Dominant-negative Zeta-associated Protein 70 Inhibits T Cell Antigen Receptor Signaling

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Summary

Zeta-associated protein (ZAP)-70 is a cytoplasmic protein tyrosine kinase required for T cell antigen receptor (TCR) signaling and development. Mutations in ZAP-70 result in severe combined immunodeficiency in humans. ZAP-70 interacts with the TCR by binding to tyrosine-phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) present in the invariant subunits of the TCR complex. Here we report that two ZAP-70 mutants devoid of kinase activity, generated either by a point mutation in the kinase domain to create an inactive kinase, or by truncation of the entire kinase domain (SH2[N+C]), functioned as dominant-negative mutants to specifically suppress TCR-mediated activation of NFAT, a nuclear factor essential for inducible interleukin 2 gene expression. Biochemical studies with the SH2(N+C) mutant showed that it also blocked early TCR signaling events, such as p95vav tyrosine phosphorylation, extracellular signal-regulated kinase 2 activation, and the association of a number of tyrosine-phosphorylated proteins with growth factor receptor-binding protein 2 (GRB2). The inhibitory effects of the SH2(N+C) mutant revealed that it requires an intact phosphotyrosine-binding site in its COOH-terminal SH2 domain. Using a CD8-ε chimeric receptor to analyze the interaction of the SH2(N+C) mutant with ITAMs of TCR-ε, we found that this mutant was constitutively bound to the hyperphosphorylated CD8-ε chimera. These results indicate that tyrosine-phosphorylated ITAM is the target for the action of this dominant-negative mutant, suggesting that the assembly of a functional receptor signaling complex on ITAMs is a critical proximal TCR signaling event leading to downstream activation.
of several signaling pathways, including phospholipid metabolism, elevation of cytoplasmic free calcium, Ras-GTP accumulation, and an extracellular signal-regulated kinase cascade involving several cytoplasmic serine/threonine kinases (1–3). These signaling events ultimately lead to T cell differentiation, proliferation, and induction of effector functions. The requisite role of ZAP-70 in TCR signaling has been established by recent studies (13–16) on human patients with an immunodeficiency disease caused by loss-of-function mutations of ZAP-70. CD4+ T cells from these patients are defective in both early and late signaling through the TCR. Moreover, these patients do not have CD8+ T cells because of a developmental arrest. These findings provide strong genetic evidence that ZAP-70 is a critical molecule involved in T cell signaling and development.

Although null mutations in patients or animals can provide insights into the importance of molecules involved in signal transduction, the resultant phenotype is a consequence of a complex developmental program and adaptive mechanisms so that the precise function of the affected molecules is often obscured. The use of dominant-negative mutants in signaling molecules is an alternative approach to investigate signal transduction pathways. In fact, dominant-negative mutants of Src family PTKs (Lck and Fyn), Ser/Thr kinases (MAP kinase or ERK kinase [MEK]) and, the small GTP-binding protein Ras, have all been successfully used to dissect T cell signaling pathways in T cell lines or in transgenic mice (17–24). In this report, we show that two ZAP-70 mutants can specifically suppress TCR-mediated early and late signal transduction leading to nuclear factor of activated T cells (NFAT) activation in Jurkat T cells.

Materials and Methods

Cells. Large T antigen (TAg) Jurkat cells, described previously (25), are human leukemia Jurkat T cells stably transfected with SV40 TAg (kindly provided by Dr. G. Crabtree, Stanford University, Palo Alto, CA). J.HM1.2.2 cells have been described previously (25). Large T antigen Jurkat cells (TAgJurkat) were used as positive controls for TCR-mediated early and late signaling pathways (25). J.HM1.2.2 cells have been described (25). 40 h later, transfected cells were aliquoted into a U-bottom 96-well plate (104/well) and cultured with various stimuli (1:500 dilution of C305 ascites, 200 mM KPO4, pH 7.8, and 1.0 mM dithiothreitol. Lysates (0.1 ml) were mixed with 0.1 ml assay buffer (200 mM KPO4, pH 7.8, 10 mM ATP, 20 mM MgCl2 and 0.1 ml 1.0 mM lucrefin; luciferase activity was measured in the MONOLIGHT 2001 Luminometer (Analytical Luminescence Laboratory, Inc., San Diego, CA). For biochemical analysis, transfected cells were resuspended in PBS, equilibrated at 37°C for 15 min, stimulated at 37°C with a 1:500 dilution of C305 ascites, 50 ng/ml PMA, or a 1:1,000 dilution of OKT8 ascites for 2 min, and lysed immediately in a buffer containing 1% NP-40, 10 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, protease, and phosphatase inhibitors, as previously described (35).

