Tyrosine 474 of ZAP-70 Is Required for Association with the Shc Adaptor and for T-cell Antigen Receptor-dependent Gene Activation*

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The protein tyrosine kinase ZAP-70 plays a central role in T-cell activation. Following receptor engagement, ZAP-70 is recruited to the phosphorylated subunits of the T-cell antigen receptor (TCR). This event results in ZAP-70 activation and in association of ZAP-70 with a number of signaling proteins. Among these is the Shc adaptor, which couples the activated TCR to Ras. Shc interaction with ZAP-70 is mediated by the Shc PTB domain. The inhibitory effect of a Shc mutant containing the isolated PTB domain suggests that Shc interaction with ZAP-70 might be required for TCR signaling. Here, we show that a point mutation (Phe474) of the putative Shc binding site on ZAP-70, spanning tyrosine 474, prevents ZAP-70 interaction with Shc and the subsequent binding of Shc to phospho-ζ. Neither ZAP-70 catalytic activity nor the pattern of protein phosphorylation induced by TCR triggering was affected by this mutation. However, expression of the Phe474 ZAP-70 mutant resulted in impaired TCR-dependent gene activation. ZAP-70 could effectively phosphorylate Shc in vitro. Only the CH domain, which contains the two Grb2 binding sites on Shc, was phosphorylated by ZAP-70. Both Grb2 binding sites were excellent substrates for ZAP-70. The data show that Tyr474 on ZAP-70 is required for TCR signaling and suggest that Shc association with ZAP-70 and the resulting phosphorylation of Shc might be an obligatory step in linking the activated TCR to the Ras pathway.

Since its identification as a tyrosine phosphoprotein associated with the ζ chain of the activated T-cell antigen receptor (TCR),1 the protein tyrosine kinase (PTK) ZAP-70 has emerged as a critical molecule in the generation and propagation of critical early signaling events initiated by TCR triggering and culminating in T-cell proliferation and differentiation (1–5). In contrast to Src family PTKs, ZAP-70 is found in resting peripheral T-cells as a cytosolic protein. Activation of ZAP-70 requires plasma membrane localization, which is achieved through a complex interaction of the tandem SH2 domains of ZAP-70 with specific tyrosine-phosphorylated motifs, termed immunoreceptor tyrosine-based activation motifs (ITAMs) (6), on the CD3-bound ζ chain (7, 8). The importance of this step is highlighted by recent reports showing that the SH2 domains become dispensable if ZAP-70 is artificially targeted to the plasma membrane (9). A key role in ZAP-70 recruitment to the activated TCR is played by Src family PTKs, the most prominent one being Lck, which are responsible for ζ chain phosphorylation (10–12). Lck is also implicated in the subsequent catalytic activation of ZAP-70 through phosphorylation of tyrosine 493, a site required for TCR function (13). Activated ZAP-70 autophosphorylates a number of tyrosine residues, which become potential docking sites for a number of signaling proteins, including Lck itself, Fyn, Ras-GAP, abl, Shc, Cbl, and Vav (14–18). However, with the exception of Vav (19), the relevance of these interactions has as yet not been established.

The interaction with Shc links ZAP-70 to the Ras activation pathway. Ras activity is strictly controlled by a differential subcellular localization of Ras itself and of nucleotide exchange factors, such as Sos, responsible for guanine nucleotide exchange on Ras. Activation of Ras is achieved through recruitment of Sos from the cytosol to the plasma membrane, an event that permits its interaction with Ras (reviewed in Ref. 20). Shc plays a key role in this process by binding either to activated receptor PTKs or to other membrane localized PTKs, such as Src. This interaction, which involves the SH2 domain of Shc, results in the generation by these PTKs of two binding sites for Grb2, a second adapter molecule, which in turn can recruit Sos to the plasma membrane through its double SH3 domain (reviewed in Ref. 21). Shc has been implicated in both T-cell and B-cell antigen receptor signaling to Ras (16, 22–24). Upon TCR triggering, Shc binds to the phosphorylated TCR ζ chain through its carboxyl-terminal SH2 domain and becomes in turn phosphorylated, an event that results in recruitment of Grb2/Sos (22, 23).

