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Characterization of the Roles of SH2 Domain-Containing Proteins in T-Lymphocyte Activation by Using Dominant Negative SH2 Domains

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Activation of the T-cell antigen receptor initiates a complex signaling cascade leading to changes in cytokine gene transcription. Several proteins containing Src homology 2 (SH2) domains, capable of interacting with phosphotyrosine-containing sequences within other proteins, are involved in the activation of signaling intermediates such as p21^{ras} and phospholipase Cγ1. In this study, we used dominant negative SH2 domains to determine the importance of SH2 domain-containing proteins for T-cell activation. We show that tandem SH2 domains of either Zap70 or Syk tyrosine kinase are potent inhibitors of signaling initiated by the T-cell receptor ζ chain in vivo while individual SH2 domains are ineffective. Thus, while only the C-terminal SH2 domains appear to have significant affinity for immunoreceptor tyrosine-based activation motifs in vitro, the N-terminal SH2 domains are necessary in vivo. We find the spacing between the tandem SH2 domains of Zap70 to be critical for in vivo interactions. The SH2 domain of the adapter protein Grb2 is an effective inhibitor in our dominant negative assay, although it has little affinity for immunoreceptor tyrosine-based activation motifs. A single point mutation that abolishes phosphotyrosine binding renders the Grb2 SH2 domain incapable of this inhibition. In contrast, the SH2 domain of Shc does not inhibit this signaling cascade. We conclude that Grb2, but not Shc, is involved in T-cell receptor signaling.

The transmission of signals generated at the T-cell antigen receptor (TCR) to nuclear transcriptional events is critical for the production of an effective immune response to foreign antigen. While the multisubunit TCR contains chains associated with antigen recognition and with signal transduction, there are no identifiable components with enzymatic activity. Thus, the invariant chains CD3γ, CD3δ, CD3ε, and ζ transmit signals with the assistance of several nonreceptor protein tyrosine kinases including Zap70, Lck, and Fyn (reviewed in reference 7). Zap70 was isolated by its association with the TCR ζ chain and contains two Src homology 2 (SH2) domains (8). The interaction of Zap70 with TCR ζ or other invariant chains occurs through immunoreceptor tyrosine-based activation motifs (ITAMs), which have the general structure YXX(L/I)$X_{(6–8)}$YXX(L/I) (23, 45, 46). Tyrosines within the ITAM become phosphorylated following TCR engagement and mediate interactions with the two tandem SH2 motifs of Zap70 (26). The presence of Src family members, Lck and Fyn, is necessary for the subsequent phosphorylation and activation of Zap70 kinase activity (8, 26). While several signaling events such as phospholipase Cγ1 activation, increased cytosolic free Ca^{2+}, and activation of p21^{ras} (Ras-GTP) occur in response to TCR stimulation (55), the mechanisms by which these events are coupled to Zap70 activation remain unknown. Furthermore, no substrates for the Zap70 tyrosine kinase activity have been identified as yet, although several proteins in addition to phospholipase Cγ are rapidly tyrosine phosphorylated following TCR stimulation. The Zap70-related tyrosine kinase Syk (protein tyrosine kinase 72) is found in association with activated membrane immunoglobulin receptors on B cells (22) and high-affinity immunoglobulin E receptors (FcεRI) (21) on mast cells. This kinase is thought to play an analogous signaling role in these cell types to that of Zap70 in T cells.

We have overexpressed isolated SH2 domains in T cells to determine the importance of various kinases and adapter proteins in transmission of signals from the TCR. In this report, we find that the tandem SH2 domains of both Zap70 and Syk can inhibit T-cell activation and that the spacing between these domains is critical while individual SH2 domains have no effect. We further show that the adapter protein Grb2 plays a key role in TCR signaling while Shc does not play a role. These studies define necessary interactions occurring in vivo during early TCR signaling events.

MATERIALS AND METHODS

Cells. The human T-cell line Jurkat derivatized to express simian virus 40 T antigen (Jurkat TagC15) has been previously described (38). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum in a 5% CO_2 humidified atmosphere.

Reporters and expression vectors. Reporter constructs responsive to nuclear factor of activated T cells (NF-ATSEAP) and to AP1 (AP1SEAP) have previously been described (12, 52). The Tacζ chimeric signaling molecule TTZ has also been described (33). All other constructs were derived by PCR using cDNA from Jurkat T cells and appropriate primers. PCR-derived constructs were sequenced to ensure the absence of mutations. The eukaryotic expression vector used for expression of SH2 domains in Jurkat cells was pyDF30 (made by David Fiorentino), a derivative of pBJ5 (36) designed to incorporate an amino-terminal FLAG epitope, DYKDDDDK, into expressed proteins. The amino acid residues present in various Zap70 and Syk expression constructs are as follows: Zap70Δkin, 1 to 331; Zap2XN-C, 5 to 263; ZapN, 1 to 170; ZapC, 158 to 263; Syk2XN-C, 10 to 270; SykN(A), 10 to 117; SykN(P), 10 to 153; SykC, 163 to 270. Hybrid Zap70 tandem SH2 domain expression vectors were made from combinations of the above constructs by using natural restriction sites. Zap2XN-C(+8) is identical to Zap2XN-C except that the eight residues HERMPWIL are inserted between residues 157 to 158. Zap2XN-N(+8) contains Zap70 residues 5 to 157, HERMPWIL, and residues 5 to 112, and Zap2XC-C(+6) contains residues 158 to 263, LNFE, and residues 110 to 263. Although there are only 4 additional non-Zap70 residues in Zap2XC-C(+6), the actual linker between the tandem CSH2 domains is 6 residues longer than that of Zap2XN-C. Zap2XC-C contains residues 158 to 259 and 108 to 263. Expression vectors for Grb2 and Shc were also constructed in pyDF30 and contain the following residues: Grb2SH2,

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45 to 164; ShcSH2, 365 to 473; ShcPTB, 46 to 232; Shcwt, 46 to 473; ShcY-F, 46 to 473 (Tyr-317→Phe); Grb2SH2-PTB(wt), Grb2SH2 residues 45 to 164 fused to ShcPTB residues 46 to 232; Grb2SH2-PTB(R-K), Grb2SH2-PTB(wt) with Arg-86→Lys.

