

# Inhibition of ZAP-70 Kinase Activity via an Analog-sensitive Allele Blocks T Cell Receptor and CD28 Superagonist Signaling<sup>\*[S]</sup>

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ZAP-70 is a cytoplasmic protein tyrosine kinase that is required for T cell antigen receptor (TCR) signaling. Both mice and humans deficient in ZAP-70 fail to develop functional T cells, thus demonstrating its necessity for T cell development and function. There is currently no highly specific, cell-permeable, small molecule inhibitor for ZAP-70; therefore, we generated a mutant ZAP-70 allele that retains kinase activity but is sensitive to inhibition by a mutant-specific inhibitor. We validated the chemical genetic inhibitor system in Jurkat T cell lines, where the inhibitor blocked ZAP-70-dependent TCR signaling in cells expressing the analog-sensitive allele. Interestingly, the inhibitor also ablated CD28 superagonist signaling, thereby demonstrating the utility of this system in dissecting the requirement for ZAP-70 in alternative mechanisms of T cell activation. Thus, we have developed the first specific chemical means of inhibiting ZAP-70 in cells, which serves as a valuable tool for studying the function of ZAP-70 in T cells.

Stimulation of the T cell antigen receptor (TCR)<sup>3</sup> initiates a cascade of signal transduction events that leads to transcriptional alterations and cell activation. Protein phosphorylation is one of the key forms of chemical modification that controls TCR signaling. Two families of proximal protein tyrosine

kinases initiate TCR signaling. Src family kinases, predominantly Lck in T cells, phosphorylate component chains of the TCR complex following cross-linking of the antigen receptor. Dual tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD3 chains and  $\zeta$ -chain homodimer of the TCR leads to recruitment of the Syk family kinase ZAP-70 to these doubly phosphorylated ITAMs, where it binds via its two tandem Src homology 2 (SH2) domains. ZAP-70 undergoes a conformational change and then becomes activated by Lck-mediated phosphorylation and, potentially, by trans-autophosphorylation (1). Once activated, ZAP-70 can propagate the TCR signal by phosphorylating key downstream signaling molecules, including the adaptor molecules SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) and the linker for activation of T cells (LAT) (2, 3).

The importance of ZAP-70 in T cell development and function is evident from the immunodeficiency that develops in both mice and humans deficient in the gene. Transgenic ZAP-70 knock-out mice fail to develop any peripheral CD3<sup>+</sup> T cells and exhibit a block at the double-positive CD4<sup>+</sup>CD8<sup>+</sup> stage of T cell development (4). In addition, human severe combined immunodeficiency patients have been identified that do not express ZAP-70 protein (5–8). These patients fail to develop peripheral CD8<sup>+</sup> T cells; however, they do develop nonfunctional CD4<sup>+</sup> T cells. These nonfunctional T cells have a specific TCR signaling defect.

Alterations in ZAP-70 function and expression have been associated with other disease phenotypes in addition to severe combined immunodeficiency in both mice and humans. For example, a spontaneous mutation in murine ZAP-70 causes an autoimmune rheumatoid arthritis-like disease in BALB/c mice (9). These mice have a single point mutation in the C-terminal SH2 domain of ZAP-70, which results in a hypomorphic mutant and alterations in TCR repertoire selection. ZAP-70 has also been associated with the human disease chronic lymphocytic leukemia. Although ZAP-70 expression is primarily restricted to T cells, nearly half of all patients with chronic lymphocytic leukemia express ZAP-70 in their leukemic B cells (10). This altered expression is associated with a poor prognosis for these patients (11, 12).

The restricted expression of ZAP-70 and its requirement for proper T cell signaling and function make it an interesting therapeutic target for transplantation, autoimmunity, asthma, and chronic lymphocytic leukemia. Protein kinases, of which there

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<sup>3</sup> The abbreviations used are: TCR, T cell antigen receptor; ITAM, immunoreceptor tyrosine-based activation motif; SH2, Src homology 2; LAT, linker for activation of T cells; PP1, 4-amino-1-tert-butyl-3-(p-methylphenyl)pyrazolo[3,4-d]pyrimidine; 3-MB-PP1, 3-methylbenzyl-pyrazolopyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; DGK $\zeta$ , diacylglycerol kinase  $\zeta$ ; IL-2, interleukin 2; AS1 and -2, analog-sensitive allele 1 and -2; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; PLC $\gamma$ 1, phospholipase C $\gamma$ 1; PMA, phorbol 12-myristate 13-acetate; FACS, fluorescence-activated cell sorter; ITK, inducible T cell kinase; WT, wild type; NFAT, nuclear factor of activated T cells.

are in excess of 500 in the human genome, are attractive targets for disease treatments and comprise a large group of drug targets, second only to G protein-coupled receptors (13). The success of the kinase inhibitor Gleevec (imatinib, STI-571, Glivec) in treating chronic myelogenous leukemia serves as a powerful example of the value of targeting kinases for drug therapies. In addition to malignancies, kinases are also being studied for the treatment of autoimmunity and asthma to inhibit pathogenic pathways that are hyperactivated in these patients. For instance, Syk inhibitors have already been shown to be an effective therapy in rat asthma models (14).