Immunoprecipitation and Immunoblot Analysis. Immunoprecipitation was carried out as described earlier (36). Immunoblot analysis using the commercial enhanced chemiluminescence (ECL) (Amersham Corp., Arlington Heights, IL) detection kit was performed as previously described (37). Stripping and reprobing blots was carried out according to the ECL kit instruction provided by Amersham Corp. Reprobed blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium colorimetric development reagents as described (36).

Results

Expression of Mutant ZAP-70 Lacking Kinase Activity Inhibited TCR-mediated NFAT Activation. We used two mutants of ZAP-70 to examine the ability of inactive forms of ZAP-70 to inhibit TCR signaling (Fig. 1). The KI mutant has a point mutation in the ATP-binding site (Lys890 to Ala) of the kinase domain that results in an inactive kinase (8). The SH2(N+C) mutant is a kinase domain truncation mutant (deletion of the COOH-terminal 343 amino acids), which leaves only the two SH2 domains of ZAP-70. As a means to assess functional T cell activation, the transcriptional activity of NFAT, a well-characterized nuclear factor
that facilitates the transcription of the IL-2 gene (38), was measured. The induction of NFAT has been shown to be responsive to TCR-mediated signaling (38-40). Plasmids containing different ZAP-70 mutant cDNAs and the NFAT-luciferase reporter were cotransfected into TAg Jurkat cells, and luciferase activity was measured after treatment with different stimuli. Stimulation with an anti-TCR mAb C305 resulted in a greater than 10-fold increase in the luciferase activity in control cells cotransfected with the empty vector (Fig. 2 A). However, this TCR-stimulated luciferase activity was markedly inhibited by cotransfection of a cDNA construct encoding either the KI or the SH2(N+C) mutant (Fig. 2 A). The inhibitory effect of either mutant on NFAT-luciferase induction appeared to be dose dependent (Fig. 3). Expression of the KI mutant also inhibited TCR-mediated transcriptional activation of NF-IL2A (data not shown), another nuclear factor involved in IL-2 gene expression (38). However, expression of the KI mutant had little or no effect on the constitutive activation of a reporter construct containing the RSV promoter (data not shown), suggesting that the KI mutant did not randomly suppress transcription. Moreover, cotransfection of wild-type ZAP-70 failed to inhibit NFAT activation (data not shown). Cotransfection of the SH2(N+*C) mutant, in which the Arg190 of the FLVRE sequence in the phosphotyrosine-binding pocket in the COOH-terminal SH2 domain was mutated to Lys (Fig. 1), abolishing its ability to bind to an ITAM, did not inhibit anti-TCR-mediated NFAT induction (Fig. 2 A); despite similar levels of expression as the SH2(N+C) mutant (Fig. 2 C). Taken together, these results demonstrate that two ZAP-70 mutants devoid of kinase activity functioned as dominant-negative mutants to suppress TCR-mediated signaling leading to NFAT activation. Moreover, the inhibitory effect of the SH2(N+C) mutant requires an intact phosphotyrosine-binding site in its COOH-terminal SH2 domain.

