems likely that understanding the mechanism of ITAM signalling will be tightly intertwined with understanding the functional role(s) of ZAP-70.

Critical unanswered questions are how ZAP-70 is activated and what the substrates for ZAP-70 are. One possibility is that ZAP-70 becomes activated when it binds to the phosphorylated ITAM. This hypothesis is supported by evidence that PI-3 kinase activation occurs when the SH2 domains of its 85-kDa subunit become engaged on phosphorylated receptors (2, 44). The engagement of both ZAP-70 SH2 domains with phosphorylated TCR subunits suggests that a similar mechanism might function here.

To test whether binding of ZAP-70 to the phosphorylated ITAM could activate its kinase activity, we measured the kinase activity of ZAP-70 both when it was bound to proteins with phosphorylated ITAMs and when it was unbound. Bound to a chimeric protein containing the cytoplasmic domain of ζ, ZAP-70 demonstrated an enhanced ability to phosphorylate itself in vitro. We were, however, unable to demonstrate that the bound ZAP-70 had enhanced kinase activity towards any of the exogenous substrates that we tested. Importantly, enhanced autophosphorylation of ZAP-70 was seen only when it was bound to proteins containing multiple ITAMs, namely, ζ and η. Interestingly, it was also seen when ZAP-70 was bound to an engineered protein that contained two copies of the ε ITAM. These results suggest that the binding of ZAP-70 to proteins that contain multiple ZAP-70 binding sites (ITAMs) does not activate ZAP-70 but rather facilitates aggregation of ZAP-70 molecules, thus enhancing the ability of ZAP-70 to phosphorylate itself. Thus, in addition, our results suggest that

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ZAP-70 may serve as the major substrate for itself. When bound to ζ, ZAP-70 acquired multiple tyrosine phosphorylation sites that were capable of binding multiple different SH2 domains in vitro. Phosphorylated ZAP-70 could serve as a tyrosine-phosphorylated scaffolding that functions to recruit SH2 domain-containing signalling proteins during T-cell activation.

MATERIALS AND METHODS

DNA constructs and mutagenesis. The DNA constructs for the ZAP-70–myc, GZeta (3), G eta, G epsilon, and G gamma proteins were described previously (17). Inverse PCR (18) was performed to generate the kinase-inactive form of ZAP-70 by using the oligonucleotides AAG CAG GGC ACG GAG AAG GC (sense) and CAG GAC **CCG** GAT GGC CAC GT (antisense). The codon encoding lysine at position 368 was changed to encode arginine (boldface). The GepsilonX2 construct was generated by PCR and encodes a protein with a duplication of the ε ITAM sequence DRPPPVPNPDYEPIRKGQRDLYSGL NQRAV. A *Hin*dIII restriction site (underlined) was inserted into the Gepsilon cDNA at the end of the coding sequence by PCR with the oligonucleotides T7 sequencing primer and 5′ TATCAAGCTTACTGCTCTCTGATTGAGG 3′. The fragment was digested with *Bam*HI and *Hin*dIII and ligated into the *Bam*HI and *Hin*dIII sites of pGepsilon to generate pGepsilon-HindIII. A DNA fragment encoding the ε ITAM (see above) with flanking *Hin*dIII restriction sites was generated by PCR using the oligonucleotides 5′ TATCAAGCTTACTGCTC TCTCTGATTGAGG 3′ and 5′ GTAAGCTTAGAGCGGCCACCACCTGTTC 3′. After digestion with *Hin*dIII, the fragment was ligated into the *Hin*dIII site of pGepsilon-HindIII. The accuracy of the construct was confirmed by DNA sequencing (40).

Protein expression and analysis. Methods for expressing proteins transiently in HeLa cells by using the vaccinia virus T7 system (15) have been previously described (17). For the ZAP-70 experiments, HeLa cells were lysed on ice with lysis buffer (1% Triton X-100, 25 mM Tris [pH 7.4]), 150 mM NaCl, 25 mM NaF, 100 μM sodium orthovanadate, aprotinin). Insoluble material was removed by centrifugation at 4°C for 5 min, and lysates were precleared for 30 min by using preimmune rabbit serum and protein A-Sepharose. Generally, 5×10^5 cells were used and these were lysed in a volume of 1 ml. For mixing experiments, 250-μl aliquots of precleared lysates were used. Antibodies and kinase reactions were as described previously (17). Kinase assays were performed in a total volume of 15 μl of kinase buffer containing 20 μCi of [γ-^{32}P]ATP (NEN, Dupont). After 5 min, the reactions were stopped with Laemmli sample buffer and the mixtures were boiled. Phosphorylated proteins were separated by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (SDS–8% PAGE). Immunoblotting was performed as described previously (17). The construction, expression, and purification of the glutathione *S*-transferase (GST)–p68 fusion protein were described previously (36). A 1-μg portion of this protein was added to the kinase reaction mixtures.

Phosphoamino acid analysis and tryptic peptide mapping. Phosphoamino acid analysis was performed as described previously (43). Tryptic phosphopeptide mapping of ZAP-70 was performed essentially as described by Luo et al. (29). ^{32}P-labelled ZAP-70 was transferred to nitrocellulose and excised after autoradiography. Digestion was performed with 5 μg of TPCK (tosylphenylalanine chloromethyl ketone; Worthington, Freehold, N.J.)-treated trypsin overnight and then with an additional 5 μg of trypsin for 2 h. After lyophilization and desalting by dialysis, phosphopeptides were mapped in two dimensions on cellulose plates (Merck) by electrophoresis at pH 4.72 and ascending chromatography using isobutyric acid buffer (1,250 ml of isobutyric acid, 38 ml of *n*-butanol, 96 ml of pyridine, 58 ml of glacial acetic acid, 558 ml of deionized water).