Transfections, stimulations, and SEAP assays. Jurkat TagC15 cells were transiently transfected by electroporation as described elsewhere (38) with 3 μg of reporter construct and 5 μg of FLAG expression construct plus or minus 3 μg of the Tacζ chimera TTZ. Stimulations were initiated 24 h after transfection and were allowed to proceed for 14 to 16 h to generate secreted alkaline phosphatase (SEAP). Phorbol ester plus calcium ionophore stimulation consisted of 20 ng of phorbol 12-myristate 13-acetate (PMA) (Sigma) per ml and 2 μM ionomycin (Calbiochem), while lectin stimulation consisted of 15 μg of phytohemagglutinin (PHA) (Sigma) per ml. For anti-Tac stimulations, cells at 10^7/ml were incubated for 10 min on ice in phosphate-buffered saline (PBS) with 2 μg of anti-Tac antibody B-B10 (Biosource) per ml followed by 10 min on ice with 10 μg of rabbit anti-mouse immunoglobulin G (Zymed) per ml and then diluted 10-fold with growth medium at 37°C. SEAP activity was measured in 100-μl aliquots of heated (68°C, 1 h) growth medium as described elsewhere (12).

Immunoprecipitations and immunoblotting. Transiently transfected cells were assayed for expression of FLAG epitope-tagged proteins by lysing whole cells in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer, followed by electrophoresis and immunoblotting with anti-FLAG M2 antibody (Kodak). The association of Zap70Δkin or Grb2SH2 with Tacζ was shown by electroporating 10^8 Jurkat TagC15 cells with 15 μg of Zap70Δkin or Grb2SH2 plus or minus 20 μg of TTZ or 20 μg of TTZ alone, followed by activation in PBS with 4 μg of anti-Tac antibody and 4 μg of rabbit anti-mouse immunoglobulin G per ml as described above except that cells were heated to 37°C for 2 min and then quickly lysed. Cells were lysed in 700 μl of ice-cold buffer (25 mM Tris-HCl [pH 7.4]–140 mM NaCl–3 mM KCl [TBS], 1% Nonidet P-40, 1 mg of bovine serum albumin [BSA] per ml, 1 mM Na_3VO_4, 5 mM NaF, 10 μg of leupeptin per ml, and 5 μg of aprotinin per ml), and particulate matter was removed by centrifugation at 100,000 × *g* for 10 min. Aliquots, 600 μl, of extract were immunoprecipitated by adding 20 μl of protein A-Sepharose beads (Sigma) preblocked in lysis buffer. After 1.5 h at 4°C, beads were washed three times with ice-cold wash buffer (TBS, 0.1% Nonidet P-40, 1 mM Na_3VO_4) and analyzed by SDS-PAGE and immunoblotting as above with anti-FLAG or antiphosphotyrosine antibody PY20 (ICN Biomedicals).

Fusion proteins. Glutathione *S*-transferase (GST) fusion proteins were generated by insertion of the appropriate DNA fragments from the above eukaryotic expression vectors into the vector pGEX-3X (Pharmacia) and expression in *Escherichia coli*. The constructs ZapCSH2, Zap2XSH2(N-C), Grb2SH2, and ShcSH2 contain the same residues indicated above. ZapNSH2 contains Zap70 residues 5 to 112, not 1 to 170. Fusion proteins were expressed in *E. coli* by induction for 2 to 3 h at 37°C with 0.4 mM isopropylthiogalactopyranoside (IPTG). Cells were lysed by sonication in buffer PBS, 100 mM EDTA, 1% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride, and proteins were isolated from the soluble fraction [with the exception of the Zap2XSH2(N-C) protein] with glutathione-agarose beads. Zap2XSH2(N-C) was isolated from the particulate fraction (inclusion body), refolded after dissolving in 8 M urea, and finally passed over glutathione-agarose beads.

Peptides and flow cytometry. Peptides used for competition were as follows: ITAM-1N, NH_2-ANQL(pY)NELN-$CONH_2$; ITAM-1C, NH_2-AREE(pY)DVLD-$CONH_2$; ITAM-1(N-C), Ac-NH-QL(pY)NELNLGRREE(pY)DVLDKR-$CONH_2$ (pY is phosphotyrosine). ITAM-1N and -1C have a free N terminus and an Ala spacer, while ITAM-1(N-C) has an acetylated N terminus. All three have an amidated C terminus. Monodisperse 10-μm-diameter amine beads have been previously described (37). Beads (20 mg) were treated with 0.11 M 9-fluorenylmethoxycarbonyl (Fmoc)-protected linker Fmoc-PEG 15 (Fmoc-NH-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-NHCO$CH_2CH_2CH_2$COOH), 0.1 M HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate], and 0.3 M diisopropylethylamine in dimethylformamide (500 ml) for 30 min. This process was repeated, and the beads were then treated with a tetrahydrofuran solution of acetic anhydride and 1-*methyl*-imidazole to cap any unreacted amines. The Fmoc protecting group was removed with 10% (vol/vol) piperidine in dimethylformamide for 10 min. Sequential amino acid coupling followed by Fmoc deprotection was repeated until the decapeptide (octapeptide-S-V) sequences were assembled. Octapeptide sequences derived from the human ζ-chain ITAMs were of the form XXX(pY)XXXX. The N-terminal amines were acetylated. Side chain group deprotection was carried out with 95% trifluoroacetic acid–5% H_2O for 1 h at room temperature. The sequence of the epidermal growth factor receptor (EGFR) pY-1068 control sequence was VPE(pY)INQSV. Peptide-derivatized or negative-control beads, 10^5 per sample, were washed in TBS and blocked in 400 μl of buffer (TBS, 1% BSA, 0.05% Tween 20) for 1 h at room temperature. After recovery by centrifugation, beads were stained for 45 min at room temperature in 500-μl volumes containing 1 μM each GST fusion protein and anti-GST monoclonal antibody directly conjugated to the cyanine fluor Cy3 in TBS–0.1% BSA–0.005% Tween 20. Peptide competitions to measure nonspecific binding were done in 150-μl volumes in the presence of 150 μM peptide. Beads were pelleted and kept on ice until analysis, at which time they were diluted with 300 μl of fluorescence-activated cell sorter sheath fluid and analyzed for fluorescence on a FACScan (Becton Dickinson). Clustered beads were gated out of the analysis by using forward-scatter measurements. An anti-GST monoclonal antibody, 75GST.141.4, was generated by immunization of an IRCF1 mouse (Simonsen Labs) with purified GST and subsequent polyethylene glycol fusion of splenocytes to the hypoxanthine phosphoribosyltransferase-negative mouse myeloma cell line P3 X63Ag8.653. Hybridomas were selected for growth in medium containing 8-azaserine and assayed for their ability to secrete GST-specific antibodies in an enzyme-linked immunosorbent assay format with GST directly coated onto polystyrene wells. Coupling of Cy3 *N*-hydroxysuccinimide (NHS) ester (Biological Detection Systems) to the purified antibody was performed in 0.1 M sodium carbonate buffer, pH 9.3, at room temperature for 60 min followed by dialysis. The fluorophore/protein ratio was calculated to be between 4 and 6.