We have recently shown that Shc binds to ZAP-70 in response to TCR triggering (16). This interaction is mediated by a second phosphotyrosine binding domain, termed PBT, which is located in the amino-terminal region of Shc and which binds tyrosine-phosphorylated proteins with a different specificity as compared with the SH2 domain (25). Overexpression of the isolated PBT domain inhibits downstream events induced by TCR engagement, suggesting that Shc interaction with ZAP-70 might be required for TCR signaling. We have identified a putative binding site for the Shc PBT domain at tyrosine 474 of ZAP-70 (16). In this study, we used a point mutant of ZAP-70 to

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1The abbreviations used are: TCR, T-cell antigen receptor; PTK, protein tyrosine kinase; mAb, monoclonal antibody; ITAM, immunoreceptor tyrosine-based activation motif; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase.

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investigate the role of Tyr\textsuperscript{474} in the interaction of ZAP-70 with Shc and the relevance of this interaction to TCR signaling. We show that mutation of Tyr\textsuperscript{474} impairs TCR-dependent gene activation and prevents ZAP-70 interaction with Shc, suggesting a crucial role for Tyr\textsuperscript{474} in ZAP-70 function and supporting the relevance of ZAP-70 interaction with Shc in TCR signaling. Furthermore, we provide evidence for a role of ZAP-70 in linking the TCR to the Ras activation pathway through phosphorylation of the Grb2 binding sites of Shc.

**MATERIALS AND METHODS**

**Plasmids—** NF-AT/luc contains a trimer of the NF-AT binding site of the interleukin-2 promoter upstream of the gene encoding firefly luciferase (19). LTR/CAT, containing the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the HIV-1 long terminal repeat (26), was used as transfection control. A Shc deletion mutant containing the isolated PTB domain under the control of the CMV enhancer was described previously (16). A ZAP mutant containing a tyrosine to phenylalnine substitution at position 474 (Phe\textsuperscript{474}ZAP-70) was generated by M13-based, oligonucleotide-directed, site-specific in vitro mutagenesis of ZAP-70 cDNA using standard procedures. The sequence of the mutagenized cDNA was checked by automated DNA sequencing. The cDNA was subcloned as an EcoRI fragment into the mammalian expression vectors pcDNAamp and RcCMV (Invitrogen, Leek, Netherlands).

**Antibodies, GST Fusion Proteins and Peptides—** IgG from OKT3 (American Type Culture Collection) hybridoma supernatants were purified on Mabtrap (Amersham Pharmacia Biotech). The anti-TCR mAb BMA031 (27) was kindly provided by R. Kurile. Anti-Shc antibodies included rabbit polyclonal antibodies raised against a Shc CH-GST fusion protein (28) and a PTB-GST fusion protein (16) and a mouse mAb raised against a Shc SH2-GST fusion protein (23). Anti-phenylalanine and anti-ZAP-70 mAbs were purchased from Upstate Biotechnology, Inc. Anti-NP-70 polyclonal antibodies and an anti-\gamma-tubulin mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Soluble or Sepharose 4B-immobilized GST fusion proteins containing either the SH2 domain, or the PTB domain or the CH domain of Shc were previously described (29). Furthermore, GST fusion proteins containing a 20-amino acid region of Shc spanning residues 231–248 or 309–325, as previously described (29). Furthermore, GST fusion proteins containing the isolated Shc PTB domain, suggesting that no significant NF-AT luciferase activity was detected in the absence of stimulation in cells transfected with either control vector or the test expression constructs (see under “Materials and Methods”).

