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 $p95^{vav}$ 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-ζ chimeric receptor to analyze the interaction of the SH2(N+C) mutant with ITAMs of TCR-ζ, we found that this mutant was constitutively bound to the hyperphosphorylated CD8-ζ 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.

The TCR is a multimolecular complex consisting of clonotypic antigen-binding subunits (α and β in most T cells) and invariant signal–transducing subunits (γ, δ, and ε chains of the CD3 complex, and ζ family proteins) (1). Engagement of the TCR by antigen bound to MHC molecules or by anti-TCR mAbs triggers a signal transduction cascade culminating in the induction of a variety of T cell functions (1). One of the earliest detectable signaling events after TCR stimulation is the tyrosine phosphorylation of a number of protein substrates, including the invariant subunits of the TCR complex, phospholipase C (PLC)[1]γ1, $p95^{vav}$, zeta-associated protein (ZAP)-70, Shc, and valosin-containing protein (VCP) (1). However, unlike many growth factor receptors, the TCR lacks intrinsic protein tyrosine kinase (PTK) activity. Numerous biochemical and genetic studies have shown that the TCR initiates signal transduction by interacting with and activating at least three cytoplasmic PTKs, Lck, Fyn, and ZAP-70 (1–3). Lck and Fyn, two members of the Src family PTK, have been suggested to initially tyrosine phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) contained within the cytoplasmic domains of the invariant TCR subunits. This phosphorylation leads to the recruitment of the Syk family PTK, ZAP-70, to the TCR (4–6). The association of ZAP-70 with the TCR is mediated by a high affinity interaction between the two tandemly arranged SH2 domains of the ZAP-70 molecule and the two phosphorylated tyrosines in the ITAM (5, 7–9). Both SH2 domains of ZAP-70 are required for this association. Tyrosine phosphorylation of ZAP-70 itself also takes place after its binding to the TCR, a process dependent on the Src family PTKs (5, 8, 10). The functional significance of ZAP-70 tyrosine phosphorylation is still not clear, but may relate to its catalytic activation and interaction with other proteins (11, 12). The combined activation of both Src and Syk family PTKs results in tyrosine phosphorylation of cellular substrates, which in turn contributes to the activation

[1]*Abbreviations used in this paper:* ERK2, extracellular signal-regulated kinase 2; GRB2, growth factor receptor–binding protein 2; HM1, human muscarinic subtype 1 receptor; ITAM, immunoreceptor tyrosine-based activation motif; KI, inactive kinase; NFAT, nuclear factor of activated T cells; PLC, phospholipase C; PTK, protein tyrosine kinase; TAg, large T antigen; ZAP-70, zeta-associated protein 70.

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 mutations in signaling molecules is an alternatitive 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 Raf), 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 (26). Cells were maintained as described (25, 26).

Plasmids. The NFAT-luciferase reporter construct, in which the expression of luciferase is driven by multiple copies of NFAT DNA binding element, was a gift from Dr. G. Crabtree. The Lys^{369} to Ala point mutant (inactive kinase [KI]) of ZAP-70, described previously (8), was subcloned into pBJ1, a mammalian expression vector containing the SRα promoter (27). The SH2(N+C) mutant was generated by digesting the wild-type ZAP-70 cDNA with HindII at nucleotide position 1039 and then adding a stop codon next to this HindII site. The resulting cDNA fragment, which encodes the amino acids 1–276 of ZAP-70, was also subcloned into pBJ1. The SH2(N+*C) was constructed by replacing the KpnI (nucleotide position 701)-ScaI (nucleotide position 870) fragment of the SH2(N+C) mutant in the pBJ1 vector with the same fragment in the full-length ZAP-70 containing the point mutation Arg^{190} to Lys which has been previously described (8). CD8-ζ, described earlier (28), was subcloned into pEF-BOS (29). Myc epitope-tagged $p95^{vav}$ and extracellular signal-regulated kinase 2 (ERK2) have been described previously (30, 31) and were subcloned into pEF-BOS. FLAG epitope-tagged growth factor receptor–binding protein 2 (GRB2), subcloned into pEF-BOS, was kindly provided by D. Motto and Dr. G. Koretzky (University of Iowa, Iowa City, IA). All expression plasmids contain an SV40 origin of replication to allow high level gene expression in TAg Jurkat cells (25).