SH2 domain binding studies. Recombinant fusion proteins containing the SH2 domains of p59^{fyn}, p56^{lck}, and Grb2 have been described previously (36). SH2 domains for phospholipase C-γ1 (PLC-γ) and p120 ras-GAP (both N- and C-terminal SH2 domains) were purchased from Santa Cruz Biotechnology. SH2 domains for SHC and PI-3 kinase (both N- and C-terminal SH2 domains) were kindly provided by Louise Larose and Tony Pawson. SH2 domains for ZAP-70 and c-abl were provided by L. Samelson and A. M. Pendergast, respectively. GST-SH2 domain fusion proteins were purified and coupled to Affi-Gel 10 (Bio-Rad) as described previously (36).

When we tested for association of SH2 domains with in vitro-^{32}P-labelled ZAP-70 from HeLa cells, ZAP-70 immunoprecipitates from 3×10^6 transfected HeLa cells were mixed with an equal amount of GZeta-fyn lysates. After in vitro kinase reactions were performed, the protein A beads were washed extensively with phosphate-buffered saline (PBS) to remove free [γ-^{32}P]ATP. The ^{32}P-labelled reaction products were then removed from the beads with 25 μl of a 0.5% SDS solution in PBS and by boiling. The sample was diluted to 1 ml with PBS, divided into 12 aliquots, and incubated with 1 to 2 μg of immobilized protein. After washing of the beads three times with lysis buffer, bound proteins were analyzed by SDS-PAGE and detected by autoradiography.

For T-cell experiments, 2×10^7 Jurkat T cells were either left untreated or stimulated with a monoclonal antibody (C305) against the TCR (7) for 3 min before lysis. Prior to activation, cells were washed twice with PBS and incubated at a concentration of 10^7 cells per ml in PBS for 15 min at 37°C before addition of the antibody (10 μl/ml). After 3 min at 37°C, cells were pelleted and lysed on ice for 30 min. After removal of insoluble material, the lysate was incubated with immobilized SH2 domains as described above. ZAP-70 binding was assessed by immunoblotting with a monoclonal ZAP-70 antibody (21).

Coprecipitation studies. Jurkat T cells (2×10^7) were either left untreated or activated by using pervanadate. Cells were lysed after 3 min as described above, and immunoprecipitates were prepared with antibodies to fyn, GAP, and abl immobilized on agarose beads (Santa Cruz Biotechnology). After washing of the beads, bound proteins were eluted, analyzed by SDS-PAGE, and immunoblotted with antibodies to ZAP-70.

RESULTS

Binding of ZAP-70 to phosphorylated ζ enhances ZAP-70 autophosphorylation. To test the kinase activity of ZAP-70 bound to the phosphorylated ITAM, we generated ZAP-70–phosphorylated TCR complexes in vitro. Previously, using transiently expressed proteins in HeLa cells, we demonstrated that ZAP-70 could bind tyrosine-phosphorylated TCR subunits when mixed with them in vitro (16). Formation of these complexes required prior tyrosine phosphorylation of the ITAM. For these experiments, we used chimeric proteins containing the cytoplasmic domains of the CD3 chains (ζ, η, γ, and ε) fused to the transmembrane and extracellular domains of the viral glycoprotein, G, of vesicular stomatitis virus (VSV) (17). The use of chimeric proteins allowed us to express each of the TCR subunits individually and also allowed us to use the same antibody against VSV G to detect each protein. These proteins are designated GZeta, G eta, G gamma, and G epsilon.

We compared the in vitro kinase activities of ZAP-70 immunoprecipitates from lysates that contained ZAP-70 alone or mixed with lysates containing phosphorylated GZeta. Previously we showed that when GZeta is expressed alone, it is unphosphorylated and cannot bind ZAP-70 (16). However, when GZeta is coexpressed with a src family kinase, like p59^{fyn}, it becomes tyrosine phosphorylated and is then competent to bind ZAP-70. As it has been suggested that src family kinases might activate ZAP-70 (8), we also tested the effects of p59^{fyn} on ZAP-70 kinase activity directly in our system.

Cell lysates from HeLa cells expressing an epitope-tagged form of mouse ZAP-70 that reacts with the 9E10 monoclonal antibody (13) were generated by using the vaccinia virus T7 expression system and either left untreated or mixed with cell lysates containing p59^{fyn}, GZeta alone, or GZeta coexpressed with p59^{fyn}. Immunoprecipitates prepared with the 9E10 antibody or a polyclonal ZAP-70 antibody (data not shown) were tested for in vitro kinase activity. When ZAP-70 was incubated with a lysate from cells expressing GZeta alone or p59^{fyn} alone, no difference in ZAP-70 kinase activity was detected (Fig. 1A; compare lanes 3 and 4 with lane 1). However, when ZAP-70 was mixed with a lysate from cells coexpressing GZeta and p59^{fyn}, immunoprecipitates prepared with both the 9E10 and anti-ZAP-70 antibodies demonstrated a significant increase in ZAP-70 autophosphorylation (Fig. 1A, lane 5). This suggests that the binding of ZAP-70 to the phosphorylated ζ chain might activate its kinase activity.