RESULTS

Zap70 and Syk SH2 domains inhibit T-cell activation. To examine the possibility of using SH2 domain overexpression as a means of generating dominant negative mutants for T-cell activation, we constructed a truncated Zap70 molecule lacking the kinase domain. This construct retains both the SH2 domains as well as the natural spacing sequence between them. We initially tested this Zap70Δkin in Jurkat cells transfected with a chimeric TCR ζ chain (33) and activated either by specific antibody cross-linking or by phorbol ester and calcium ionophore (Fig. 1A). When coexpressed with a T-cell-specific reporter gene construct, NF-ATSEAP (12), this kinase-deficient Zap70 molecule acted as a potent inhibitor of signal transduction mediated by the TCR ζ chain (Fig. 1A). In contrast, the Zap70Δkin protein was an ineffective inhibitor when reporter gene activity was induced by PMA and ionomycin, agents expected to bypass its inhibitory effect by stimulating protein kinase C and increased intracellular Ca^{2+}, respectively. We presumed that the Zap70Δkin molecule functioned by binding to the ζ-chain chimera, thus preventing binding of endogenous Zap70 in a competitive manner. To test this, we attempted to immunoprecipitate Zap70Δkin with an anti-Tac antibody in the presence or absence of the Tacζ chimera previously activated by antibody cross-linking. As shown in Fig. 1B, Zap70Δkin is precipitated and tyrosine phosphorylated only in the presence of the Tacζ chimera, although it is expressed at equally high levels with and without the chimera. When the Tacζ chimera is expressed in the absence of Zap70Δkin (Fig. 1B), no FLAG-reactive or phosphotyrosine-containing protein of this size is detected. Furthermore, the material that precipitates appears more highly phosphorylated than the bulk of the Zap70Δkin protein (Fig. 1B). Taken together, these data indicate that Zap70 is an essential component of the signal transduction pathway activated by the T-cell antigen receptor.

The Zap70-related tyrosine kinase Syk has also been shown to associate with TCR components (10). To determine if the tandem SH2 domains of Syk could also serve as a dominant negative protein, we designed a construct to express Syk residues 10 to 270 (Syk2XN-C), containing only the SH2 domains and intervening residues. As a control, a corresponding construct was made for Zap70, Zap2XN-C. These proteins were expressed in Jurkat cells, and their abilities to inhibit lectin-induced NF-ATSEAP activity were determined (Fig. 1C). Like Zap70Δkin, both proteins were effective inhibitors while having no effect on PMA- and ionomycin-induced reporter activity. All three proteins are expressed at quite high levels, as shown in Fig. 1D.

Binding of Zap70 single and tandem SH2 domains to ITAM sequences in vitro. Previous studies have indicated that, for Zap70, both SH2 domains must be functional for a stable interaction with doubly phosphorylated ITAM motifs of the TCR ζ chain to occur in vitro (26). To determine which SH2 domain has greater intrinsic affinity for individual phosphotyrosine-containing ITAM sequences, we synthesized peptides