Dominant-negative ZAP-70 Acts Proximally in the TCR Signaling Pathway and Is Specific to TCR-mediated Signaling. The induction of NFAT represents a late activation event that is a summation of both proximal and distal TCR signaling. To investigate the specificity of the effect of dominant-negative ZAP-70 mutants and whether they operate at a step(s) proximally or distally in the TCR signaling pathway leading to NFAT activation, two different types of experiments were performed. In the first series of experiments, we cotransfected plasmids containing ZAP-70 mutant cDNA and the NFAT-luciferase reporter into TAg Jurkat cells. We then assayed luciferase activity after treatment with PMA plus ionomycin, pharmacological agents that can bypass proximal TCR signaling to activate the IL-2 gene through the activation of protein kinase C (PKC) and the increase of cytoplasmic free calcium, respectively. In the second group of experiments, we employed JHM1.2.2 cell, a Jurkat T cell that had been stably transfected with a heterologous receptor, the seven-transmembrane domain human muscarinic subtype 1 receptor (HM1). HM1 is known to activate PLC through a heterotrimeric G-pro-
tein–coupled mechanism (41). Stimulation of either the TCR or HM1 on J.HM1.2.2 cells leads to IL-2 gene expression (41). Mutant ZAP-70 and the NFAT-luciferase reporter were cotransfected into J.HM1.2.2 cells and the luciferase activity was measured after stimulation with anti-TCR mAb C305 or carbachol, an agonist for HM1. It is interesting to note that in contrast to their effects on NFAT activation induced by TCR stimulation (Figs. 2 A and 4 A), coexpression of either the KI or the SH2(N+C) mutant failed to inhibit NFAT activation induced by PMA plus ionomycin treatment in TAg Jurkat cells (Fig. 2 B) or by carbachol stimulation in J.HM1.2.2 cells (Fig. 4 B). These results strongly suggest that both dominant-negative ZAP-70 mutants acted proximally in the TCR signaling pathway, upstream of PLC activation, and that their dominant-negative effect is specific to the TCR-mediated signaling pathway.

The biological properties of both dominant-negative ZAP-70 mutants appear to be similar. Therefore, we focused our attention on one of the dominant-negative mutants, the SH2(N+C) mutant, which also has a smaller molecular mass, hence allowing us to distinguish it from endogenous ZAP-70 on SDS-PAGE. The remaining experiments described in this report were performed with the SH2(N+C) mutant.

Expression of the SH2(N+C) Mutant Inhibited TCR-stimulated p95fork Tyrosine Phosphorylation, ERK2 Activation, and the Association of a Number of Tyrosine-phosphorylated Proteins with GRB2. To provide direct biochemical evidence that the dominant-negative ZAP-70 blocks early TCR signaling, we examined three early TCR signaling events, tyrosine phosphorylation of p95fork, the activation of ERK2, and the association of a number of tyrosine-phosphorylated proteins with GRB2. p95fork is one of the major substrates of PTK activity after TCR stimulation (42, 43). The involvement of p95fork in TCR signaling has been implicated by recent “knockout” and overexpression studies (30, 44–46). To assess the effect of expression of the SH2(N+C) mutant on p95fork tyrosine phosphorylation, we cotransfected the SH2(N+C) mutant and a Myc epitope-tagged p95fork into TAg Jurkat cells. The transfected cells were stimulated with the anti-TCR mAb C305 and the epitope-tagged p95fork was isolated by anti-Myc epitope immunoprecipitation and analyzed by immunoblotting with the phosphotyrosine-specific mAb, 4G10 (Fig. 5). Expression of the SH2(N+C) mutant greatly inhibited TCR-stimu-
phosphorylation, this mutant also failed to inhibit TCR-
association of a number of tyrosine-phosphorylated pro-
teins, including pp36-38, pp76, and pp116-120, with the
adaptor protein GRB2 (49-51). pp36-38 and pp76 also
associate with PLCγ1 (51, 52), whereas pp120 also associ-
ates with PI 3-kinase (53). Thus, these tyrosine-phosphory-
lated proteins have been proposed to have important roles
in linking TCR-regulated PTKs to Ras signaling, PLC ac-
tivation, and other signaling pathways (49-53). To exam-
ine the effect of expression of the SH2(N+C) mutant on
the association of tyrosine-phosphorylated proteins with
GRB2, we cotransfected the SH2(N+C) mutant with a
FLAG epitope-tagged GRB2 into TAg Jurkat cells and
stimulated transfected cells with the anti-TCR mAb C305.
Epitope-tagged GRB2 was subsequently isolated by anti-
FLAG epitope immunoprecipitation, and the immunopre-
cipitates were analyzed by immunoblotting with the antiphosphotyrosine-specific mAb 4G10 (top). The blot was then stripped and reprobed with
9E10 (bottom).