In addition to serving as potential disease therapies, kinase inhibitors provide a powerful means of dissecting protein function. Yet, alternative methods for inactivating protein kinases are commonly used because specific inhibitors are not readily available for all kinases. For example, the ZAP-70-deficient Jurkat T cell line, P116, has been studied extensively in order to understand ZAP-70 function. However, P116 cells may compensate for the lack of ZAP-70 expression by altering other factors. Moreover, without a small molecule inhibitor, the adaptor function of the kinase can be studied only with a kinase-inactive mutant, which eliminates the opportunity to study function after signaling is initiated. ZAP-70-deficient mice have been useful in the study of the role of ZAP-70 in thymocyte development, but because of the complete block at the double-positive stage, it has been impossible to study the role of ZAP-70 in mature thymocytes or in peripheral T cell responses. Furthermore, methods such as RNA interference can be used to down-regulate protein expression; but they often take hours to days to have a sufficient effect, and these effects are commonly transient and incomplete. Thus, small molecule inhibitors are attractive because they usually function within seconds and often do not require any other cell manipulation. Selective inhibitors of several TCR proximal cytoplasmic kinases such as Lck have been reported (15). Yet, despite much interest, no selective cell-permeable ZAP-70 inhibitors, other than a peptide inhibitor, have been reported, suggesting that this kinase presents a significant challenge to inhibitor development (16).

The biggest drawback of inhibitors is the difficulty in ensuring kinase specificity. This problem has been addressed by the development of a genetically controlled system whereby a given kinase is mutated to render it uniquely susceptible to a designed analog of 4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)pyrazolo[3,4-*d*]pyrimidine (PP1), a competitive inhibitor of ATP binding (17). The unmodified ("targetless") cells, containing only wild-type kinases, serve as the specificity control for the mutant-specific small molecule inhibitor. The system takes advantage of a conserved residue in protein tyrosine kinases termed the "gatekeeper," which is usually a bulky amino acid that forms close contact with ATP. A larger space is generated in the kinase active site by mutating this residue to a smaller amino acid, and this pocket can then accommodate an enlarged PP1 analog. Conversely, wild-type kinases present in the cell will not bind the inhibitor because of the steric clash between their bulky gatekeeper residues and the enlarged group in the PP1-derived inhibitor. Because the kinase of interest has to be mutated in this technique, this inhibitor system cannot be used

for direct clinical use; however, it enables analysis of protein function as well as *in vivo* testing to determine the clinical utility of an inhibitor for the kinase of interest.

We used this inhibitor strategy successfully to develop two ZAP-70 analog-sensitive (ZAP-70<sup>AS</sup>) alleles that retains catalytic activity and are also sensitive to the PP1 analog 3-methylbenzyl-pyrazolopyrimidine (3-MB-PP1). We validated the general utility of this membrane-permeable inhibitor system in the Jurkat T cell system. We also show that, in addition to inhibiting TCR antibody-mediated signaling and superantigen stimulation, 3-MB-PP1 inhibits ZAP-70 analog-sensitive expressing cells from responding to a CD28 superagonist, where the role of ZAP-70 has been controversial. As no ZAP-70-specific small molecule inhibitor currently exists, this inhibitor system provides a valuable tool for studying the role of ZAP-70 in T cell signaling and peripheral T cell responses *in vivo* and for dissecting other potential ZAP-70-dependent pathways.

## EXPERIMENTAL PROCEDURES

**Generation of 3-MB-PP1**—3-MB-PP1 was synthesized using a previously described route with slightly modified conditions (18).

**Cell Lines and Transfections**—P116, a ZAP-70-deficient Jurkat-derived T cell line, was obtained from R. Abraham (The Burnham Institute, La Jolla, CA). 293 cells, a kidney epithelial cell line, were obtained from the American Type Culture Collections. Cells were grown as described previously. For transient transfections of ZAP-70 constructs into P116,  $20 \times 10^6$  cells were transfected with 5  $\mu$ g of expression construct and 25  $\mu$ g of empty vector. Cells were transfected as described previously (1). Stable P116 clones expressing ZAP-70 constructs were selected with blasticidin (10  $\mu$ g/ml; Invitrogen). Transient transfections of 293 cells were carried out in 24-well plates using Lipofectamine PLUS reagent (Invitrogen) according to the manufacturer's instructions.