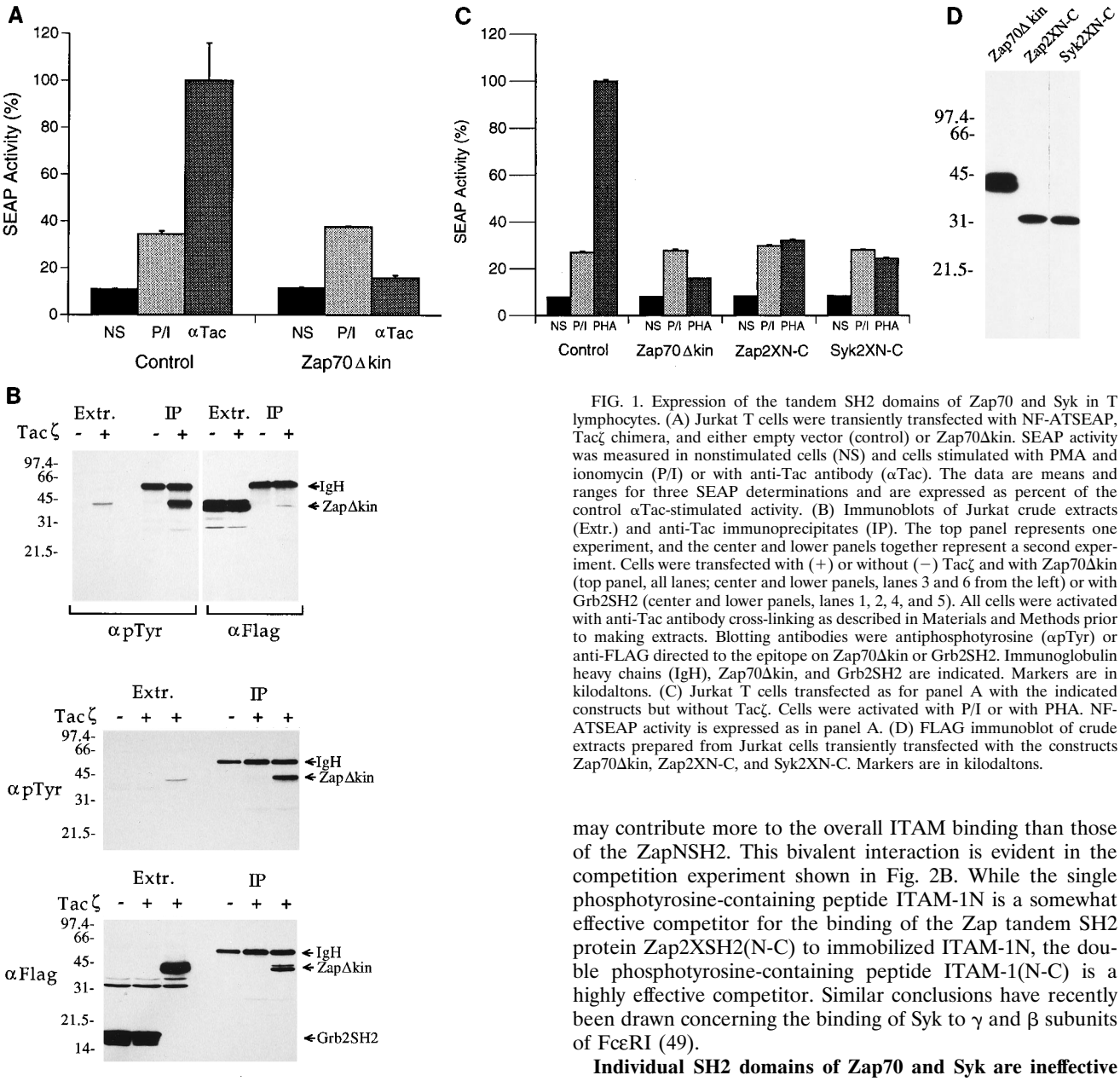


FIG. 1. Expression of the tandem SH2 domains of Zap70 and Syk in T lymphocytes. (A) Jurkat T cells were transiently transfected with NF-ATSEAP, Tacζ chimera, and either empty vector (control) or Zap70Δkin. SEAP activity was measured in nonstimulated cells (NS) and cells stimulated with PMA and ionomycin (P/I) or with anti-Tac antibody (αTac). The data are means and ranges for three SEAP determinations and are expressed as percent of the control αTac-stimulated activity. (B) Immunoblots of Jurkat crude extracts (Extr.) and anti-Tac immunoprecipitates (IP). The top panel represents one experiment, and the center and lower panels together represent a second experiment. Cells were transfected with (+) or without (−) Tacζ and with Zap70Δkin (top panel, all lanes; center and lower panels, lanes 3 and 6 from the left) or with Grb2SH2 (center and lower panels, lanes 1, 2, 4, and 5). All cells were activated with anti-Tac antibody cross-linking as described in Materials and Methods prior to making extracts. Blotting antibodies were antiphosphotyrosine (αpTyr) or anti-FLAG directed to the epitope on Zap70Δkin or Grb2SH2. Immunoglobulin heavy chains (IgH), Zap70Δkin, and Grb2SH2 are indicated. Markers are in kilodaltons. (C) Jurkat T cells transfected as for panel A with the indicated constructs but without Tacζ. Cells were activated with P/I or with PHA. NF-ATSEAP activity is expressed as in panel A. (D) FLAG immunoblot of crude extracts prepared from Jurkat cells transiently transfected with the constructs Zap70Δkin, Zap2XN-C, and Syk2XN-C. Markers are in kilodaltons.

on beads (37) and assessed, by flow cytometry, the ability of bacterially expressed SH2 fusion proteins to bind these sequences. In this assay the Zap C-terminal SH2 domain, ZapCSH2, binds well to the N-terminal phosphotyrosine-containing sequence of the ζ-chain first ITAM, ITAM-1N (Fig. 2A). In contrast, ZapCSH2 binds significantly less well to ITAM-1C, while there is little binding to control beads. The sequence preference of ZapCSH2 is further illustrated by the differential ability of these two sequences in solution to compete for binding to immobilized ITAM-1N (Fig. 2B). This binding pattern is qualitatively the same for the second and third ITAMs of the ζ chain (data not shown). Surprisingly, the Zap N-terminal SH2 domain, ZapNSH2, appears to have little affinity on its own for either ITAM sequence (Fig. 2A). These data indicate that ZapCSH2 binds preferentially to the N-terminal half of an ITAM motif and that these interactions may contribute more to the overall ITAM binding than those of the ZapNSH2. This bivalent interaction is evident in the competition experiment shown in Fig. 2B. While the single phosphotyrosine-containing peptide ITAM-1N is a somewhat effective competitor for the binding of the Zap tandem SH2 protein Zap2XSH2(N-C) to immobilized ITAM-1N, the double phosphotyrosine-containing peptide ITAM-1(N-C) is a highly effective competitor. Similar conclusions have recently been drawn concerning the binding of Syk to γ and β subunits of FcεRI (49).

Individual SH2 domains of Zap70 and Syk are ineffective dominant negative proteins for T-cell activation. We wished to determine whether any of the individual SH2 domains of Zap70 and Syk, regardless of whether they had high or low apparent affinities for ITAM sequences in vitro, could bind to ITAM motifs in vivo with sufficient affinity to act as dominant negative proteins for T-cell activation. Coexpression of these four individual SH2 domains with the NF-ATSEAP reporter in all cases failed to effectively inhibit T-cell activation compared with Zap2XN-C (Fig. 2C). Although there are large differences in expression levels for some constructs (ZapC, for instance) (Fig. 2D), we feel that this conclusion is valid for two reasons. First, while there is minimal inhibition with some Syk SH2 domain constructs, their expression is as high as or higher than that of the highly effective Zap70Δkin (Fig. 2D). Second, SH2 domains with similar low-level expression have vastly different dominant negative potential (compare ZapN with the SH2 domain of Grb2; Fig. 2D; also, see below). While there is little correlation between dominant negative potential and expres-