The activation of ERK2 is known to be another early
TCR signaling event (47). Although both anti-TCR mAb
and the PKC activator PMA stimulate rapid activation of
ERK2 in T cells, they appear to activate ERK2 via differ-
ent mechanisms (22). To examine the effect of expression
of the SH2(N+C) mutant on the activation of ERK2, we
cotransfected the SH2(N+C) mutant with Myc epitope-
tagged ERK2 into TAg Jurkat cells and stimulated trans-
fected cells with either anti-TCR mAb C305 or PMA.
Cellular lysates were then subjected to immunoblot analysis
using the antiepitope mAb 9E10. Results shown in Fig. 6
indicate that expression of the SH2(N+C) mutant mark-
edly inhibited the TCR-stimulated mobility shift of ERK2,
a commonly used marker for ERK2 activation (48). How-
ever, such inhibition was not apparent with the mobility
shift of ERK2 induced by PMA stimulation, suggesting that
the inhibition is specific to the TCR-mediated signaling
pathway. Correlating with the failure of the SH2(N+C)
mutant to antagonize NFAT induction and p95 tyrosine
phosphorylation, this mutant also failed to inhibit TCR-
mediated ERK2 activation.

One well-documented early TCR signaling event is the
association of a number of tyrosine-phosphorylated pro-
teins, including pp36-38, pp76, and pp116-120, with the
adaptor protein GRB2 (49-51). pp36-38 and pp76 also
associate with PLCγ1 (51, 52), whereas pp120 also associ-
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ine the effect of expression of the SH2(N+C) mutant on
the association of tyrosine-phosphorylated proteins with
GRB2, we cotransfected the SH2(N+C) mutant with a
FLAG epitope-tagged GRB2 into TAg Jurkat cells and
stimulated transfected cells with the anti-TCR mAb C305.
Epitope-tagged GRB2 was subsequently isolated by anti-
FLAG epitope immunoprecipitation, and the immunopre-
cipitates were analyzed by immunoblotting with the antiphosphotyrosine mAb 4G10 (Fig. 7) or an anti-GRB2
antibody (data not shown). Consistent with previous re-
ports, TCR activation resulted in the appearance of several
tyrosine-phosphorylated proteins, including pp36-38, pp76,
and pp116-120, that were communoprecipitated with the
antiepitope mAb in control cells cotransfected with the
empty vector. However, the recovery of these tyrosine-
phosphorylated proteins by anti-FLAG-GRB2 immuno-
precipitation in TCR-stimulated cells cotransfected with
the SH2(N+C) mutant was greatly reduced. Anti-GRB2
immunoblotting showed that the amount of FLAG-GRB2
in all the immunoprecipitates was similar. Since the asso-
ciation of pp36-38 with GRB2 was demonstrated to be
mediated by the SH2 domain of GRB2 (51), these results
strongly suggest that expression of the SH2(N+C) mutant
inhibited the tyrosine phosphorylation of pp36-38. The
association of pp76 and pp116-120 with GRB2 is not medi-
at by the SH2 domain but by the SH3 domain of GRB2
(49, 50, 52). Therefore, a reduction in the level of pp76
and pp116-120 in anti-FLAG-GRB2 immunoprecipitates
could be a result of inhibition in tyrosine phosphorylation
and/or association with GRB2 by the expression of the
SH2(N+C) mutant.