**Plasmids**—A QuikChange site-directed mutagenesis kit (Stratagene) and standard PCR techniques were used to prepare ZAP-70 mutations M414A (ZAP-70<sup>AS1</sup>) and M414A/C405V (ZAP-70<sup>AS2</sup>) in the plasmid pBlueScript (Invitrogen). The mutated versions and wild-type human ZAP-70 were then subcloned into expression vector pEF6.A (Invitrogen) via EcoRI digest. For transfections, the following previously described plasmids were used: Lck (19), FLAG-tagged LAT (20), CD8- $\zeta$  (21), hemagglutinin-tagged rat PLC $\gamma$ 1 (22), and hemagglutinin-tagged murine Tec kinase (22). The diacylglycerol kinase  $\zeta$  (DGK $\zeta$ ) construct was a gift from Gary Koretzky. An enhanced green fluorescent protein plasmid from Invitrogen was used in cotransfection experiments.

**Antibodies**—Ascites of C305, an anti-Jurkat TCR  $\beta$ -chain monoclonal antibody was used for TCR stimulations (23). For stimulation via CD28, the stimulating antibody ANC28.1/5D10 was purchased from Ancell. The following antibodies were used for Western blotting: PLC $\gamma$ 1-pY783, LAT-pY132 (BIO-SOURCE), ZAP-70-pY319, Thr<sup>202</sup>/Tyr<sup>204</sup> for phospho-p44/42 MAPK (Cell Signaling), Lck (1F6 from J. B. Bolen), anti-phosphotyrosine (4G10; Upstate Biotechnology),  $\alpha$ -tubulin (Sigma), LAT (Abcam), SLP-76 (Santa Cruz Biotechnology), active p38, active JNK (Promega), PLC $\gamma$ 1 mixed monoclonal antibodies,

and Tec antibody (Upstate Biotechnology). The following antibodies have been described previously: 2F3.2 (anti-ZAP-70) (24) and 6B10.2 (anti-TCR- $\zeta$ ) (25).

**Flow Cytometry Assays**—For CD69 experiments, 6 h after transfection, cells were washed in RPMI and resuspended at  $1 \times 10^6$  cell/ml. Cells were incubated with DMSO (vehicle) or 3-MB-PP1 and stimulated with anti-TCR antibody (1:1000 C305) or phorbol 12-myristate 13-acetate (PMA) (25 ng/ml). Cells were left overnight at 37 °C with 5% CO<sub>2</sub> and then stained with allophycocyanin-conjugated CD69 (BD Biosciences). Cells were then fixed with BD CytoFix (BD Biosciences), washed twice with FACS buffer, and then permeabilized in Caltag Fix/Perm Medium B and stained for intracellular ZAP-70 (Caltag). For intracellular flow cytometry analysis of pERK, P116 stable lines were incubated with anti-TCR antibody (1:2000 C305) for a 30-min time course, with vehicle (DMSO) or 3-MB-PP1, in a 96-well round bottom plate. Cells were fixed by adding BD CytoFix, washed twice with FACS buffer, and then permeabilized with 100% ice-cold methanol. Cells were then washed three times, stained with primary pERK antibody, washed twice, and then stained with allophycocyanin-conjugated goat anti-rabbit secondary (Jackson ImmunoResearch).

**Measurement of Free Intracellular Calcium**—Free intracellular calcium was measured as described previously (1). Cell were stimulated with anti-TCR antibody (1:2000 C305) in the presence of DMSO, 3-MB-PP1, or PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) (Calbiochem).

**Luciferase Assays**—Stable cell lines were transfected with 20  $\mu$ g of NFAT/AP-1 luciferase and 20  $\mu$ g of vector only. Approximately 16 h after transfection, cells were stimulated with anti-TCR antibody (1:2000 C305) or PMA (50 ng/ml) and ionomycin (1  $\mu$ M). Six hours later, the cells were harvested, lysed, and assayed for luciferase activity using a Mithras LB 940 (Berthold Technologies).