It was possible that this activation was secondary to the effects of a kinase, such as p59^{fyn}, on ZAP-70 in the lysate. This possibility was tested in several ways. First, the experiment was performed in the presence of EDTA, which should block any kinases in the lysate from phosphorylating ZAP-70. Addition of 10 mM EDTA to all of the lysates before mixing had no effect on the subsequent enhancement of ZAP-70 autophosphorylation (Fig. 1A, lane 6). Second, we coexpressed p59^{fyn} with ZAP-70. Although ZAP-70 exhibited a decreased mobility, suggesting that ZAP-70 was indeed phosphorylated by

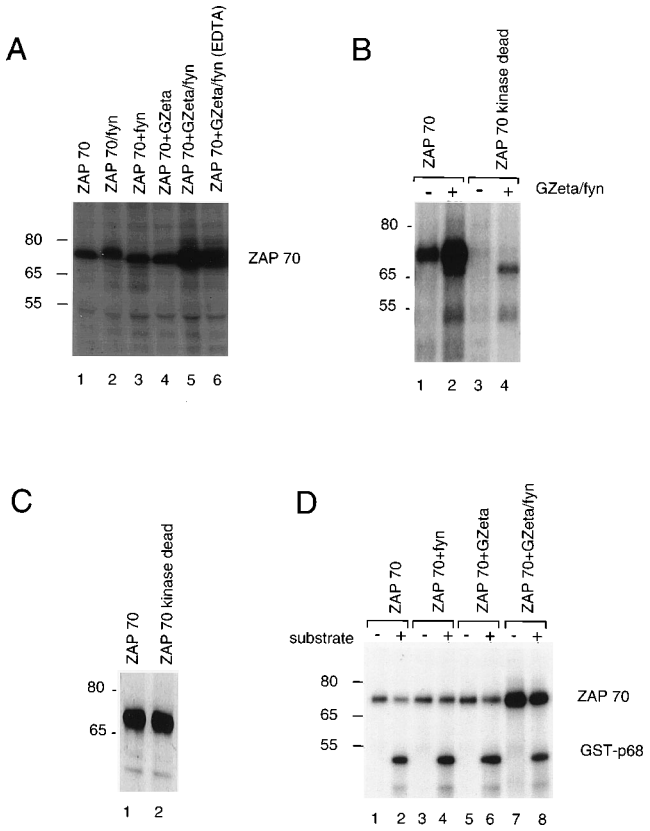

FIG. 1. Association of ZAP-70 with phosphorylated ζ enhances its autophosphorylation in vitro. (A) A cell lysate containing epitope-tagged ZAP-70 expressed by using the vaccinia virus T7 expression system was divided into four equal fractions and mixed with buffer alone (lane 1), lysates containing p59^{fyn} (lane 3), GZeta (lane 4), or GZeta coexpressed with p59^{fyn} with (lane 6) or without (lane 5) 10 mM EDTA. A lysate from HeLa cells transfected with cDNAs for both ZAP-70 and p59^{fyn} was used for lane 2. Immunoprecipitates were prepared with an antibody that recognizes the epitope tag (9E10), and in vitro kinase assays were performed. Phosphoproteins were separated on SDS–10% polyacrylamide gels and analyzed by autoradiography. Molecular mass markers (in kilodaltons) are denoted on the left. (B) Cell lysates containing either wild-type ZAP-70 (lanes 1 and 2) or kinase-inactive ZAP-70 (lanes 3 and 4) were divided in half and either incubated with a lysate containing GZeta and p59^{fyn} (lanes 2 and 4) or left untreated (lanes 1 and 3). Kinase reactions were performed as described above. (C) ZAP-70 immunoblot of whole-cell lysates containing ZAP-70 prepared as described for panel B. (D) A cell lysate containing ZAP-70 was divided into equal fractions and incubated with buffer alone (lanes 1 and 2) or lysates containing p59^{fyn} alone (lanes 3 and 4), GZeta alone (lanes 5 and 6), or GZeta and p59^{fyn} (lanes 7 and 8). ZAP-70 immunoprecipitates were divided, and in vitro kinase reactions were performed in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of an exogenous substrate, GST-p68. Figures were generated by scanning autoradiographs with a Microtek scanner and using Photoshop and Canvas software.

p59^{fyn} in vivo, there was no apparent change in the ZAP-70 autokinase activity from these lysates (Fig. 1A, lane 2). It is possible, however, that increased prephosphorylation of ZAP-70 obscured our ability to see enhanced ZAP-70 autophosphorylation. Third, it was important to prove that the phosphorylation of ZAP-70 was autophosphorylation and was not due to another kinase. It was possible, for example, that p59^{fyn} associated with GZeta was coprecipitating in the ZAP-70 immunoprecipitates. Although this was unlikely, as we have shown previously that GZeta-p59^{fyn} complexes are unstable in the detergent used in all of these experiments, Triton X-100 (17), the experiment was repeated with a kinase-inactive form of ZAP-70 (Fig. 1B). No phosphorylation of ZAP-70 was observed when the lysate containing the kinase-inactive form of ZAP-70 was incubated with a lysate containing GZeta and p59^{fyn} (Fig. 1B, lane 4). ZAP-70 immunoblotting demonstrated that similar amounts of both active and inactive ZAP-70 were expressed (Fig. 1C). Therefore, the ZAP-70 phosphorylation observed in this assay was due exclusively to ZAP-70 kinase activity and not to any contaminating kinase. Lastly, the enhanced ZAP-70 phosphorylation was still seen when ZAP-70 was incubated with immunopurified phosphorylated GZeta, which should be free of contaminating proteins (data not shown). Altogether, these data demonstrate that simple binding of ZAP-70 to phosphorylated ζ enhances its ability to phosphorylate itself and that apparently no other kinase is required.

We analyzed the enhanced autophosphorylation of ZAP-70 by testing whether the TCR-bound ZAP-70 had any increased kinase activity towards exogenous substrates. Traditional substrates like angiotensin and enolase were relatively poor substrates for ZAP-70 (data not shown). Moreover, they did not demonstrate increased phosphorylation when ZAP-70 was bound to phosphorylated GZeta (data not shown). Finally, we found that a GST fusion protein containing the C terminus of the src-associated protein p68 (36) could be efficiently phosphorylated by ZAP-70 in vitro (Fig. 1D, lanes 2, 4, 6, and 8). However, when GST-p68 was incubated with the ZAP-70 bound to phosphorylated ζ, no enhancement of p68 phosphorylation was detected even though enhanced ZAP-70 autophosphorylation in the same reaction was seen (Fig. 1D; compare lanes 7 and 8). Although these data do not rule out the possibility that ZAP-70 is catalytically activated when it is bound to phosphorylated ζ, they suggest, instead, that the binding of ZAP-70 to ζ might be facilitating its ability to be phosphorylated by itself. This facilitation might occur by the induction of a conformational change in ZAP-70 when it is bound to a phosphorylated ITAM, or it might be secondary to the clustering of ZAP-70 molecules when they are bound to the tandem ITAMs of ζ.