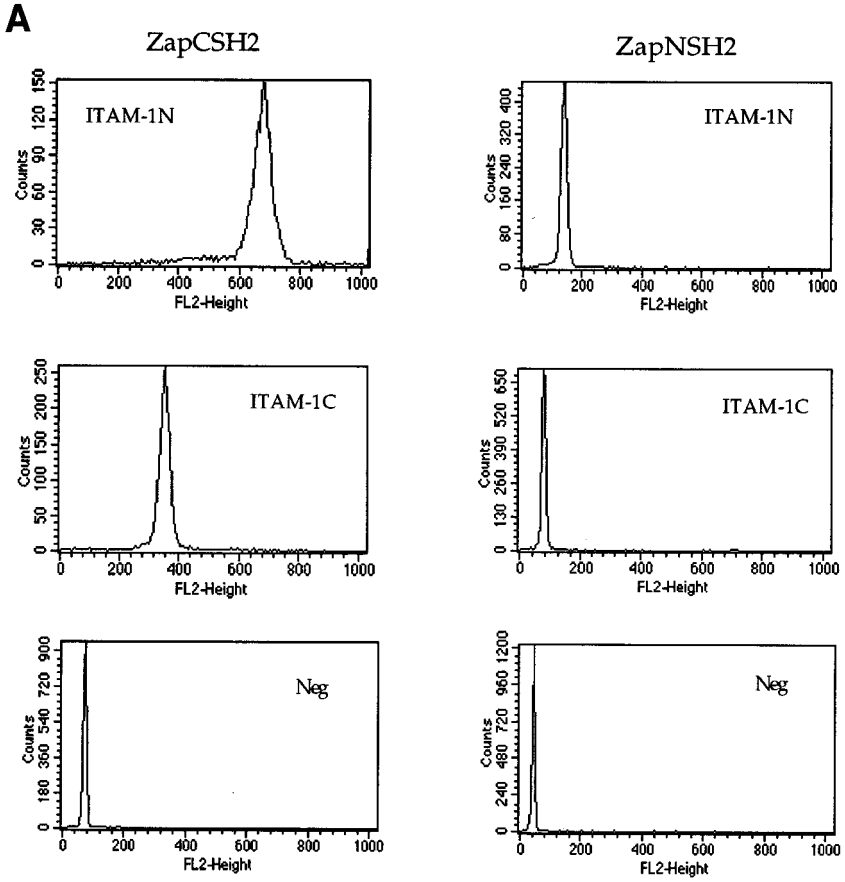


FIG. 2. Analysis of individual SH2 domains of Zap70 and Syk in vitro and in vivo. (A) Flow-cytometric analysis of 10-μm beads derivatized with phosphotyrosine-containing peptides and stained with individual SH2 domains of Zap70 expressed as GST fusion proteins. ITAM-1N and -1C, immobilized peptides representing the N-terminal and C-terminal halves of the first ITAM motif of the ζ chain, respectively (see Materials and Methods). Neg, beads with no peptide. *y* axis, bead counts; *x* axis, fluorescence intensity. (B) Competition for binding of ZapCSH2 or the tandem SH2 domains of Zap70, Zap2XSH2(N-C), to the ITAM-1N sequence on 10-μm beads. Competing peptides in solution (150 μM) are labeled as in panel A. ITAM-1(N-C), peptide representing the whole first ITAM of the ζ chain; none, no competing peptide. Histogram axes are the same as in panel A. (C) Expression of individual SH2 domains of Zap70 and Syk in Jurkat cells. Cells were transfected as for Fig. 1C with vector (control) or with the indicated SH2 domain constructs. NF-ATSEAP is expressed as PHA-stimulated activity in relation to control when normalized to activity stimulated by PMA and ionomycin. The data are means and ranges for two independent transfections, except for SykN (P) (one transfection). (D) FLAG immunoblot of crude extracts prepared from the transiently transfected cells described for panel C. Two exposures of 10 and 100 s are shown. Lane 1, Grb2 SH2; lane 2, ZapN SH2; lane 3, ZapC SH2; lane 4, Zap2XN-C; lane 5, SykN (A) SH2; lane 6, SykN (P) SH2; lane 7, SykC SH2. Markers are in kilodaltons.

sion levels, we cannot rule out the possibility that ZapCSH2 would have an effect if expressed at higher levels.

Dominant negative effects of tandem Zap70 SH2 domains are highly dependent on their spacing. ZapCSH2 has greater apparent affinities for ITAM sequences than ZapNSH2, yet the latter is absolutely required for ITAM interactions in vivo. To investigate whether ZapCSH2 and ZapNSH2 are interchangeable, we constructed molecules with either two CSH2s of two NSH2s. The initial constructs failed to inhibit T-cell activation (Fig. 3). These constructs encode several extra residues in the linker between the repeated N-N or C-C SH2 domains compared with the wild-type N-C construct (see Materials and Methods), and this altered spacing could account for their lack of function. To test this, we constructed an N-C tandem SH2 fusion with eight additional residues in the linker segment. This protein was also nonfunctional in the dominant negative assay, indicating that a bivalent interaction with the ITAM motifs was prohibited in vivo. Interestingly, a second tandem C-C SH2 construct encoding no extra residues between the SH2 domains was also an ineffective dominant negative mutant (Fig. 3). All four of these tandem SH2-containing proteins were expressed at levels identical to that of the Zap2XN-C wild-type protein (data not shown). These results demonstrate the need for a distinct distance and/or conformation between the two SH2 domains for binding to the closely spaced YXX(L/I) motifs of the ITAM.

The SH2 domain of Grb2, but not of Shc, inhibits T-cell activation. While several mechanisms have been proposed for the activation of the p21^{ras} guanine nucleotide-binding protein during T-cell activation (14, 20, 42), the exact mechanism and proteins involved remain unknown. Several SH2 domain-containing proteins including Grb2 (35), Shc (42), the proto-oncogenic protein Vav (20), and the uncharacterized protein p36 (6, 44) may have roles in promoting p21^{ras} activation. To define the roles of Grb2 and Shc in T-cell activation, we expressed their isolated SH2 domains in Jurkat cells. The results (Fig. 4A) clearly show that the SH2 domain of Grb2, but not that of Shc, is capable of inhibiting signal transduction in T cells. As shown in Fig. 4D, the Shc SH2 domain is expressed at higher levels than the Grb2 SH2 domain, ruling out the possibility that insufficient expression underlies this difference. Although the Shc SH2 domain is capable of binding to tyrosine-phosphorylated TCR ζ-chain sequences (42), the so-called phosphotyrosine binding (PTB) domain of Shc has recently been shown