Together, these results provide direct biochemical evi-
dence that the SH2(N+C) mutant blocked, at least some,
early TCR signaling events.

**Association of the SH2(N+C) Mutant with a CD8-ζ Chi-
mera.** The SH2(N+C) mutant requires an intact phos-
Figure 7. Expression of the SH2(N+C) mutant in TAg Jurkat cells inhibits TCR-stimulated association of several tyrosine-phosphorylated proteins with GRB2. TAg Jurkat cells were cotransfected with 5 μg of FLAG epitope-tagged GRB2 expression vector together with 40 μg of empty expression vector or the SH2(N+C) expression plasmid. 40 h later, transfected cells were either left unstimulated or stimulated for 2 min with anti-TCR mAb C305. Cells were then lysed, and FLAG epitope-tagged GRB2 was isolated by immunoprecipitation with the anti-FLAG epitope mAb, followed by immunoblotting with the phosphotyrosine-specific mAb 4G10.

Figure 8. Association of SH2(N+C) mutant and endogenous ZAP-70 with the CD8-ζ chimera. TAg Jurkat cells were cotransfected with 5 μg of CD8-ζ expression vector together with 40 μg of empty expression vector or the SH2(N+C) expression plasmid. 40 h later, transfected cells were either left unstimulated or stimulated for 2 min with anti-CD8 mAb OKT8. Cells were then lysed, and CD8-ζ was isolated by immunoprecipitation with mAb OKTS, followed by immunoblotting with anti-ZAP-70 mAb 2F3.2 (top), anti-phosphotyrosine mAb 4G10 (middle), or anti-ζ mAb 6B10.2 (bottom).
SH2(N+C) mutant. A substantial amount of the SH2(N+C) molecule was found to associate constitutively with the CD8-ξ chimera at levels that were much greater than endogenous ZAP-70. Anti-CD8 stimulation only induced a slight increase in CD8-ξ tyrosine phosphorylation, suggesting that this phosphorylation may be close to saturating. The basal association of endogenous ZAP-70 with CD8-ξ was also increased in cells cotransfected with the SH2(N+C) mutant. However, this increase in association is not proportional to the large increase in tyrosine phosphorylation of CD8-ξ. These results, combined with a requirement for an intact phosphotyrosine-binding site in the COOH-terminal SH2 domain of the SH2(N+C) mutant to demonstrate its dominant-negative activity, indicate that the SH2(N+C) mutant mediates its effect on TCR signaling by binding to tyrosine-phosphorylated TCR subunits.

Discussion

In this report, we show that two mutants of the ZAP-70 PTK functioned as dominant-negative mutants to inhibit TCR-mediated signal transduction leading to NFAT activation. The inhibitory effect of these mutants demonstrated remarkable specificity. First, TCR-mediated transcriotional activation of NFAT and NF-IL2A was inhibited (Figs. 2, 3, 4, and data not shown), whereas the constitutive activation of a reporter construct containing the RSV promoter was not inhibited (data not shown). Second, the inhibition of TCR-mediated NFAT activation was a dose-dependent effect (Fig. 3). Third, only NFAT activation mediated through the TCR was inhibited; little or no inhibition was observed on NFAT activation induced by PMA plus ionomycin treatment or by HM1 receptor agonist carbachol stimulation (Figs. 2 and 4). Fourth, the inhibition of NFAT activation was due to a block proximal but not distal in the TCR signaling pathway (Figs. 2 and 4). These apparent selectivities strongly argue that a signaling pathway in which ZAP-70 is involved was specifically blocked.