These two possibilities were analyzed by testing whether the enhanced autophosphorylation of ZAP-70 could be detected when ZAP-70 was bound to proteins which contain only single ITAMs like CD3 γ and ε (Fig. 2). We reasoned that if the enhanced autophosphorylation of ZAP-70 was due to a conformational change, simple binding of ZAP-70 to an ITAM should result in enhanced ZAP-70 autophosphorylation. On the other hand, if the enhanced autophosphorylation of ZAP-70 was due to clustering of ZAP-70 molecules by binding to a protein with multiple ITAMs, enhanced ZAP-70 phosphorylation would require ZAP-70 binding to proteins which contain multiple ITAMs like ζ and η. Chimeric proteins containing the cytoplasmic domains of the CD3 ζ, η, γ, and ε chains were coexpressed with p59^{fyn} and incubated with a ZAP-70-containing cell lysate (Fig. 2). Enhanced ZAP-70 phosphorylation was seen when ZAP-70 was incubated with tyrosine-phosphorylated GZeta and G eta (Fig. 2A; compare lane 1 with lanes 2 and 3) but not when it was bound to tyrosine-phosphorylated G epsilon or G gamma (Fig. 2A, lanes 4 and 5). Immunoblotting with an antibody against the common VSV G protein confirmed that all of the chimeric proteins were expressed (Fig. 2B, lanes 1 to 4). Immunoblotting with an antibody against phosphotyrosine showed that all of the proteins were tyrosine phosphorylated (Fig. 2B, lanes 5 to 8). G epsilon and G gamma were not as heavily tyrosine phosphorylated as GZeta and G eta were (Fig. 2B; compare lanes 5 and 6 with lanes 7 and 8), because they contain fewer potential tyrosine phosphorylation sites. These results suggest that binding of ZAP-70 to proteins with multiple ITAMs can enhance its ability to phosphorylate itself.

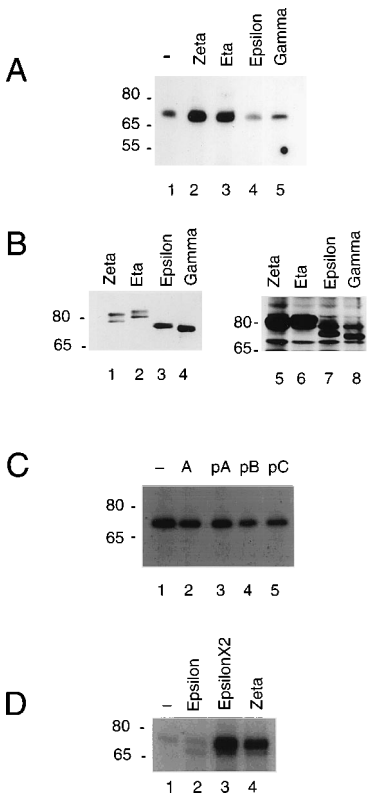

FIG. 2. Enhancement of ZAP-70 autokinase activity occurs only when ZAP-70 is bound to proteins with multiple ITAMs. (A) A ZAP-70-containing cell lysate was divided into five portions and incubated with cell lysates containing GZeta and p59^{fyn} (lane 2), G eta and p59^{fyn} (lane 3), G epsilon and p59^{fyn} (lane 4), or G gamma and p59^{fyn} (lane 5) or left untreated (lane 1). Immunoprecipitates were prepared with the 9E10 antibody, and in vitro kinase assays were performed. Phosphoproteins were separated on SDS–10% polyacrylamide gels and analyzed by autoradiography. (B) VSV and anti-phosphotyrosine immunoblots of lysates from panel A. Cell lysates from cells expressing GZeta and p59^{fyn} (lanes 1 and 5), G eta and p59^{fyn} (lanes 2 and 6), G epsilon and p59^{fyn} (lanes 3 and 7), or G gamma and p59^{fyn} (lanes 4 and 8) were immunoblotted with antibodies to VSV (lanes 1 to 4) or phosphotyrosine (lanes 5 to 8). (C) A ZAP-70-containing cell lysate was divided into five portions, and immunoprecipitates were prepared with the 9E10 antibody. Immunoprecipitates were preincubated with phosphorylated (lanes 3 to 5) or unphosphorylated (lane 2) peptides (1 μM) or left untreated (lane 1) for 15 min before in vitro kinase assays were performed. Phosphoproteins were separated on SDS–10% polyacrylamide gels and analyzed by autoradiography. (D) Binding of ZAP-70 to a protein containing two ε ITAMs can enhance ZAP-70 autophosphorylation. A ZAP-70-containing cell lysate was divided into four fractions and incubated with cell lysates containing G epsilon and p59^{fyn} (lane 2), G epsilonX2 and p59^{fyn} (lane 3), or GZeta and p59^{fyn} (lane 4) or left untreated (lane 1). Immunoprecipitates were prepared with the 9E10 antibody, and in vitro kinase assays were performed. Phosphoproteins were separated on SDS–10% polyacrylamide gels and analyzed by autoradiography.