Antibodies. The following mAbs (and their specificity) were used: C305 (mouse anti–Jurkat Ti β chain) (32), 9E10 (mouse anti–Myc epitope) (kindly provided by J. M. Bishop, University of California, San Francisco) (33), 4G10 (mouse antiphosphotyrosine) (purchased from Upstate Biotechnology, Inc., Lake Placid, NY), OKT8 (mouse anti–human CD8) (obtained from American Type Culture Collection, Rockville, MD), 2F3.2 (mouse anti–human ZAP-70) (8), 6B10.2 (mouse anti-TCRζ chain) (34), and M2 (mouse anti-FLAG epitope) (obtained from Eastman Kodak Co., New Haven, CT). The rabbit polyclonal anti-GRB2 serum was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Transfections, Stimulations, Luciferase Assay, and Solubilization for Biochemical Analysis. Cells (10^7) were transiently transfected by electroporation using the Gene Pulser (Bio-Rad Laboratories, Hercules, CA) as previously described (25). 40 h later, transfected cells were aliquoted into a U-bottom 96-well plate (10^5/well) and cultured with various stimuli (1:500 dilution of C305 ascites, 500 μM carbachol, or 50 ng/ml PMA plus 1.0 μM ionomycin) in RPMI 1640 medium supplemented with 10% FCS in a final volume of 90 μl. After a 6-h stimulation period at 37°C, cells were lysed in a buffer containing 1% Triton X–100, 100 mM KPO_4, pH 7.8, and 1.0 mM dithiothreitol. Lysates (0.1 ml) were mixed with 0.1 ml assay buffer (200 mM KPO_4, pH 7.8, 10 mM ATP, 20 mM $MgCl_2$) and 0.1 ml 1.0 mM luciferin; 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 (Lys^{369} 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

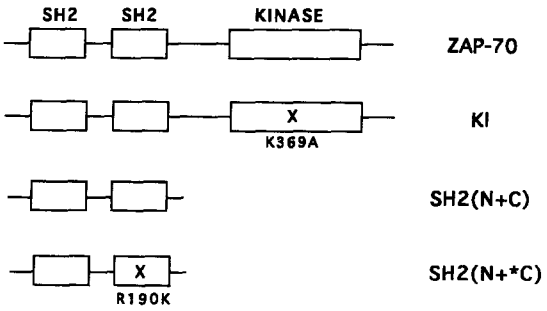

Figure 1. Schematic representation of mutant ZAP-70 constructs.

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 Arg^{190} 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 J.HM1.2.2 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 PLCβ through a heterotrimeric G-pro-