**Transfections, Luciferase and CAT Assays, and Flow Cytometry—** Transfections were carried out as described using a modification of the DEAE-dextran procedure (30). To minimize variability among samples, activations were carried out on aliquots of a single pool of transfected cells. In addition, all samples were in duplicate. A phasimide encoding bacterial CAT under the control of the HIV long terminal repeat (0.4 mg/sample) was included in all cotransfections as a control of transfection efficiency. Cells were allowed to recover for 22 h before activation. Cells were collected 8–10 h after activation and assayed for luciferase activity using a modification of the Promega (Madison, WI) protocol (31). Luciferase activity in the absence of stimulation was barely detectable (0.005–0.010 relative luciferase units), whereas luciferase activity in control samples activated with anti-TCR mAb ranged from 1.000 to 2.000 relative luciferase units. Test samples were compared with control samples within the same experiment. CAT assays were carried out as described (32) using equal amounts of proteins for each sample. Thin layer chromatograms were scanned, and chloramphenicol acetylation was quantitated using a PhosphorImage (Molecular Dynamics, Sunnyvale, CA). Luciferase values were adjusted to the test protein concentration and normalized to the CAT values to correct for variations in transfection efficiency. Each experiment was repeated 3–5 times. The RcCMV-Phe\textsuperscript{474}ZAP vector was introduced into Jurkat cells by electroporation, and stably transfected cells were selected in medium containing 1 mg/ml G418 (Life Technologies, Inc.). Expression of Phe\textsuperscript{474}ZAP-70 was evaluated by sequential immunoblotting of the cell lysates with anti-ZAP-70 and anti-p-\gamma-tubulin mAbs. Cells were analyzed for TCR/CD3 surface expression using fluorescein isothiocyanate-labeled anti-CD3 mAb and a FACS\textsuperscript{3} flow cytometer (Becton Dickinson, San Jose, CA).

**Activation, Immunoprecipitations, Immunobots, and Kinase Assays—** Activations were carried out as described (16). Cells were lysed in 1% (v/v) Triton X-100 in 20 mM Tris-HCl, pH 7.4, 10 mM MgCl\textsubscript{2}, 10 mM MnCl\textsubscript{2}, 50 mM ATP, 5 mM [gamma-\textsuperscript{32}P]ATP for 20 min at 37 °C using 10 units of Lck (Upstate Biotechnology) or 125 ng of GST-ZAP-70 per reaction. The reaction products were extensively washed in PBS/0.2% sodium orthovanadate before either scintillation counting or gel analysis. ZAP-70 autophosphorylation activity in Phe\textsuperscript{474}ZAP-70-expressing cells was tested under the same experimental conditions on ZAP-70-specific immunoprecipitates from cells lysed in 3% Triton X-100.

**RESULTS**

**Mutation of Tyr\textsuperscript{474} on ZAP-70 Results in Impaired TCR-dependent Gene Activation—** TCR-dependent gene activating signals can be conveniently traced using a reporter construct under the control of the NF-AT transcription factor (reviewed in Ref. 33). To assess the relevance to TCR signaling of residue Tyr\textsuperscript{474} on ZAP-70, a ZAP-70 mutant was generated by site-specific mutagenesis, carrying a tyrosine to phenylalanine substitution at position 474 (Phe\textsuperscript{474}ZAP-70). The mutant cDNA was subcloned in a mammalian expression vector under the control of the CMV enhancer and cotransfected in Jurkat cells together with a NF-AT/luciferase reporter construct. As shown in Fig. 1, a significant reduction of TCR-induced NF-AT activation was observed in the presence of Phe\textsuperscript{474}ZAP-70, suggesting a role for Tyr\textsuperscript{474} of ZAP-70 in TCR signal transduction. As reported (16) and as shown in Fig. 1, a similar inhibition was also detected following expression of a dominant negative mutant encoding the isolated Shc PTB domain, suggesting that the inhibitory effect of Phe\textsuperscript{474}ZAP-70 might result from a failure of this mutant to interact with Shc. No significant NF-AT activation was detected in the absence of stimulation in cells transfected with either control vector or the test expression constructs (see under “Materials and Methods”).