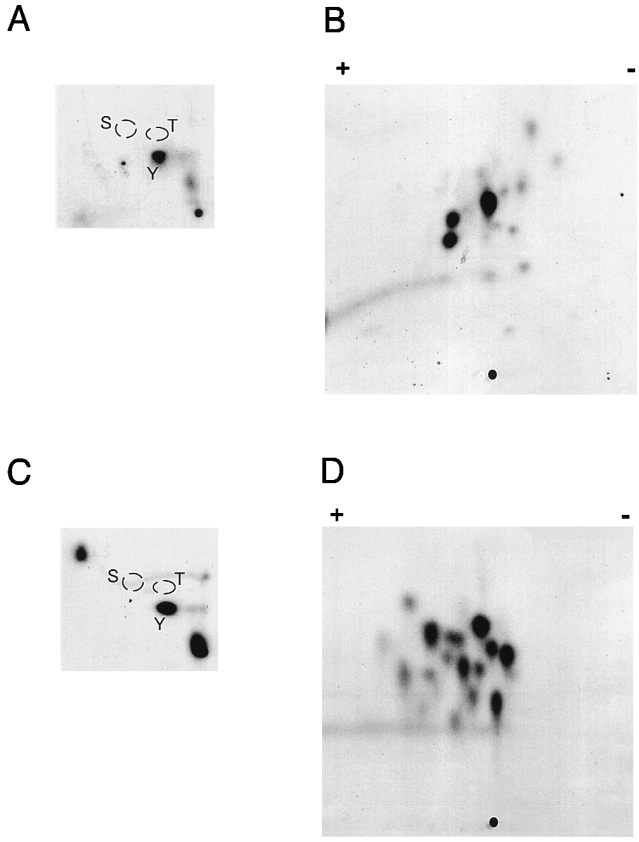

FIG. 3. Phosphoamino acid analysis and tryptic peptide maps of in vitro-autophosphorylated ZAP-70 alone or bound to phosphorylated GZeta. (A) Phosphoamino acid analysis of autophosphorylated ZAP-70 not bound to GZeta. (B) Tryptic peptide map of ZAP-70 autophosphorylation in the absence of GZeta. Immune complex kinase reactions were analyzed by SDS-PAGE, and products were visualized by autoradiography after transfer to a nylon membrane. The autophosphorylated ZAP-70 band was excised from the gel and digested overnight with trypsin. Phosphopeptides were separated in the first dimension by electrophoresis in a buffer with a pH of 4.72 and in the second dimension by chromatography. Phosphopeptides were visualized by fluorography. (C) Phosphoamino acid analysis of autophosphorylated ZAP-70 bound to GZeta. (D) Tryptic peptide map of autophosphorylation of ZAP-70 bound to phosphorylated ζ. Peptides were prepared as described above and analyzed in two dimensions.

As each ITAM has a unique sequence, it was still possible that one of the ζ or η ITAMs was responsible for enhancing ZAP-70 autokinase activity. Tyrosine-phosphorylated peptides derived from each of the ζ ITAMs were, therefore, tested for their ability to enhance ZAP-70 autophosphorylation (Fig. 2C). Incubation of ZAP-70 with phosphopeptides derived from the first (pA), second (pB), or third (pC) ITAM had no effect on ZAP-70 kinase activity (Fig. 2C, lanes 3 to 5). The unphosphorylated peptide (A; Fig. 2C, lane 2), which cannot bind ZAP-70, also had no effect on ZAP-70 kinase activity. These results suggest that the enhanced ZAP-70 autophosphorylation is not sequence specific but is due to multiple tandem ITAMs in ζ and η. To prove this point, we generated a G epsilon chimeric protein that contains two epsilon ITAMs, G epsilonX2 (Fig. 2D). Lysates from cells coexpressing G epsilon, G epsilonX2, or GZeta with p59^{fyn} were mixed with a lysate containing ZAP-70. ZAP-70 kinase activity was enhanced when the protein was bound to G epsilonX2 and GZeta but not when it was bound to G epsilon (Fig. 2D, lanes 2 to 4). The binding of ZAP-70 to proteins containing multiple tandem ITAMs therefore mediates enhanced ZAP-70 autophosphorylation in vitro.

Distinct ZAP-70 autophosphorylation sites are induced when ZAP-70 is bound to phosphorylated ζ. To determine whether the enhanced autophosphorylation of ZAP-70 was simply quantitative (with increased phosphorylation of the same sites) or whether it was qualitative (resulting in the formation of novel tyrosine phosphorylation sites), tryptic phosphopeptide maps of autophosphorylated ZAP-70, both unbound and bound to ζ, were generated. ZAP-70 was purified by SDS-PAGE, eluted, and digested to completion with trypsin. Phosphopeptides were separated by electrophoresis in one dimension and by chromatography in the second dimension (Fig.

3). Surprisingly, both maps were relatively complex, but more importantly, they were distinct from each other. Tryptic digestion of unbound autophosphorylated ZAP-70 resulted in three major phosphopeptides (Fig. 3B). In contrast, digestion of ζ-bound, autophosphorylated ZAP-70 resulted in more than nine distinct phosphopeptides (Fig. 3D). As the phosphoamino acid analysis of both proteins demonstrated only phosphotyrosine (Fig. 3A and C), these peptides are all tyrosine phosphorylated. Therefore, when ZAP-70 is bound to ζ, its ability to phosphorylate itself is enhanced and this enhancement results in the generation of multiple distinct tyrosine-phosphorylated sites.

In vitro- and in vivo-tyrosine-phosphorylated ZAP-70 binds to a distinct set of SH2 domains in vitro. Recent progress has demonstrated that one of the major functions of phosphotyrosine is to bind to SH2 domain-containing proteins. Given the complex phosphopeptide maps of ZAP-70, we tested the ability of the ζ-bound autophosphorylated ZAP-70 to specifically bind a panel of different SH2 domain-containing fusion proteins (Fig. 4A). This panel included the SH2 domains of abl, fyn, ras-GAP, Grb2, lck, PI-3 kinase, PLC-γ, and ZAP-70. Each of the SH2 domains was generated as a GST fusion protein and was covalently coupled to Sepharose beads. Autophosphorylated ZAP-70 alone or bound to ζ was washed, eluted by boiling in SDS, divided equally, and incubated with the Sepharose beads coupled to the various SH2 domains. Significant binding of ZAP-70 to the SH2 domains of abl, fyn, GAP (N terminal), and lck was detected (Fig. 4A, lanes 3 to 5 and 8). Less binding was detected with the C-terminal SH2 domain of GAP (lane 6) and a construct containing both of the SH2 domains PLC-γ1 (lane 11). No significant binding to the Grb2 (Fig. 4A, lane 7), SHC (data not shown), PI-3 kinase (lanes 9 and 10), and ZAP-70 (lane 12) SH2 domains was detected. In addition, no significant binding to any of the SH2 domains was detected with autophosphorylated ZAP-70 not bound to phosphorylated GZeta (data not shown). These results suggest that one function of ZAP-70 association with phosphorylated ζ is to facilitate autophosphorylation of ZAP-70, permitting SH2 domain-containing signalling proteins to be recruited to the activated receptor complex.