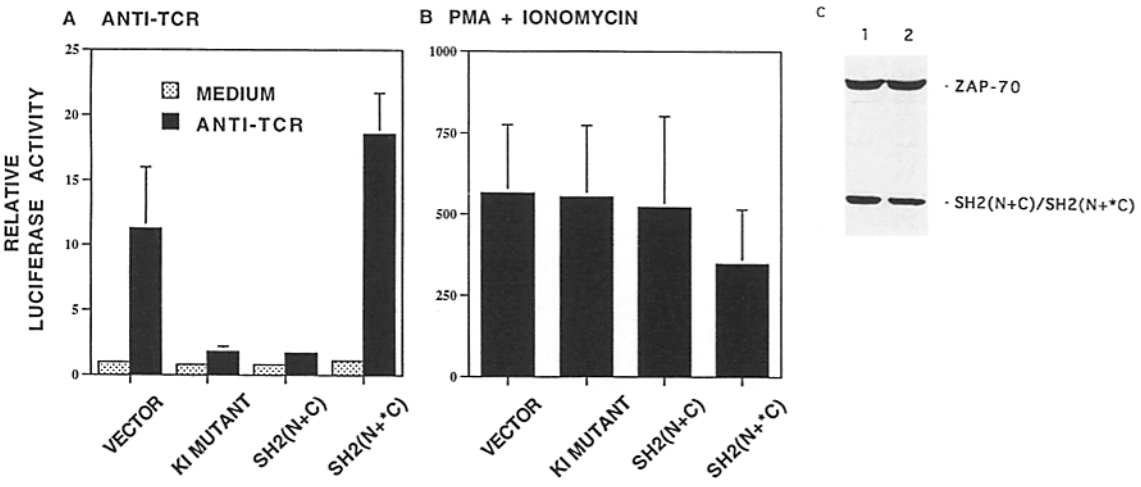

Figure 2. Effects of different ZAP-70 mutants on NFAT activation in TAg Jurkat T cells. TAg Jurkat cells were cotransfected with NFAT-luciferase reporter (20 μg) together with 40 μg of empty expression vector or expression plasmids containing cDNAs encoding different ZAP-70 mutants. 40 h later, transfected cells were stimulated with: (*A*) culture medium, anti-TCR mAb C305, or (*B*) PMA plus ionomycin. 6 h after stimulation, cells were lysed, and luciferase activity was determined. Luciferase activity produced in cells transfected with mutant ZAP-70 expression vectors was divided by the activity produced in the presence of the empty expression vector. Results represent the averages of five independent experiments. An aliquot of cells was analyzed for the expression of the SH2(N+C) (lane *1*) and SH2(N+*C) (lane *2*) mutant proteins by immunoblotting using an anti-ZAP-70 mAb (*C*).

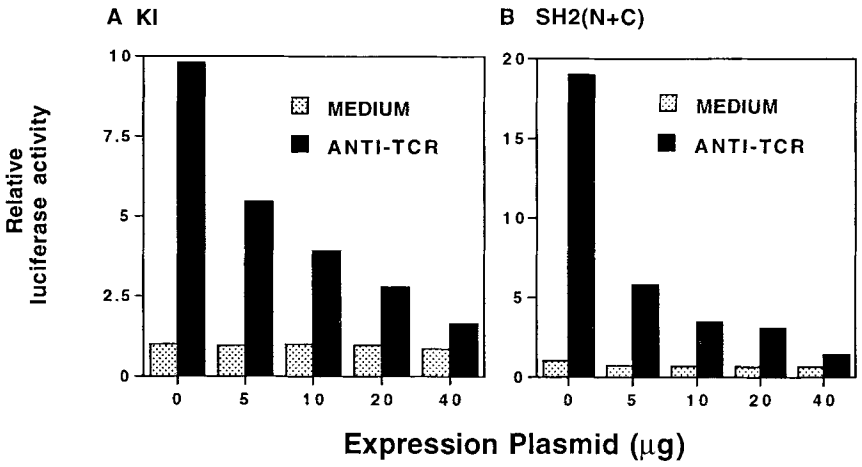

Figure 3. The inhibitory effect of KI and SH2(N+C) mutants on TCR-mediated NFAT activation in TAg Jurkat cells is dose dependent. TAg Jurkat cells were cotransfected with indicated amounts of KI (*A*) or SH2(N+C) (*B*) expression vector together with the NFAT-luciferase reporter (20 μg). The amount of DNA was adjusted to 60 μg with the empty expression vector. 40 h later, transfected cells were stimulated with culture medium or anti-TCR mAb C305. Relative luciferase activity was determined as described in Fig. 2. Results are representative of two separate experiments.

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 p95^{vav} 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 p95^{vav}, the activation of ERK2, and the association of a number of tyrosine-phosphorylated proteins with GRB2. p95^{vav} is one of the major substrates of PTK activity after TCR stimulation (42, 43). The involvement of p95^{vav} 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 p95^{vav} tyrosine phosphorylation, we cotransfected the SH2(N+C) mutant and a Myc epitope-tagged p95^{vav} into TAg Jurkat cells. The transfected cells were stimulated with the anti-TCR mAb C305 and the epitope-tagged p95^{vav} 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-