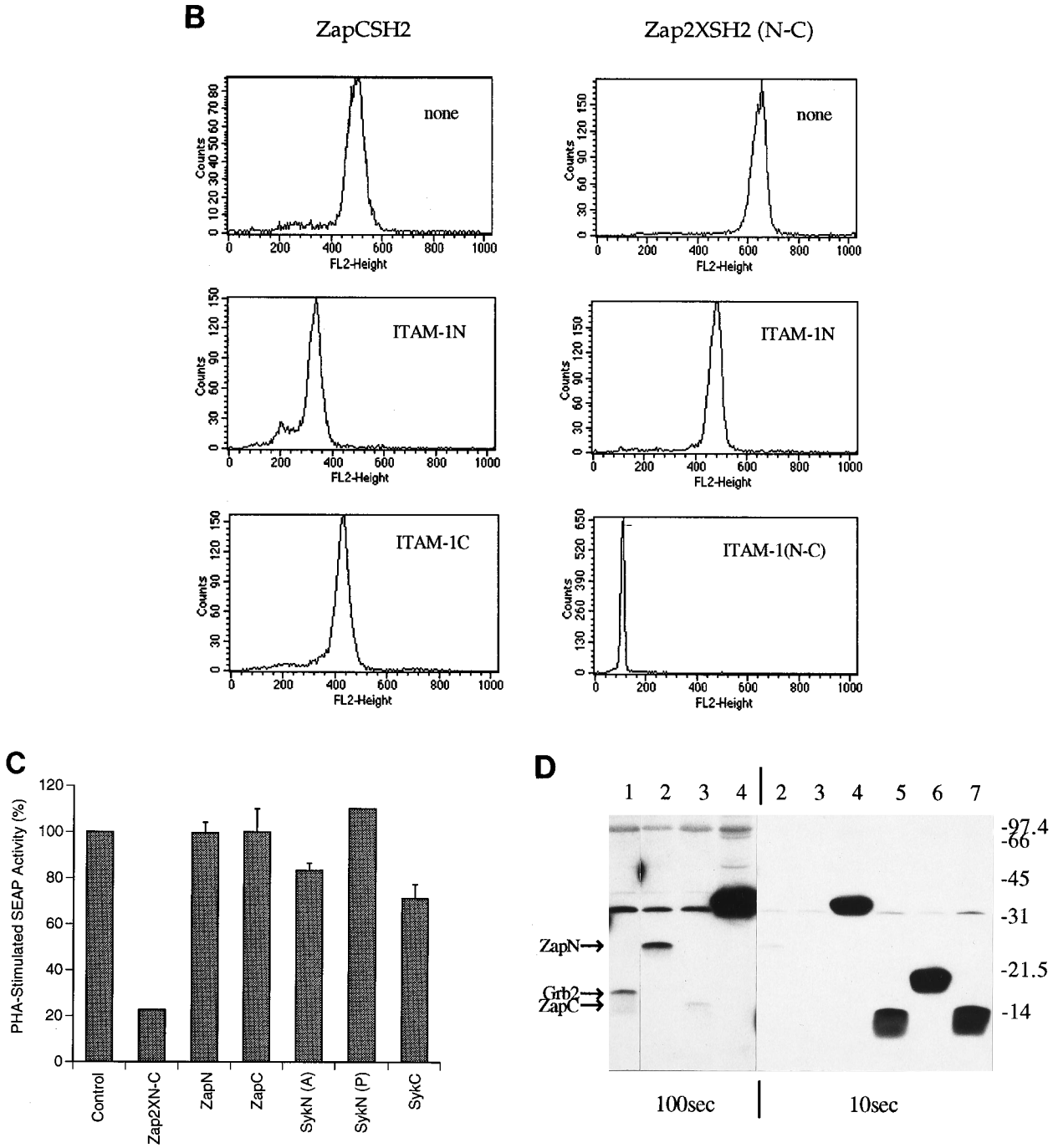


FIG. 2—*Continued.*

to also bind to phosphotyrosine-containing sequences (4, 29). We found that the PTB domain, either alone or in the context of the complete Shc molecule, could not inhibit T-cell activation even when the ability of Shc to associate with Grb2 was eliminated by mutating the critical tyrosine in the Grb2 binding sequence (Fig. 4A). These proteins were expressed at levels comparable to that of the Zap2XN-C dominant negative mutant (Fig. 4D). To test the specific nature of the Grb2 SH2 inhibitory effect, we mutated the critical Arg in the phosphotyrosine binding pocket to Lys (34). Although we observed no inhibitory effect on T-cell activation (data not shown), we were unable to verify expression of this protein. We therefore fused both wild-type and R-K mutant Grb2 SH2 domains to the Shc PTB domain to obtain expression. The wild-type construct is an effective dominant negative protein, while the R-K mutant protein fails to inhibit T-cell activation (Fig. 4A). Both proteins are expressed at high levels (Fig. 4D). The results suggest that, while Grb2 has a distinct role in T-cell activation, the Shc molecule appears to have little if any role.

To determine the intrinsic abilities of the Grb2 and Shc SH2 domains to bind to ITAM sequences, we made GST fusion proteins of each and assessed their abilities to bind to ITAM

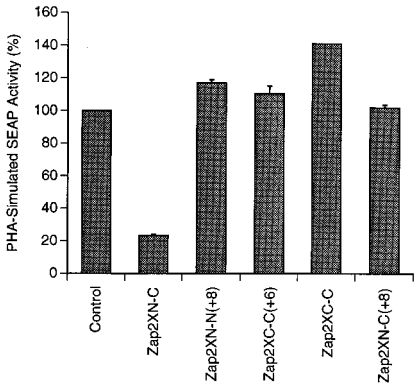


FIG. 3. Inhibition of NF-ATSEAP induction by tandem SH2 domains of Zap70. Jurkat cells were transfected as for Fig. 1C with the indicated constructs. Numbers in parentheses indicate the number of extra amino acids in the linker piece between the SH2 domains compared with the wild-type protein. NF-ATSEAP is expressed as in Fig. 2C. The data are means and ranges for two or three independent transfections, except for Zap2XC-C (one transfection).

peptides immobilized on beads. The results for Grb2 and Shc SH2s are shown in Fig. 4B and C, respectively. Although the Grb2 SH2 domain has slight affinity for ITAM-1N and -3N, the binding is significantly lower than that of ZapCSH2 for the same sequences and far below the affinity for an EGFR control sequence. These interactions could not account for the dominant negative effect of the Grb2 SH2 domain. Furthermore, we have been unable to detect the Grb2 SH2 domain in immunoprecipitates of the activated Tacζ chimera when they are coexpressed (Fig. 1B). Grb2 can, however, be coimmunoprecipitated with the activated EGFR (5, 11, 16, 19, 34, 39, 47, 51; also, data not shown). We found that the Shc SH2 domain had somewhat higher affinity for the ITAM sequences and, consistent with previous reports (42), gave the highest signals with sequences derived from the third ITAM motif of the TCR ζ chain.

Alternative methods of p21^{ras} activation override some dominant negative effects of the Grb2 SH2 domain. We found that the SH2 domain of Grb2 inhibited T-cell activation. It has been proposed that Grb2, through its interaction with the guanine nucleotide exchange protein Sos, is directly involved in coupling the TCR to activation of p21^{ras}. As a downstream readout for activated p21^{ras}, we used an AP1-driven reporter gene. To test whether the Grb2 SH2 domain was able to prevent the activation of p21^{ras}, we attempted to overcome the inhibitory effect by activating p21^{ras} with alternative methods. As the Zap70Δkin inhibits signaling events proximal to p21^{ras} activation, we used this as a positive control for inhibition of AP1-dependent transcription. While PHA-stimulated AP1 activity was significantly decreased by both Zap70Δkin and the Grb2 SH2 domain, stimulation by activation of protein kinase C overcame these inhibitory effects (Fig. 5). In addition, coexpression of a constitutively active p21^{ras} was also sufficient to overcome the inhibitory effects of these proteins (data not shown). As expected, adding protein kinase stimulation to T-cell activation by PHA did not overcome the inhibitory effect of Zap70Δkin on NF-ATSEAP activity (Fig. 5). Surprisingly, this treatment also did not permit activation of NF-ATSEAP activity in the presence of the Grb2 SH2 domain. Taken together, these results indicate that the Grb2 SH2 domain, like the Zap70Δkin protein, exerts some of its inhibitory effects upstream of p21^{ras} activation, yet at a signaling step different from the Zap70-ζ interaction.