Current models of T cell activation suggest that ZAP-70 participates proximally in TCR signaling, in part, through its interaction with tyrosine-phosphorylated ITAMs contained within the invariant subunits of the TCR complex (1, 54). Upon TCR stimulation, the ITAMs become tyrosine phosphorylated, creating high affinity binding sites for the two SH2 domains of ZAP-70. The recruitment of ZAP-70 from the cytosol to the TCR leads to tyrosine phosphorylation and activation of ZAP-70. Activated ZAP-70 can then act on its targets to elicit subsequent biochemical responses. The exact mechanism by which ZAP-70 is activated has yet to be elucidated. Nevertheless, it is clear that interfering with ZAP-70 function could have severe consequences for TCR signaling. T cells from individuals lacking ZAP-70 exhibit markedly reduced or no tyrosine phosphorylation and calcium influx, as well as no detectable IL-2 production, upon TCR ligation, reflecting an early block in TCR signaling (13-15). A kinase-defective ZAP-70 was unable to induce tyrosine phosphorylation of cellular proteins in COS cells (8), nor could it reconstitute B cell receptor-mediated activation in B cell lines lacking the homologous Syk PTK (55). Efforts to inhibit ZAP-70 binding to the TCR by introducing a phosphatase-resistant, tyrosine-phosphorylated peptide derived from the COOH-terminal ITAM of the TCRξ chain into permeabilized T cells prevented TCR-stimulated tyrosine phosphorylation and activation of ZAP-70 and reduced tyrosine phosphorylation of other substrates (56). Unfortunately, the effect of disrupting ZAP-70 binding to the TCR on late activation events, such as IL-2 production, was not evaluated in this system, probably because of the toxicity of permeabilization to the cells. These results collectively support the notion that the assembly of a functional receptor signaling complex on the tyrosine-phosphorylated TCR ITAMs is an essential early biochemical event after TCR stimulation. This receptor signaling complex, which contains ZAP-70 as a critical component, allows the coupling of ligand-binding signal to be transmitted into downstream activation.

Our biochemical studies focused on the SH2(N+C) mutant show that this mutant bound constitutively to the tyrosine-phosphorylated CD8-ξ chimera (Fig. 8). In addition, the dominant-negative activity of this mutant on both early and late TCR signaling requires it to maintain an intact phosphotyrosine-binding site in its COOH-terminal SH2 domain (Figs. 2, 4, 5, and 6). These results indicate that the SH2(N+C) mutant exerts its effect by binding to tyrosine-phosphorylated TCR ITAMs. This conclusion is also based on previous studies demonstrating a highly restricted repertoire of tyrosine-phosphorylated proteins to which the two SH2 domains of ZAP-70 bind (7). When the two SH2 domains of ZAP-70 were expressed as a fusion protein and used as an affinity precipitation reagent to elute activated T cell lysates, only tyrosine-phosphorylated TCRξ and CD3e chains were found to bind to the fusion protein, in sharp contrast to the binding specificity of other SH2 domains. Binding of the SH2(N+C) mutant to tyrosine-phosphorylated CD8-ξ chimera correlated with diminished tyrosine phosphorylation of p95vav (Fig. 6) as well as blocks in other signaling events, such as ERK2 activation (Fig. 5), the association of several tyrosine-phosphorylated proteins with GRB2 (Fig. 7), and NFAT induction (Fig. 2). Since p95vav becomes rapidly tyrosine phosphorylated (42, 43) and binds to ZAP-70 (57), it has been suggested to be a downstream substrate for ZAP-70 (57). Lack of p95vav tyrosine phosphorylation implies that ZAP-70 activation was blocked by the SH2(N+C) mutant.