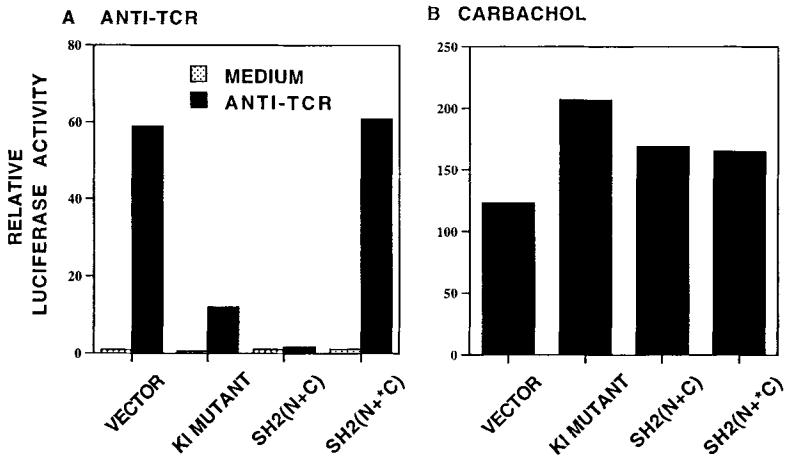

Figure 4. Effects of different ZAP-70 mutants on NFAT activation in J.HM1.2.2 cells. J.HM1.2.2 cells were cotransfected with the NFAT-luciferase reporter (20 μg) together with 40 μg of empty expression vector or expression plasmids containing cDNAs encoding different ZAP-70 mutants. 40 h later, transfected cells were stimulated for 6 h with: (*A*) culture medium, anti-TCR mAb C305, or (*B*) carbachol. Relative luciferase activity was determined as described in Fig. 2. Results are the averages of two separate experiments.

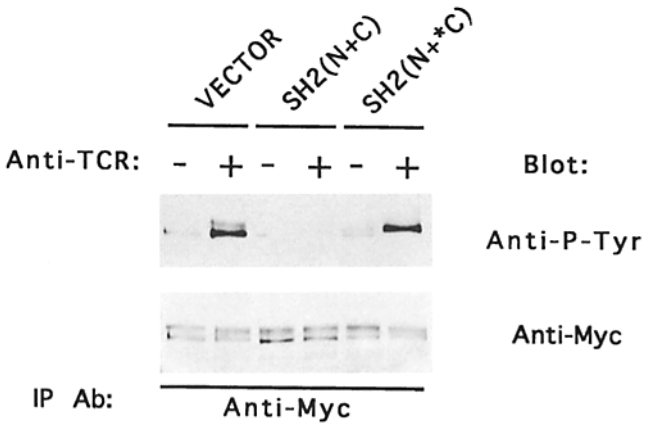

Figure 5. Expression of the SH2(N+C) mutant in TAg Jurkat cells inhibits TCR-mediated p95^{vav} tyrosine phosphorylation. TAg Jurkat cells were cotransfected with 5 μg of Myc epitope-tagged p95^{vav} expression vector together with 40 μg of empty expression vector or expression plasmids containing cDNAs encoding different ZAP-70 mutants. 40 h later, transfected cells were either left unstimulated or stimulated for 2 min with anti-TCR mAb C305. Cells were then lysed, and Myc epitope-tagged p95^{vav} was isolated by immunoprecipitation with the anti-Myc epitope mAb 9E10, followed by immunoblotting with the phosphotyrosine-specific mAb 4G10 (*top*). The blot was then stripped and reprobed with 9E10 (*bottom*).

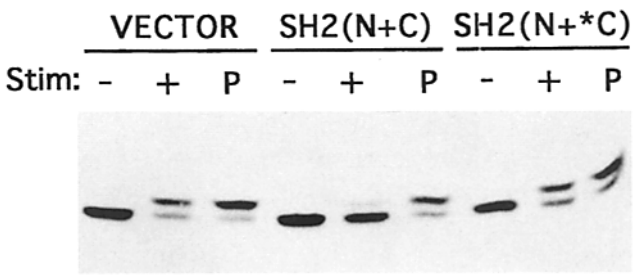

Figure 6. Expression of the SH2(N+C) mutant in TAg Jurkat cells inhibits TCR-mediated ERK2 kinase activation. TAg Jurkat cells were cotransfected with 5 μg of Myc epitope-tagged ERK2 expression vector together with 40 μg of empty expression vector or expression plasmids containing cDNAs encoding different ZAP-70 mutants. 40 h later, transfected cells were left unstimulated (−), or stimulated for 2 min with anti-TCR mAb C305 (+) or PMA (P). Cells were then lysed, and the whole cell lysates were analyzed by immunoblotting with the anti-Myc epitope mAb 9E10.

lated tyrosine phosphorylation of epitope-tagged p95^{vav}. Consistent with its lack of inhibitory effect on NFAT induction, the SH2(N+*C) mutant did not block p95^{vav} tyrosine phosphorylation. The expression of Myc epitope-tagged p95^{vav} was not significantly different in all the transfectants, as determined by stripping and reprobing the blot with the anti-Myc epitope mAb.