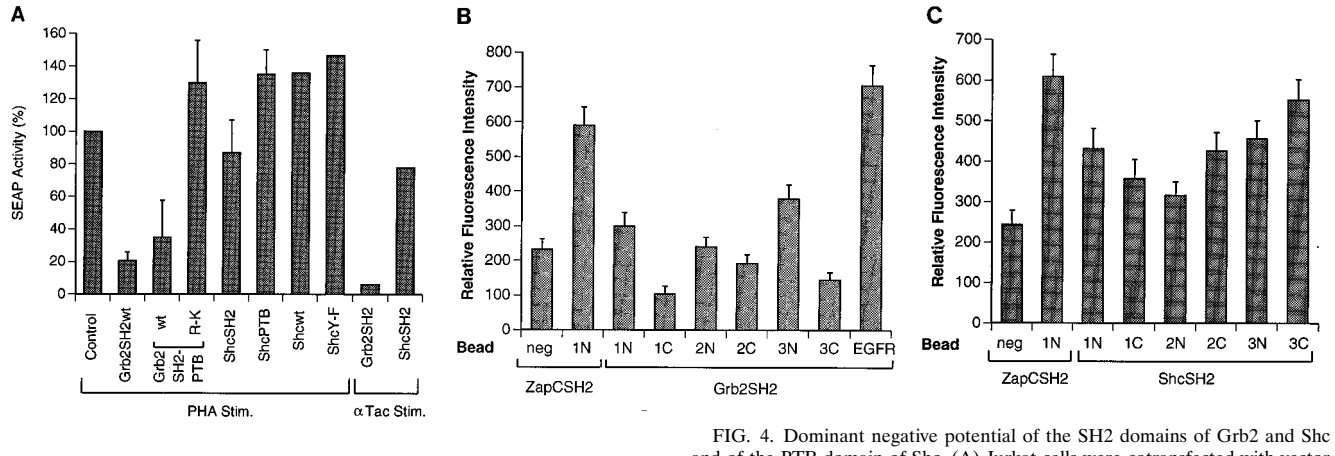
DISCUSSION

We have shown that the tandem SH2 domains of Zap70 and Syk can be potent dominant negative proteins for the TCR-mediated activation of a T-cell specific transcription factor, NF-AT. The signals required for activation of NF-AT can be generated by the TCR (48) or, more specifically, by the ζ chain alone or even isolated ITAM motifs when sufficiently aggregated (23, 24, 33, 52). As NF-AT is required for interleukin 2 gene transcription and T-cell activation (15, 48), these expressed SH2 domains would be effective inhibitors of antigen-driven T-cell activation. The role of Zap70 in T-cell activation has been inferred through studies using chimeric transmembrane proteins (32), Lck-deficient T cells (26, 53), and reconstitution of early signaling events in Cos cells (8, 26). Recently, studies of several clinical cases of severe combined immunodeficiency in humans have pinpointed the cause of the immunodeficiency to mutations in Zap70 resulting in complete lack of Zap70 expression in mature peripheral T cells as well as defects in thymocyte maturation (1, 9, 17). These findings point to an essential role for Zap70 in immune function. The Zap70Δkin dominant negative molecule described here demonstrates both the key role of Zap70 in TCR-mediated signal transduction and the necessary function of its kinase domain. This result both complements and adds to the conclusions of previous studies.

The hematopoietically expressed proto-Vav gene product p95^{vav} has been implicated as a guanine nucleotide releasing factor for p21^{ras} in T cells (20) and has more recently been suggested to associate through its SH2 domain with a specific tyrosine-containing sequence of Zap70 (28). This sequence, YESP (residues 315 to 318), is present in Zap70Δkin, and we have shown that Zap70Δkin is tyrosine phosphorylated in a ζ-chain-dependent manner. Although the sites of phosphorylation are unknown, it is possible that Vav can still associate with Zap70Δkin but remain inactive because of the lack of Zap70 kinase activity. We are currently investigating this possibility. Further truncation of Zap70, Zap2XN-C, which eliminates this putative Vav binding sequence, does not alter the inhibitory potential. Thus, lack of Vav association with or phosphorylation by Zap70 may result in a defect in signaling. Recent targeted disruption of *vav* (18, 54, 57) has shown that Vav-deficient T cells have attenuated responses to TCR stimulation; however, it is not known if Vav is involved in TCR-mediated p21^{ras} activation.

While the tandem SH2 domains of Syk can also function as dominant negative proteins for T-cell activation, tissue distribution of Syk suggests that it is involved in signaling in B cells and mast cells rather than in mature peripheral T cells, where it is expressed at relatively low levels compared with Zap70 (10). In addition, Zap70-deficient T cells fail to activate when stimulated through the TCR (1, 9, 17), indicating that Syk cannot compensate. Nevertheless, studies with Jurkat cells indicate that Syk can associate with activated TCR components (10). Thus, our results are fully consistent with previous observations. We have also examined the ability of bacterially expressed Syk SH2 domains to interact with ζ ITAM sequences and find that, like Zap70, SykCSH2 has quite high affinity while SykNSH2 has relatively little affinity on its own for these sequences (data not shown). Although Zap70 and Syk have been shown to display some preferential binding for certain ITAMs (25, 30, 49), our studies support the conclusion (10) that tissue distribution rather than ability to interact with specific receptors dictates their functional contributions in vivo.

Studies with tandem SH2 domain-containing proteins such as Zap70 (25, 26), Syk (49), phosphatidylinositol 3-kinase (13,



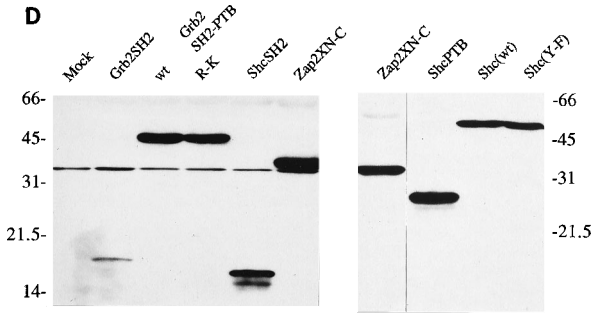


FIG. 4. Dominant negative potential of the SH2 domains of Grb2 and Shc and of the PTB domain of Shc. (A) Jurkat cells were cotransfected with vector (control) or the indicated constructs as for Fig. 1A and C and stimulated with anti-Tac antibody (αTac Stim.) or PHA as indicated. Stimulated SEAP activity is expressed as in Fig. 2C. Data with error bars represent means and ranges for three to six independent transfections; other data are mean SEAP activities from single transfections. (B and C) Summaries of flow-cytometric analyses comparing ZapCSH2 to the Grb2 and Shc SH2 domains, respectively, as indicated. Peptide sequences on beads correspond to N- and C-terminal-half ITAMs for each of the three ITAMs of the TCR ζ chain. EGFR, beads with EGFR-derived sequence containing phosphotyrosine 1068; neg, beads with no peptide. The data represent the mean fluorescent intensities of 10^4 stained beads, with error bars indicating the fluorescence range within which 90 to 95% of the analyzed beads fall. (D) FLAG immunoblot of crude extracts prepared from the transiently transfected cells described for panel A. Left, comparison of Grb2 and Shc SH2 domain and Grb2 SH2-PTB fusion expression with that of Zap2XN-C; right, comparison of Shc PTB and full-length constructs with Zap2XN-C.