Since the ZAP-70 used in our experiments was autophosphorylated in vitro, it was possible that the ZAP-70 phosphorylated in vivo during T-cell activation contained distinct tyrosine phosphorylation sites. Although tryptic maps of ZAP-70 from activated T cells demonstrate a similar level of phosphopeptide complexity, analysis of these maps is complicated by the large amount of serine phosphorylation (6). We therefore tested the ability of ZAP-70 from activated T-cell lysates to bind the SH2 domains mentioned above. Jurkat T cells were activated by using an antibody to the TCR or were left untreated. Activation was confirmed by immunoblotting cell lysates with an antibody against phosphotyrosines (Fig. 4C, lanes 1 and 2). Lysates from both activated and unactivated T cells were incubated with the immobilized SH2 domains. The bound proteins were analyzed by SDS-PAGE and immunoblotted with antibodies to ZAP-70 (Fig. 4B). In a manner similar to that of the ζ-bound ZAP-70 autophosphorylated in vitro, ZAP-70 from activated T cells bound strongly to the SH2 domains of abl, fyn, GAP (N terminal), and lck and weakly to the PLC-γ and Grb2 SH2 domains (Fig. 4B). These results suggest that multiple tyrosine phosphorylation sites of ZAP-70 are generated during T-cell activation and that these sites can bind a variety of SH2 domain-containing signalling proteins.

ZAP-70 associates with abl and GAP during T-cell activation. We next tested the validity of our hypothesis by determining whether any of the proteins suggested to bind ZAP-70 by our experiments were actually bound to ZAP-70 in vivo. The binding of the fyn SH2 domain to ZAP-70 was not unexpected, as the SH2 domains of fyn and lck have similar binding specificities and it had been reported previously that lck associates with ZAP-70 during T-cell activation via its SH2 domain (9). We therefore focused on testing the association of abl and ras-GAP with ZAP-70 during T-cell activation. Anti-abl and anti-GAP immunoprecipitates from activated or unactivated Jurkat cell lysates were analyzed for association with ZAP-70 by immunoblotting (Fig. 4D). GAP and abl immunoprecipitates from the activated T-cell lysates clearly contained abundant ZAP-70, confirming the theory that ras-GAP and abl can associate with ZAP-70 in vivo (Fig. 4D, lanes 3 and 6). No

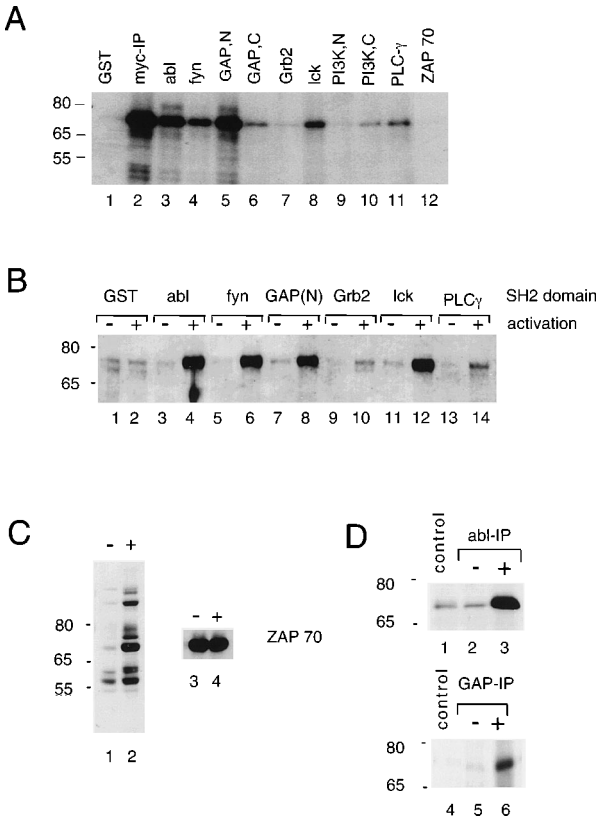

FIG. 4. Tyrosine-phosphorylated ZAP-70 prepared in vitro or in vivo binds to specific SH2 domains. (A) A ZAP-70-containing cell lysate was mixed with a lysate containing GZeta and $p59^{fyn}$. Immunoprecipitates were prepared with the 9E10 antibody, and immune complex kinase reactions were performed. After washing of the beads to remove free label, autophosphorylated ZAP-70 was eluted by treatment with SDS and boiling and then incubated with the agarose-immobilized SH2 domains indicated above lanes 3 to 12 or GST alone (lane 1). Immobilized 9E10 antibody was used as a positive control and also to indicate the maximal amount of ZAP-70 added to each sample (lane 2). After washing of the agarose beads, bound proteins were analyzed by SDS-PAGE and autoradiography. N, N terminal; C, C terminal. (B) ZAP-70 from activated T cells binds specific SH2 domains. Jurkat cells were either left untreated (−) or activated with the TCR antibody C305 (+). Cells were lysed after 5 min, cleared of insoluble debris, and incubated with the indicated agarose-immobilized SH2 domain (lanes 3 to 14) or immobilized GST alone (lanes 1 and 2). After washing of the beads, bound proteins were analyzed by SDS-PAGE and immunoblotted with antibodies to ZAP-70. (C) Anti-phosphotyrosine (lanes 1 and 2) and ZAP-70 (lanes 3 and 4) immunoblots from activated (+) and resting (−) Jurkat lysates used for panel B. (D) Tyrosine-phosphorylated ZAP-70 binds to abl and ras-GAP in vivo. Jurkat cells were left untreated (−) or activated (+) with antibodies to the TCR. Cells were then lysed and immunoprecipitated with antibodies to abl (lanes 2 and 3) or GAP (lanes 5 and 6). Immunoprecipitates (IP) were immunoblotted with antibodies to ZAP-70. Isotype-matched control antibodies were used for lanes 1 and 4.