**She Interacts with Tyr\textsuperscript{474} of ZAP-70—** To address the role of Tyr\textsuperscript{474} in the interaction of ZAP-70 with Shc, we tested the capacity of Shc to bind activated ZAP-70 in the presence of either a phosphopeptide spanning ZAP-70 Tyr\textsuperscript{474} or its non-phosphorylated analogue. Shc was immunoprecipitated from lysates of nonactivated Jurkat T-cells lysed in 3% Triton X-100 to disrupt pre-existing complexes. The Shc-specific immunoprecipitates were then incubated with lysates of nonactivated or anti-CD3 mAb activated Jurkat cells and subsequently probed by immunoblot for the presence of ZAP-70. As shown in Fig. 2, ZAP-70 bands in activated cells, but not those from nonactivated cells, specifically bound to Shc. Incubation of a Shc-specific immunoprecipitate with a lysate of activated cells, in the presence of the phosphorylated ZAP-70 peptide encompassing Tyr\textsuperscript{474} resulted in a significant reduction of ZAP-70 binding, whereas the nonphosphorylated peptide had little effect (Fig. 2, upper panel). Similar amounts of Shc were detected in all immunoprecipitates after immunoblotting with an anti-Shc mAb (Fig. 2, lower panel). These results show that ZAP-70

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\[ g \textsuperscript{474} \text{ZAP-70} \]
Phosphorylation of Tyr 474 of ZAP-70 by Lck

In vitro phosphorylation of a ZAP-70 peptide spanning Tyr 474 by recombinant Lck or ZAP-70.

| PTK       | Phosphorylation (n = 4) |
|-----------|------------------------|
| None      | cpm ± S.D.              |
| Lck       | 202 ± 79               |
| ZAP-70    | 7125 ± 340             |
|            | 525 ± 475              |

The inhibitory effect of Phe 474ZAP-70 on TCR-dependent NF-AT activation was confirmed by transient transfection of the line expressing Phe 474ZAP-70 with the NF-AT reporter (40 ± 8.7% reduction as compared with parental cells). Mutation of Tyr 474, which is located in the kinase domain, could, however, potentially impair ZAP-70 kinase activity, resulting in defective TCR signal transduction, including NF-AT activation. To rule out a defect in Phe 474ZAP-70 kinase activity, ZAP-70-specific immunoprecipitates from parental Jurkat cells or cells expressing Phe 474ZAP were subjected to an in vitro kinase assay in the presence of [γ-32P]ATP. The reaction products were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and exposed to a PhosphorImager. The filter was subsequently immunoblotted with an anti-ZAP mAb. As shown in Fig. 3C, the increased level of total ZAP-70 in cells expressing Phe 474ZAP-70 was reflected in a parallel increase in the basal activity of Phe 474ZAP-70, suggesting that ZAP-70 kinase activity is unaffected by mutation of Tyr 474. Furthermore, the degree of ZAP-70 activation in

![Fig. 1. Inhibition of TCR-dependent NF-AT activation by expression of Phe 474ZAP-70. Relative luciferase activity in Jurkat cells cotransfected with NF-AT/luc and either control vector or the same vector expressing either Phe 474ZAP-70 (P474ZAP) or the isolated Shc PTB domain. Cells were activated with anti-TCR mAb. The reporter plasmid LTR/CAT was included in all transfections as a control of transfection efficiency. Luciferase activities, adjusted to the protein concentration of each sample, were normalized to CAT values and are expressed as a percentage of the values obtained with cells transfected with the empty vector and activated with anti-TCR mAb. Bars represent the SD for multiple experiments (n = 3), each with duplicate samples. Luciferase activity was below detection in the absence of stimulation both in cells transfected with control vector and in cells transfected with the Phe 474ZAP-70 or the Shc-PTB expression constructs.

![Fig. 2. Shc interacts with tyrosine-phosphorylated Tyr 474 of ZAP-70. Upper panel, immunoblot with anti-ZAP-70 mAb of an in vitro binding assay of Shc-specific immunoprecipitates with lysates of resting or activated Jurkat cells, as such or in the presence of either a non-phosphorylated (Y-pept.) or a phosphorylated (pY-pept.) peptide spanning residues 467-479 of ZAP-70. A control lysate from nonactivated cells is included. Lower panel, immunoblot with anti-Shc mAb of the same filter after stripping. Proteins were immunoprecipitated from 1 mg of lysate.

associates with Shc through Tyr 474 in a phosphorylation-dependent manner.