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 different 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 transfected 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 markedly inhibited the TCR-stimulated mobility shift of ERK2, a commonly used marker for ERK2 activation (48). However, 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^{vav} 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 proteins, 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 associates with PI 3-kinase (53). Thus, these tyrosine-phosphorylated proteins have been proposed to have important roles in linking TCR-regulated PTKs to Ras signaling, PLC activation, and other signaling pathways (49–53). To examine 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 immunoprecipitates were analyzed by immunoblotting with the antiphosphotyrosine mAb 4G10 (Fig. 7) or an anti-GRB2 antibody (data not shown). Consistent with previous reports, TCR activation resulted in the appearance of several tyrosine-phosphorylated proteins, including pp36-38, pp76, and pp116-120, that were coimmunoprecipitated with the antiepitope mAb in control cells cotransfected with the empty vector. However, the recovery of these tyrosine-phosphorylated proteins by anti-FLAG-GRB2 immunoprecipitation 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 association 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 mediated 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 evidence 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 Chimera. The SH2(N+C) mutant requires an intact phos-

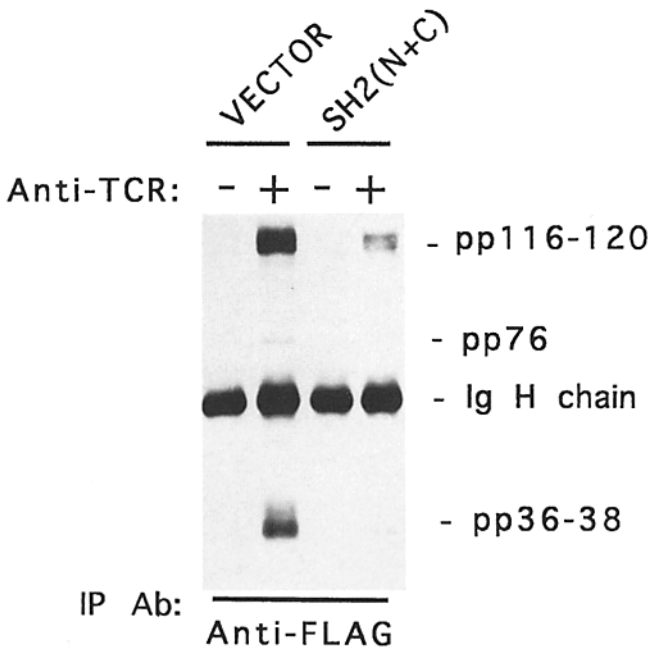

Figure 7. Expression of the SH2(N+C) mutant in TAg Jurkat cells inhibits TCR-stimulated association of several tyrosine-phosphorylated proteins with GRB2. TAg Jurkat cells were cotransfected with 5 μg of FLAG epitope-tagged GRB2 expression vector together with 40 μg of empty expression vector or the 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.