association of ras-GAP or abl with ZAP-70 was detected for the unstimulated T-cell extracts, demonstrating that formation of the complex is regulated by T-cell activation. The lack of ZAP-70 binding to ras-GAP and abl in the unactivated samples was not due to the absence of ZAP-70 (Fig. 4C, lanes 3 and 4). These studies confirmed our previous observations and demonstrated that both abl and ras-GAP bind to activated ZAP-70 in vivo.

DISCUSSION

During T-cell activation, ZAP-70 is recruited to the TCR. Although the function of this recruitment step is not known, the severe immunodeficiency that results in patients who lack ZAP-70 suggests that the recruitment and activation of ZAP-70 play a critical role in T-cell activation and development (1, 9, 12). Important unresolved issues, therefore, are understanding how ZAP-70 is activated and identifying its substrates. As it has been demonstrated that PI-3 kinase can be activated when its SH2 domains are engaged on phosphorylated receptors (2, 44), we asked whether binding of ZAP-70 to tyrosine-phosphorylated TCRs was able to activate ZAP-70 kinase activity.

We found that binding of ZAP-70 to phosphorylated cytoplasmic domains of ζ and η results in enhanced autophosphorylation of ZAP-70 in vitro. Although these results suggest that binding stimulates ZAP-70 kinase activity, our data suggest for several reasons that this is not true enzymatic activation of ZAP-70. First, we were unable to demonstrate increased ZAP-70 kinase activity towards any exogenous substrate. Second, the enhanced kinase activity was detected only when ZAP-70 was bound to the phosphorylated ζ and η chains. We were unable to observe enhanced kinase activity when ZAP-70 was bound to proteins that contain only a single ITAM or when ZAP-70 was incubated with phosphorylated ITAM peptides. These results suggest that it is unlikely that the enhanced ZAP-70 autophosphorylation is due to a specific ITAM but rather that it is the close arrangement of multiple ITAMs in ζ and η which is responsible. The ability of a G epsilon chimera containing duplicated ε ITAMs to enhance ZAP-70 autophosphorylation confirmed that it is the multiple tandem ITAMs that are responsible. The enhanced phosphorylation that we detected was not unique to the VSV G chimeras. Binding of ZAP-70 to a tyrosine-phosphorylated bacterially expressed ζ fusion protein was also able to enhance ZAP-70 autophosphorylation (data not shown).

One possible mechanism for this effect is that multiple ZAP-70 molecules can be bound side-by-side to the tandem ITAMs of ζ or η. This would help to present ZAP-70 as a kinase substrate to an adjacent ZAP-70 molecule, thus enhancing transphosphorylation by adjacent ZAP-70 molecules. Using differentially tagged ZAP-70 molecules, we were unsuccessful in our attempts to demonstrate that multiple ZAP-70 molecules could bind to the same ζ chain. But we suspect that the efficiency of higher-order complex formation was low in our system and that our detection methods may not be sensitive enough.

Because chimeric proteins containing single ITAMs are able to efficiently activate T cells (19, 20, 27, 28, 37, 38, 51) and because T cells lacking all of the ζ ITAMs can develop normally (45), it is unlikely that multiple-ITAM-containing proteins like ζ and η have any unique functions. Rather, the multiple ITAMs of ζ and η probably function to amplify signals from the TCR (19, 20, 27, 28, 37, 38). We propose therefore that transphosphorylation of ZAP-70 can be achieved either by aggregation via TCR oligomerization or by recruitment of multiple ZAP-70 molecules to a ζ chain which is extensively phosphorylated. Either or both could function to recruit and concentrate ZAP-70 molecules, allowing transphosphorylation to occur. It is interesting to speculate that distinct mechanisms to promote ζ phosphorylation and TCR aggregation might exist and that each mechanism might result in different patterns of ZAP-70 tyrosine phosphorylation.

For example, it has recently been shown that the pattern and intensity of TCR ζ-chain phosphorylation differ when T cells are stimulated with wild-type peptide ligand versus stimulation by an altered peptide ligand (47). As stimulation by the altered peptide ligand leads to differential signalling and the induction of anergy, the altered phosphorylation of the ζ chain has an important functional consequence (46). Interestingly, ζ-chain immunoprecipitates from T cells stimulated with the altered peptide ligand lack ZAP-70 kinase activity (47). One possible interpretation of these results is that the binding of multiple ZAP-70 molecules in close proximity is required for efficient ZAP-70 signal transduction and full T-cell activation.

A direct corollary from our experiments is the idea that ZAP-70 is the major tyrosine-phosphorylated substrate for itself. This idea is supported by the fact that, compared with ZAP-70, routine tyrosine kinase substrates like angiotensin and enolase were relatively poor in vitro substrates. In addition, the complex phosphopeptide maps of ZAP-70 either bound or unbound to phosphorylated ζ support the idea that ZAP-70 prefers to phosphorylate itself. In this way, ZAP-70 behaves more like receptor tyrosine kinases than like nonreceptor tyrosine kinases, such as the src family of kinases.