Although Tyr 474 has not been identified as one of the major tyrosine-phosphorylated residues on ZAP-70, not all the phosphorylation sites observed by two dimensional peptide mapping have as yet been identified (13, 34). The failure of ZAP-70 to interact with Shc in the presence of a phosphopeptide encompassing Tyr 474 strongly supports the possibility that Tyr 474 is a phosphorylation site in vivo. Two PTKs, the Src family kinase Lck and ZAP-70 itself, have been implicated in ZAP-70 phosphorylation (11-13, 15). We tested the capacity of both Lck and ZAP-70 to phosphorylate the Tyr 474 peptide in vitro. Affi-Gel-conjugated Tyr 474 peptide was incubated with either recombinant Lck or recombinant ZAP-70 in the presence of [γ-32P]ATP and subjected to an in vitro kinase assay. The samples were then extensively washed, and 32P incorporation was measured by scintillation counting. The values, expressed as cpm, are reported in Table I. The Tyr 474 peptide was effectively phosphorylated by Lck but not by ZAP-70. This was not due to a lower specific activity of recombinant ZAP-70, because experiments using the Shc CH domain as a substrate gave the opposite result (see below). Thus, Lck might be responsible for ZAP-70 Tyr 474 phosphorylation in vivo.

Mutation of ZAP-70 at Tyr 474 Prevents the Interaction with Shc without Affecting ZAP-70 Kinase Activity—To analyze the effect of Tyr 474 substitution on the interaction of ZAP-70 with Shc, we generated a Jurkat T cell line stably expressing Phe 474ZAP-70. As shown in Fig. 3A, the level of the ZAP-70 immunoreactive band in this cell line is significantly higher than in the parental line, indicating a level of 2-3-fold overexpression of Phe 474ZAP-70 as compared with endogenous ZAP-70. This difference was confirmed by immunoblotting the same filter with anti-β tubulin mAb, which revealed similar amounts of tubulin in the two lanes (data not shown). No significant difference could be detected in the level of surface TCR/CD3 expression, as evaluated by flow cytometry (MFI 26.4 for parental cells compared with 24.6 for Phe 474ZAP-70 expressing cells). Comparison by immunoblot with anti-phosphotyrosine mAb of total cell lysates from nonactivated and activated cells expressing Phe 474ZAP-70 with lysates of similarly treated parental cells did not show any obvious difference in either the basal or the induced pattern of tyrosine-phosphorylated proteins (Fig. 3B).
response to TCR triggering, measured as autophosphorylation, was similar for wild-type and Phe474ZAP-70. Thus mutation of Tyr474 does not affect ZAP-70 kinase activity, suggesting that the inhibition of NF-AT activation by Phe474ZAP-70 might be due to the failure of ZAP-70 to recruit downstream effectors.

The effect of Phe474ZAP-70 expression on ZAP-70 association with Shc was tested in coimmunoprecipitation experiments. Fig. 4A shows an anti-phosphotyrosine immunoblot of ZAP-70-specific immunoprecipitates from lysates of either parental Jurkat cells or Jurkat cells stably expressing Phe474ZAP-70. Although the levels of ZAP-70 phosphorylation were similar in the two lines, a significant reduction in the levels of the coprecipitating 52-kDa phosphoprotein was observed in cells expressing Phe474ZAP-70 (Fig. 4A, top left). Probing the stripped filter with anti-Shc mAb confirmed the identity of this protein with the p52 isoform of Shc (data not shown). Conversely, anti-phosphotyrosine immunoblots of Shc-specific immunoprecipitates from the same cells showed a similar ratio of phospho-p52Shc to phospho-ZAP-70 in the two lines (Fig. 4A, top right), consistent with Shc phosphorylation following its interaction with wild-type ZAP-70. Thus mutation of Tyr474 of ZAP-70 results in the failure of Shc to bind ZAP-70 and to become phosphorylated.