**Superantigen Stimulation and Interleukin-2 (IL-2) Measurement**—Superantigen-loaded antigen-presenting cells were prepared by incubating Raji B cells with 100 ng/ml staphylococcus enterotoxin (Toxin Technology). 10<sup>5</sup> Raji cells ( $\pm$  staphylococcus enterotoxin) were then incubated with an equal number of ZAP-70<sup>WT</sup> or ZAP-70<sup>AS</sup> cells per well in a 96-well plate in a total volume of 0.2 ml of medium (RPMI with 5% fetal calf serum supplemented with penicillin, streptomycin, and glutamine) treated with the indicated concentrations of 3-MB-PP1. After 18 h at 37 °C with 5% CO<sub>2</sub>, the IL-2 concentration was determined by using Human IL-2 ELISA Ready-SET-Go! enzyme-linked immunosorbent assay reagents according to manufacturer's instructions (eBioscience). Cells were treated with PMA (20 ng/ml) plus ionomycin (1  $\mu$ M) as a control.

**Stimulations and Immunoblot Analysis**—For 293 cell experiments, inhibitor or DMSO control was added at the time of transfection, and cells were incubated for 24 h. Cells were then harvested, spun down, and lysed in 2 $\times$  concentrated SDS sample buffer. Lysates were cleared by ultracentrifugation at  $\sim 440,000 \times g$  for 30 min at 24 °C. Supernatants were then collected, and dithiothreitol was added to a final concentration of 1%. P116 stable lines were stimulated ( $25 \times 10^6$ /ml) in RPMI with anti-TCR antibody (1:2000 C305) for the indicated time.

For most experiments, cells were lysed as noted in 2 $\times$  concentrated SDS sample buffer and subjected to ultracentrifugation as described above. For immunoprecipitation experiments,  $10 \times 10^6$  cells were stimulated and lysed in ice-cold lysis buffer (10 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, and a mixture of protease and phosphatase inhibitors).

Postnuclear supernatant was used for immunoprecipitation with antibody bound to protein A or protein G beads (Amersham Biosciences). 2 $\times$  SDS sample buffer containing 2-mercaptoethanol was added to the immunoprecipitated lysates. Samples were analyzed by SDS-PAGE, and immunoblotting was performed using primary and horseradish peroxidase-conjugated secondary antibodies. Proteins were detected by chemiluminescence (Western Lightning) using a Kodak Image Station (Kodak).