photyrosine-binding site in its COOH-terminal SH2 domain to demonstrate its dominant-negative activity (Figs. 2 *A*, 4 *A*, 5, and 6), suggesting that this mutant mediates its effect by binding to tyrosine-phosphorylated proteins, most likely tyrosine-phosphorylated TCR subunits. To test directly if the SH2(N+C) mutant binds to tyrosine-phosphorylated TCR subunits, we employed a surrogate TCR, the CD8-ζ chimera, in the transient cotransfection assay. Stimulation of this chimeric receptor with anti-CD8 mAb has been shown to recapitulate both proximal and distal events normally associated with stimulation of the intact TCR (28). CD8-ζ was cotransfected into TAg Jurkat cells with either the empty vector or the SH2(N+C) mutant, and transfected cells were unstimulated or were stimulated with an anti-CD8 mAb, OKT8. The CD8-ζ chimera was subsequently isolated by immunoprecipitation with OKT8 mAb and analyzed by immunoblotting with anti-ζ, anti-phosphotyrosine, or anti-ZAP-70 mAb. Results from a representative experiment are shown in Fig. 8. Consistent with previous studies, anti-CD8 stimulation resulted in enhanced association of endogenous ZAP-70 with CD8-ζ in cells cotransfected with the empty vector. This association correlates with increased tyrosine phosphorylation of CD8-ζ. It is striking that expression of the SH2(N+C) mutant resulted in a substantial increase in the basal level tyrosine phosphorylation of CD8-ζ, even though comparable amounts of chimeric protein were present in anti-CD8 immunoprecipitates from cells cotransfected with the empty vector or SH2(N+C) mutant. This suggests that the dephosphorylation of CD8-ζ was prevented by the expression of the

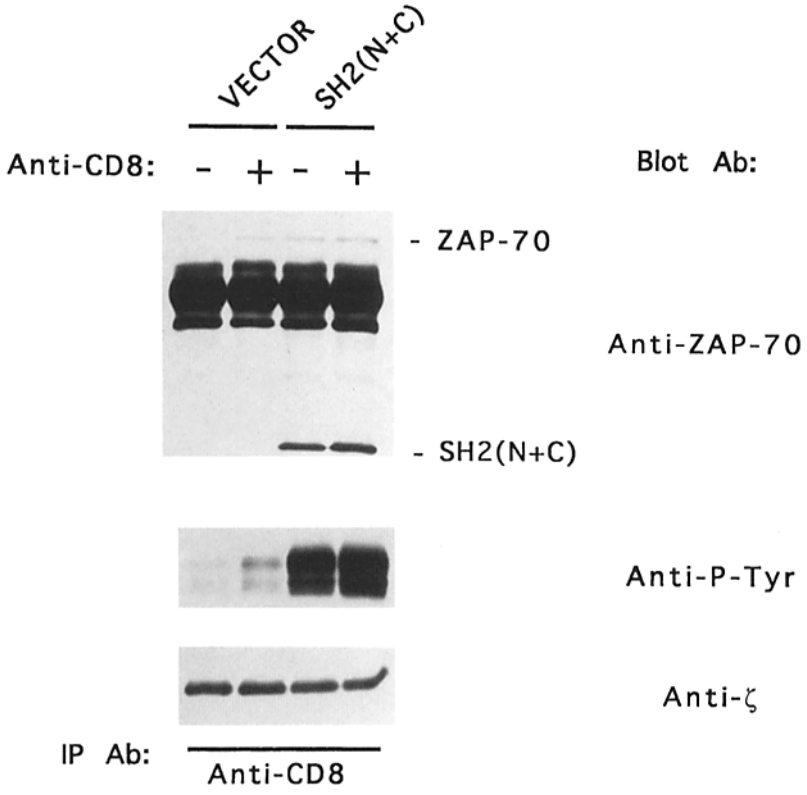

Figure 8. Association of SH2(N+C) mutant and endogenous ZAP-70 with the CD8-ζ chimera. TAg Jurkat cells were cotransfected with 5 μg of CD8-ζ expression vector together with 40 μg of empty expression vector or the SH2(N+C) expression plasmid. 40 h later, transfected cells were either left unstimulated or stimulated for 2 min with anti-CD8 mAb OKT8. Cells were then lysed, and CD8-ζ was isolated by immunoprecipitation with mAb OKT8, 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-ζ chimera at levels that were much greater than endogenous ZAP-70. Anti-CD8 stimulation only induced a slight increase in CD8-ζ tyrosine phosphorylation, suggesting that this phosphorylation may be close to saturating. The basal association of endogenous ZAP-70 with CD8-ζ 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-ζ. 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 transcriptional 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 TCRζ 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-ζ 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 TCRζ and CD3ε 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-ζ chimera correlated with diminished tyrosine phosphorylation of $p95^{vav}$ (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 $p95^{vav}$ 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 $p95^{vav}$ 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-ζ 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-ζ. 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 lymph

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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