Mutation of ZAP-70 at Tyr474 Prevents Shc Binding to the TCR ζ Chain—Both ZAP-70 and Shc bind the phosphorylated ζ chain ITAMs (7, 8, 22). We have previously shown that Shc association with ZAP-70 is required for Shc binding to phospho-ζ (16). We thus expected a reduction in the levels of Shc-associated phospho-ζ in cells expressing Phe474ZAP-70. The presence of tyrosine-phosphorylated ζ chain in ZAP-70-specific and Shc-specific immunoprecipitates was tested by immunoblotting with anti-phosphotyrosine mAb. As previously reported (16), and as shown in Fig. 4, two ζ chain isoforms differing in the levels of tyrosine phosphorylation, pp21 and pp23, were observed in cells expressing ZAP-70. Although no difference in ZAP-70-bound phospho-ζ could be detected in cells expressing Phe474ZAP-70, a significant reduction in the levels of Shc-bound phospho-ζ was observed in the presence of the ZAP-70 mutant (Fig. 4). Immunoblotting the stripped filters with anti-ZAP-70 (Fig. 4A) or anti-Shc (Fig. 4B) mAb showed that similar amounts of specific protein were present in the immunoprecipitates from the two cell lines (data not shown). This result is consistent with a priming role of ZAP-70 in the interaction between Shc and tyrosine-phosphorylated ζ.

Phosphorylation of the Shc CH Domain by ZAP-70—The association between ZAP-70 and Shc induced by TCR engagement, as well as the reduction in Shc phosphorylation in cells expressing Phe474ZAP-70, suggests that Shc phosphorylation might be the direct outcome of this interaction. To address this issue, Shc was immunoprecipitated from lysates of nonactivated Jurkat cells and subjected to an in vitro kinase assay with [γ-32P]ATP in the presence or absence of recombinant GST-ZAP-70. The reaction products were resolved by SDS-polyacrylamide gel electrophoresis and visualized on the fixed and dried gel by PhosphorImager analysis. The results are presented in Fig. 5A. When Shc immunoprecipitates were incubated with recombinant ZAP-70, a phosphorylated band mi-
The Shc Binding Site on ZAP-70 Is Required for TCR Signaling

**FIG. 4.** Tyr(P) is required for ZAP-70 interaction with Shc. A, anti-phosphotyrosine immunoblots of ZAP-70-specific immunoprecipitates of nonactivated (0) or activated (CD3) Jurkat cells or from similarly treated cells expressing Phe/F474ZAP-70 (F474ZAP-70). B, anti-phosphotyrosine immunoblot of Shc-specific immunoprecipitates of nonactivated (0) or activated (CD3) Jurkat cells or from similarly treated cells expressing Phe/F474ZAP-70 (F474ZAP-70). The migration on the same gel of ZAP-70 and p52Shc from a total cell lysate is indicated, as well as the migration of molecular mass markers (kDa). The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis on either 10% (top panels) or 15% (bottom panels) acrylamide to achieve an optimal resolution of the relevant bands.

**FIG. 5.** The Shc CH domain is a substrate of ZAP-70. A, PhosphorImager print of an *in vitro* kinase assay of Shc-specific immunoprecipitates from 3% Triton X-100 lysates of nonactivated Jurkat cells in the absence or in the presence of recombinant GST-ZAP-70. B, *upper panel*, PhosphorImager print of an *in vitro* kinase assay of similar amounts of either GST or GST fusion proteins containing the isolated Shc domains in the presence of recombinant GST-ZAP-70. Lower panel, Coomassie Blue staining of a gel with similar amounts of the GST fusion proteins used for the *in vitro* kinase assay. C, *left*, PhosphorImager print of an *in vitro* kinase assay of equal amounts of GST fusion proteins containing the isolated Grb2 binding sites of Shc either as such (Y239/240, Y317) or with tyrosine to phenylalanine substitutions (F239/240, F317). The *right panel* shows an enlargement of the same kinase assay highlighting the double band in the sample containing GST-Tyr(239/240). The migration of molecular mass markers is indicated (kDa).