31), and Ras-GTPase activating protein (13) have shown variable roles for both SH2 domains in the interaction with specific receptor sequences in vitro. These studies, however, leave unanswered the question of in vivo binding requirements. Our results show that the single SH2 domains of Syk and ZapNSH2 fail to interact with signal transduction components with sufficient affinity to compete with endogenous Zap70 and/or Syk. Although ZapCSH2 is expressed at relatively low levels and we thus cannot rule out a potential in vivo interaction, our results support the concept that a bivalent interaction with the ITAM is necessary in vivo.

We found that the spacing between the tandem SH2 domains of Zap70 was critical for functional interactions in vivo. This spacer likely allows for the concise juxtaposition of the two SH2 domains necessary for simultaneous binding to the closely spaced (10 or 11 residues) phosphotyrosines of the ITAM. Our construct designed to maintain the correct spacing also failed to interact with ITAMs in vivo, suggesting that the SH2 domains of Zap70 are not interchangeable. We cannot rule out, however, the possibility that the spacer still did not permit the correct geometry for binding. Interestingly, this type of study using phosphatidylinositol 3-kinase and GTPase-activating protein (GAP) SH2 domains has been successful (13). These experiments, indicating a high degree of SH2 domain interchangeability, likely worked because of the greater spacing between target phosphotyrosine residues in the platelet-derived growth factor receptor. Given more flexible spacing between two target phosphotyrosine residues, such as two identical peptides tethered through flexible linkers to a solid support, we suspect that a bivalent interaction could occur. In fact the Zap2XSH2(N-C) fusion protein binds to beads derivatized with ζ ITAM-1C with much greater affinity than either SH2 alone (37a), suggesting a bivalent interaction.

The activation of $p21^{ras}$ is essential for responses to TCR stimulation (2, 43). The mammalian homolog of the guanine nucleotide exchange protein son of sevenless (Sos) may promote activation of $p21^{ras}$ in T cells. Activation of $p21^{ras}$ in response to receptor tyrosine kinases involves the SH2 and SH3 domain-containing adapter protein Grb2 (5, 11, 16, 19, 34, 39, 47, 51), which couples receptor phosphotyrosine-containing sequences to Sos. The ubiquitous adapter protein Shc has been found to be phosphorylated after T-cell activation and to associate with the third ITAM of the ζ chain (42). As Shc is able to bind to Grb2 through a specific phosphotyrosine-containing sequence, Shc could adapt a Grb2-Sos complex to the ζ chain, thus leading to $p21^{ras}$ activation. The interaction of Shc with the ζ chain could occur through the SH2 domain, the PTB domain, or both. The PTB domain has no detectable affinity for ζ-chain ITAM sequences (37a) but, rather, appears to be responsible (29) for the association of Shc with a 140- to 150-kDa protein(s) in activated T cells (6, 42). Our in vivo results, and in vitro results of others (40), suggest that Shc does not function in transmission of TCR ζ-chain signals and agree with recent data presented by Baldari et al. (3). Shc may, however, play a significant role in coupling the interleukin 2 receptor (41) and CD4 (3) to $p21^{ras}$. In contrast, the SH2 domain of Grb2 is a potent inhibitor of T-cell activation while displaying little affinity for the ζ-chain ITAMs. Thus, while Grb2 may be involved in coupling the TCR to $p21^{ras}$ activation, it appears to require additional factors to do this. This is consistent with the inability of us and others (42) to demonstrate a direct interaction between the Grb2 SH2 domain and ζ-chain sequences. A recently discovered but as yet uncharacterized phosphoprotein in activated T cells, p36 (6, 44), is a likely candidate for this adapter function, and Grb2 binds to the tyrosine-phosphorylated p36 through its SH2 domain. The

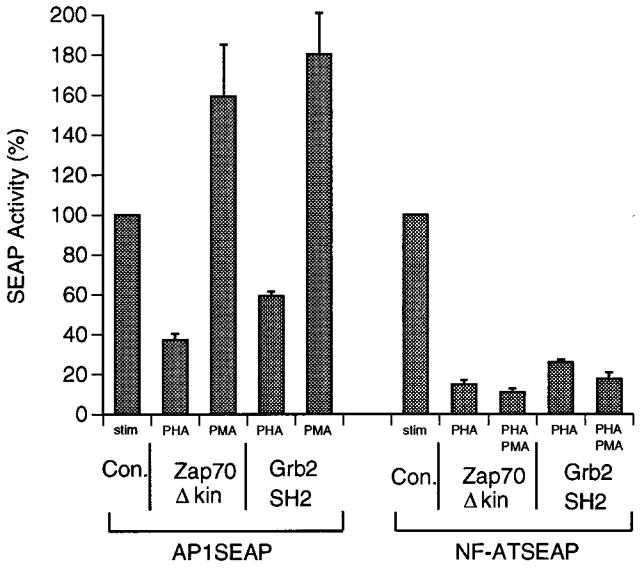


FIG. 5. Inhibition of AP1- and NF-AT-dependent reporter activity by Zap70 and Grb2 SH2 domains. Jurkat cells were transfected with either AP1SEAP or NF-ATSEAP and without (Con.) or with the indicated constructs. SEAP activities are expressed as percent of the stimulated (stim) control and are means and ranges for two independent transfections. stim, PHA or PMA treatment (AP1SEAP) or PHA or PHA-plus-PMA treatment (NF-ATSEAP). No-stimulation levels of SEAP activity in the two experiments: AP1SEAP, 10.4 and 11.3% of stimulated activity; NF-ATSEAP, 1 and 2% of stimulated activity.

molecular characterization of p36 will allow this question to be addressed.

Our results indicate that the inhibitory effects of the Grb2 dominant negative protein on p21^{ras}-dependent transcriptional events can be overcome by alternative methods of p21^{ras} activation. We determined, however, that activation of p21^{ras} was not sufficient to overcome the repression of NF-AT-dependent transcription, and our data, therefore, support the notion (50) that Grb2 participates in signals needed for increased intracellular Ca^{2+} as well. Recent findings (27) suggest an association between phospholipase Cγ1 and an SH2 domain-containing protein, SLP-76, which is itself associated with Grb2 in activated T cells. Therefore, Grb2 may couple the TCR, through p36 on one hand, to both p21^{ras} and phospholipase Cγ1 activation through its associations with Sos or Vav (56) and SLP-76, respectively.

In summary, we have shown the general utility of using dominant negative SH2 domains to investigate the role of specific proteins in T-cell activation. Importantly, while a variety of protein-protein interactions can be demonstrated in vitro, our results reasonably assess potential interactions in vivo and offer a highly complementary and functional method of studying signal transduction pathways involving SH2 domain-containing proteins.

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