Recent progress has clarified the mechanism of signalling used by the receptor tyrosine kinases (24, 26, 33, 42). A theme emerging from these studies is the idea that the major role of tyrosine phosphorylation is to create binding sites for SH2 domain-containing proteins. Central to this model is the role of a tyrosine-phosphorylated scaffolding. Phosphorylation at multiple sites of a specific substrate or scaffold generates specific sites for the recruitment of SH2 domain-containing signalling proteins, which allows the formation of a multiprotein signalling complex. For the platelet-derived growth factor and epidermal growth factor receptors, the receptor itself serves as kinase, substrate, and scaffolding. Activation of either of these receptors results in tyrosine phosphorylation of the receptor at multiple sites, and each site serves as a specific binding site for an SH2 domain-containing signalling protein. These studies suggest that understanding specific tyrosine kinase signalling pathways will require identification of the kinase substrates that will serve as the scaffolding for the recruitment of other signalling proteins.

Our data suggest that TCR signalling generates two tyrosine-phosphorylated scaffolds (schematically depicted in Fig. 5). One is generated by src family kinases, and the other is generated by ZAP-70. Interestingly, the scaffolds are assembled in layers. During TCR signal transduction, activation of src family kinases like $p59^{fyn}$ and $p56^{lck}$ and aggregation of these kinases with the TCR (25, 52) allow them to phosphorylate the ITAM. The phosphorylated ITAM serves as the first layer of the scaffold, binding to at least one specific SH2 domain-containing signalling protein, ZAP-70 (16, 21, 49). As all of the conserved features of the ITAM are also requirements for ZAP-70 binding, it seems likely that the major function of the ITAM is to bind ZAP-70 (16). There is, however, evidence that the ITAM can bind to other SH2 domain-containing signalling proteins, like SHC and PI-3 kinase (14, 35).

Recruitment of multiple ZAP-70 molecules to the phosphorylated ITAM allows ZAP-70 to generate a second tyrosine-phosphorylated scaffolding for the recruitment of other

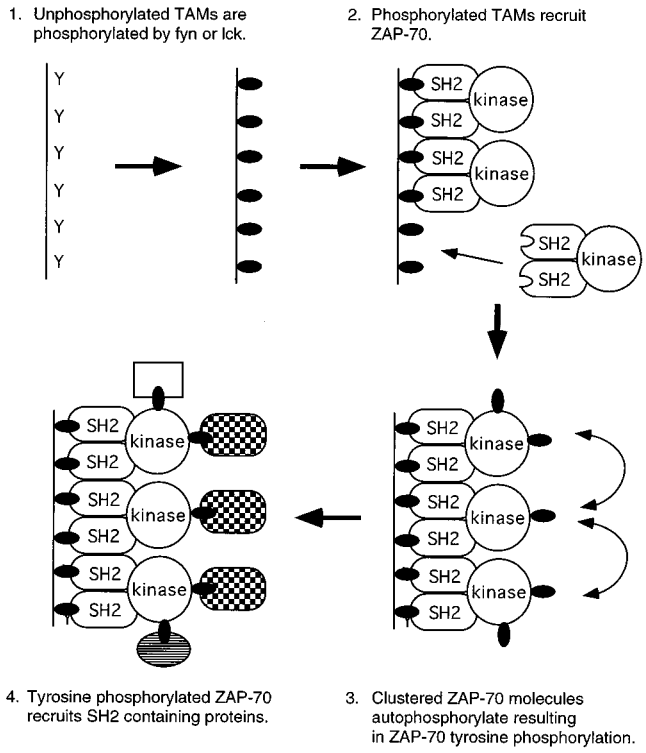

FIG. 5. Scaffolding model of T-cell activation. Phosphorylation of ITAMs by src family kinases generates binding sites for ZAP-70, recruiting ZAP-70 to the membrane. Clustering of ZAP-70 molecules at the membrane facilitates phosphorylation of ZAP-70, resulting in the generation of binding sites for SH2 domain-containing signalling proteins. TAM, tyrosine activating motif.

SH2 domain-containing proteins (Fig. 5). The substrate for ZAP-70, as for the receptor tyrosine kinases, is itself, and autophosphorylation of ZAP-70 generates multiple binding sites for SH2 domain-containing signalling proteins. Some of the SH2 domains with potential to bind ZAP-70 that we identified are those of fyn, lck, ras-GAP, and abl. Others have reported association of lck (11) and a protein related to focal adhesion kinase, fakB, with ZAP-70 (23); these associations are enhanced in activated Jurkat cells. As src family kinases, fak, ras-GAP, and abl have all been implicated in cytoskeletal regulation (10, 30, 32, 41, 48, 53), we are exploring the possibility that recruitment of ZAP-70 to a discrete part of the plasma membrane allows the cell to polarize and assemble a "contact cap."

Although we were not able to demonstrate true enzymatic activation of ZAP-70, such activation does occur after TCR cross-linking in vivo (3). Our model does not rule out the possibility that such activation is required for T-cell activation. It is possible, for example, that the ZAP-70 used in our system was already active. In fact, with baculovirus-expressed ZAP-70, phosphorylation by a src family kinase, $p56^{lck}$, can activate the kinase activity of ZAP-70 (6). Nevertheless, our results suggest that binding of ZAP-70 to ζ enhances its ability to phosphorylate itself. As the kinase-inactive form of ZAP-70 did not become labelled in our reactions, the phosphorylation measured in our experiments was due exclusively to ZAP-70 and not to another kinase. Interestingly, phosphopeptide maps of kinase-inactive ZAP-70 phosphorylated by $p59^{fyn}$ in vitro demonstrated tyrosine phosphorylation sites that were distinct from those phosphorylated by ZAP-70 (reference 50 and our unpublished data). This brings up the possibility that the number and types of ZAP-70 phosphorylation sites can be further enriched by interactions between ZAP-70 and src family kinases. Further work will be necessary to determine the significance of these phosphorylation sites and these kinase-kinase interactions.

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