Although a potential role for other tyrosine kinases in Shc phosphorylation cannot be ruled out, the results presented above suggest that Shc association with ZAP-70 in response to TCR triggering results in Shc phosphorylation by ZAP-70 on the CH domain. This domain includes Tyr(239)/Tyr(240) and Tyr(317), which are phosphorylated *in vivo* and bind Grb2 (29, 35, 36). The capacity of ZAP-70 to phosphorylate either of the two Grb2 binding sites on Shc was tested in *in vitro* kinase assays. The Shc regions spanning residues 231–248 or 309–325, containing either Grb2 binding site, were expressed as GST fusion proteins. Similar constructs with tyrosine to phenylalanine substitutions at the Grb2 binding sites (Phe(239)/Phe(240) and Phe(317)) were used as controls. The capacity of ZAP-70 to phosphorylate these proteins was tested in *in vitro* kinase assays. As shown in Fig. 5C (*left panel*), both GST fusion proteins encoding the Shc Grb2 binding sites, but not the respective substitution mutants, were effectively phosphorylated by GST-ZAP-70. Two bands could be detected in the sample containing GST-Tyr(239)/Tyr(240), corresponding to the singly and doubly phosphorylated products (Fig. 5C, *right panel*). No phosphorylation was detectable in the presence of Lck (data not shown). Similar results were obtained using Affi-Gel-conjugated 15-mers spanning Shc residues 233–247 and residues 310–324 (data not shown). Collectively, these data support a direct role for ZAP-70 in tyrosine phosphorylation of Shc on the Grb2 binding sites and in the subsequent activation of the Ras pathway induced by TCR triggering.

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3 S. Pacini, L. Lanfrancone, and C. T. Baldari, unpublished observations.
The data presented provide evidence for a role of Tyr$^{474}$ of ZAP-70 in Shc recruitment and phosphorylation and in TCR signaling. A tyrosine to phenylalanine substitution at position 474 results, in fact, in a ZAP-70 mutant able to transdominantly inhibit endogenous ZAP-70 signaling to downstream targets leading to activation of the transcription factor NF-AT, which specifically drives the response of the interleukin-2 gene promoter to TCR generated signals (reviewed in Ref. 33). To date, two other tyrosine residues involved in positive regulation of ZAP-70 have been identified. The first is Tyr$^{193}$, which is phosphorylated by Lck and which is required for catalytic activation of ZAP-70 (13, 37). The second is Tyr$^{315}$, which is the binding site for Vav, a putative guanine nucleotide exchange factor for the Rac/Rho/edc42 family of small GTP-binding proteins, which plays a key role in lymphocyte development and activation (38–42). Two other tyrosine residues, Tyr$^{292}$ and Tyr$^{492}$, are implicated in negative regulation of ZAP-70, potentially by promoting interactions with inhibitory proteins (43, 44). Furthermore, two-dimensional phosphopeptide mapping has revealed the existence of additional phosphorylation sites, which might also include Tyr$^{69}$, Tyr$^{126}$, and Tyr$^{178}$, shown by Watts et al. (34) to be phosphorylated in vitro by Lck. These phosphotyrosine residues might represent the docking sites for important signaling proteins, such as Lck itself, Fyn, Ras-GAP, abl, and Cbl, which have been reported to associate with ZAP-70 either in vitro or in vivo (14, 15, 17). Although Tyr$^{474}$ of ZAP-70 might interact with additional molecular partners, our data suggest that at least one of the functions of Tyr$^{474}$ of ZAP-70 is to recruit Shc and that this interaction might be required for productive antigen receptor signaling.