The mechanism by which the SH2(N+C) mutant blocks ZAP-70 activation is not fully understood. Examination of the association of endogenous ZAP-70 with tyrosine-phosphorylated CD8-ξ chimera yielded unexpected results (Fig. 8). Expression of the SH2(N+C) mutant did not appear to completely block the association of the endogenous ZAP-70 with tyrosine-phosphorylated CD8-ξ. There are several possibilities that might account for these results. It should be noted that the association of ZAP-70 with tyrosine-phosphorylated TCR ITAMs does not always correlate with the activation of ZAP-70. In freshly isolated lymphocytes, ZAP-70 is not expressed, but is induced after TCR stimulation. The mechanism by which ZAP-70 is induced is not known, but it appears to be independent of NFAT activation. Nevertheless, the association of ZAP-70 with tyrosine-phosphorylated ITAMs is an essential early biochemical event after TCR stimulation. This receptor signaling complex, which contains ZAP-70 as a critical component, allows the coupling of ligand-binding signal to be transmitted into downstream activation.
node T cells and thymocytes, ZAP-70 constitutively associates with tyrosine-phosphorylated TCRζ chain, yet there is no activation (37). TCR stimulation is required for the increase in tyrosine phosphorylation of ZAP-70 and for cellular activation (37). In antagonist-induced anergic T cells, ZAP-70 associated with one isoform of tyrosine-phosphorylated TCRζ chain but was neither tyrosine phosphorylated nor activated upon antagonist peptide stimulation (58). One model proposed by Neumeister et al. (12) suggests that an oligomeric complex involving multiple ZAP-70 molecules aligned tandemly on tyrosine-phosphorylated ITAMs may be a prerequisite for the transphosphorylation and activation of ZAP-70. Thus, it is possible that the SH2(N+C) mutant might interfere with the tandem alignment of the endogenous ZAP-70 on ITAMs, thereby preventing the proper transphosphorylation and activation. Consistent with this, we observed a reduction in the tyrosine phosphorylation of endogenous ZAP-70 that was associated with the CD8-ζ chimera (data not shown). Alternatively, the presence of the SH2(N+C) mutant in the same receptor complex could lock the endogenous ZAP-70 in an inactive conformation through an intermolecular interaction. Additional studies will be needed for evidence of an inter- or intramolecular interaction between the SH2 domains and the kinase domain of ZAP-70. Such interactions could normally serve as a means of keeping ZAP-70 inactive before TCR stimulation. Upon ITAM engagement, the interaction between the SH2 domains and the kinase domain of ZAP-70 may be released to give rise to an active conformation. Indeed, a recent study (59) suggested such a model with the related Syk PTK. In our studies, the SH2(N+C) mutant was present in great excess, so that the equilibrium may have been shifted toward the maintenance of an inactive conformation. Future experiments are needed to distinguish between these possibilities.

Although we favor the interpretation that the SH2(N+C) mutant inhibited TCR signaling primarily by blocking the function of ZAP-70, this interpretation is complicated by the possibility that the function of other SH2 domain-containing effector molecules capable of binding to the tyrosine-phosphorylated TCR ITAMs could also be affected by the SH2(N+C) mutant. For example, both Syk and the adapter protein Shc can bind to tyrosine-phosphorylated TCRζ chain (60, 61). However, a critical role similar to that of ZAP-70 in TCR signaling has yet to be demonstrated for Syk or Shc. Thus, the action of SH2(N+C) mutant may have been to bind tyrosine-phosphorylated ITAMs, thereby interfering with the assembly on ITAMs of a functional receptor signaling complex containing ZAP-70, and possibly Syk and Shc.

The ability of using dominant-negative ZAP-70 mutants to specifically suppress TCR-mediated signal transduction leading to functional T cell activation holds great promise for future use of this approach to dissect signaling pathways in which Syk family kinases are involved. It should be pointed out that whereas ZAP-70 has been shown to be involved primarily in TCR signaling, Syk has been implicated in the signaling pathways of a variety of receptors, including the antigen receptors (TCR, B cell antigen receptor [BCR], FcεR1, FcγRI, and FcγRII), cytokine receptors (G-CSFR and IL-2R), and the erythrocyte thrombin receptor (60, 62–67). Our preliminary results show that the two dominant-negative ZAP-70 mutants used in this study were also capable of inhibiting BCR-mediated signal transduction in a B cell line expressing Syk as the only Syk family PTK. In addition, Hirasawa et al. (68) recently used a kinase domain–truncated Syk mutant to block FcεR1-mediated signaling in mast cells. Without doubt, the dominant-negative approach, combined with other genetic and biochemical approaches, should yield a better understanding of the molecular mechanisms that regulate the activation signals transduced by these receptors.

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