The obvious consequence of Shc recruitment to the activated TCR complex is activation of the Ras pathway (reviewed in Refs. 20 and 21). Shc has been reported to directly bind tyrosine-phosphorylated ζ chain in response to TCR triggering (22). However, the low affinity of this interaction, as measured in vitro, suggests that only when bound to pp21, pp23, or pp24, only pp21ζ, would suggest a secondary role for Shc in Grb2/Sos recruitment to Ras (45). We have previously reported that binding of Shc to phospho-ζ is dependent on Shc association with ZAP-70, suggesting that ZAP-70 interaction with the Shc PTB domain is responsible for Shc recruitment in the proximity of the ζ chain, to which it can subsequently bind through its SH2 domain (16). This is strongly supported by the data presented in this paper showing a significant reduction in Shc-bound phospho-ζ in the presence of Phe$^{474}$-ZAP-70. The combined interaction of Shc with both ZAP-70 and phospho-ζ, achieved through its dual phosphotyrosine binding specificity, might stabilize Shc on the activated TCR and therefore compensate for the low affinity of the interactions between Shc and individual phosphorylated ζ chain ITAMs, thus reproposing Shc as an important player in TCR-induced Ras activation. In this respect, the capacity of Shc to simultaneously bind two Grb2 molecules has been proposed as an important means to stabilize the Ras exchange factor Sos in the Shc/Grb2/Sos complexes (46).

Shc is composed of three distinct domains, the amino-terminal PTB domain and the carboxy-terminal SH2 domain, both of which are involved in interactions with tyrosine-phosphorylated proteins, and the CH domain, which contains both Tyr$^{209}$/Tyr$^{240}$ and Tyr$^{317}$ (reviewed in Ref. 21). The dual specificity of the tyrosine binding domains, as well as the potential of each domain to bind multiple phosphoproteins in activated T-cells, suggests that, besides Ras activation, Shc might play a complex role in TCR signaling by contributing to the assembly around the activated TCR of a large multimolecular signaling complex, for which we have proposed the term “transduceosome” (16). The ζ chain is found in association with the TCR/CD3 complex predominantly as a disulfide bond-linked homodimer. Each ζ chain has three ITAMs, implying that, when fully phosphorylated, each ζ homodimer could recruit up to six molecules of ZAP-70. An important outcome of ZAP-70 recruitment to phospho-ζ is tyrosine phosphorylation of ZAP-70 both by Lck and by autophosphorylation, which results in the generation on ZAP-70 of multiple binding sites for signaling proteins, most of which have the potential to recruit additional molecular partners. Hence, ZAP-70 interaction with the activated TCR appears to be a key step in the assembly of a functional transducing complex. In this context, it is interesting to notice that overexpression of ZAP-70 does not result in significant NF-AT activation (13, 44, 47), in agreement with our finding that no NF-AT activity could be detected in the absence of stimulation in cells expressing Phe$^{474}$-ZAP-70, where the basal level of total ZAP-70 activity is higher than in parental cells. Conversely, targeting ZAP-70 to the plasma membrane, either by engineering a heterologous membrane localization site (47) or by using synthetic ligands and their binding proteins (9) is sufficient for ZAP-70 function, underlining the importance of ZAP-70 localization to the cytosolic side of the plasma membrane for productive signaling. The contribution of ZAP-70 to transduceosome assembly may be further amplified by association with Shc, with its multivalent capacity to recruit other components of the transducing complex.

A puzzling issue is the failure of ZAP-70 to become activated in response to TCR engagement by altered peptide ligands, which, despite their similarity with peptide antigens, fail to activate T-cells. When TCR is engaged by a specific altered peptide ligand, ZAP-70 is recruited to phospho-ζ, but this event is followed neither by activation nor by phosphorylation of ZAP-70 (48, 49). A clue to this question has been recently provided. Inducing alternative configurations of membrane targeted ZAP-70 using chemical inducers of dimerization has shown that orientation is an important parameter in ZAP-70 function (9). Because, of the two major phosphorylated species of ζ, pp21 and pp22, only pp22ζ can be found in T-cells presented with an altered peptide ligand (48, 49), a possibility is that only when bound to pp22ζ can ZAP-70 acquire the correct orientation. In this respect, the differential binding of Shc to pp22ζ is particularly interesting. Because Shc only binds to pp23ζ and this binding depends on a previous interaction of Shc with ZAP-70 (Ref. 16 and present results), our data suggest that the dual interaction of Shc with ZAP-70 and pp23ζ might contribute to conferring the correct orientation to ZAP-70, resulting in productive TCR signaling.

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