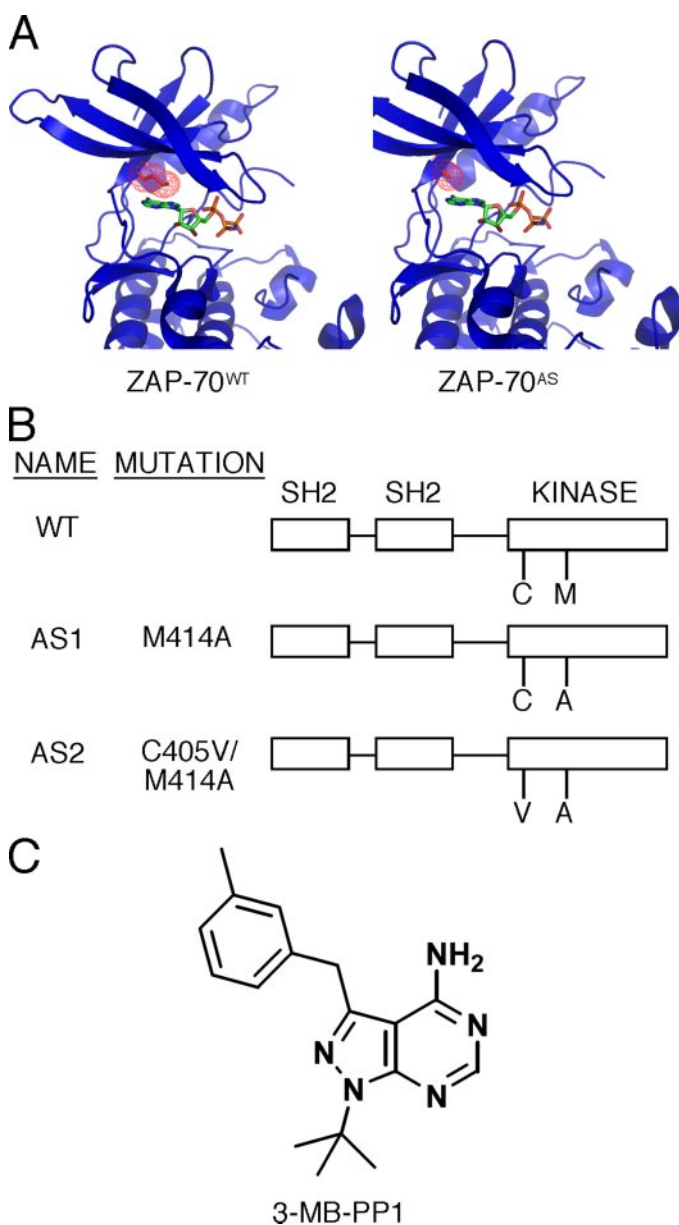
## RESULTS

**Generation of Analog-sensitive ZAP-70 Allele**—We generated an analog-sensitive ZAP-70 allele by mutating the gatekeeper methionine to alanine to create greater access in the ATP pocket of the kinase domain. The resulting M414A mutant is referred to as analog-sensitive allele 1 (AS1) (Fig. 1, A and B). In addition, we introduced a secondary mutation, C405V, in conjunction with M414A, in an attempt to restore stability to the  $\beta$ -sheet in the N-terminal kinase domain, which could potentially be compromised by the parental M414A mutation (26). We termed this mutant AS2 (Fig. 1B). As seen in Fig. 1A, the mutation of the gatekeeper methionine to the smaller alanine residue generates more space in the catalytic domain, allowing room for binding of bulky PP1 analogs. In these studies, we chose to use the PP1 analog 3-MB-PP1 over other PP1 analogs, based upon its potent inhibition of ZAP-70<sup>AS</sup> and the lack of effect on wild-type cells in a screen for T cell activation (data not shown). 3-MB-PP1 contains an extra methylene bridge to the phenyl substituent of PP1 and a 3-methyl substituent that has been shown to be important for reducing binding to wild-type kinases (Fig. 1C) (27).

To test the cellular activity of the ZAP-70<sup>AS</sup> mutants and their sensitivity to 3-MB-PP1, we transiently transfected 293 cells with ZAP-70<sup>WT</sup>, or either one of the ZAP-70<sup>AS</sup> constructs, along with Lck to activate ZAP-70 and the transmembrane adaptor LAT, which serves as a ZAP-70-specific substrate. As shown in Fig. 2A, ZAP-70<sup>WT</sup> was insensitive to the addition of 3 or 6  $\mu$ M 3-MB-PP1. In contrast, both ZAP-70<sup>AS1</sup> and ZAP-70<sup>AS2</sup> were inhibited by 3-MB-PP1 in a dose-dependent manner as measured by decreased LAT phosphorylation. Importantly, the 293 transfection data also showed that the analog-sensitive ZAP-70 exhibited reduced catalytic activity when compared with the wild-type; however, both alleles were able to phosphorylate LAT in the absence of inhibitor. We estimate the analog-sensitive ZAP-70 mutant has an average 2.1-fold reduction in cellular activity relative to wild-type, based upon quantification of the intensity of the total level of ZAP-70 relative to that of the phosphorylated LAT in a series of immunoblots (data not shown).

To further characterize the sensitivity of ZAP-70<sup>AS</sup> to 3-MB-PP1, we transiently transfected the ZAP-70 constructs into the ZAP-70-deficient Jurkat-derived T cell line, P116. P116 fails to





**FIGURE 1. ZAP-70<sup>AS</sup> generation.** *A*, ribbon structure of a portion of the kinase domain of ZAP-70<sup>WT</sup> (left) (Protein Data Bank code 2OZO (35)) and ZAP-70<sup>M414A</sup> (M414A was manually introduced in PyMOL) (right). Gatekeeper residues are represented as red sticks with a meshed surface. An ATP analog, AMPPNP is shown. *B*, diagrammatic representation of ZAP-70<sup>WT</sup> and ZAP-70<sup>AS</sup> constructs. *C*, structure of 3-MB-PP1.

effectively initiate signaling events downstream of ZAP-70, including protein tyrosine phosphorylation, Ca<sup>2+</sup> mobilization, Ras/MAPK activation, NFAT-directed transcription, and expression of a variety of downstream genes such as CD69 (28, 29). We tested the transiently transfected cells for surface CD69 expression, mediated via the Ras/MAPK pathway, after overnight stimulation with an anti-TCR antibody (C305) in the presence or absence of 10  $\mu$ M 3-MB-PP1. Both ZAP-70<sup>WT</sup>- and ZAP-70<sup>AS1</sup>-expressing cells efficiently up-regulated CD69 following TCR stimulation in the presence of vehicle (DMSO). However, the activation of cells expressing ZAP-70<sup>AS1</sup> was markedly impaired in the presence of 3-MB-PP1 (Fig. 2*B*). Additionally, only the highest ZAP-70<sup>AS1</sup>-expressing cells up-

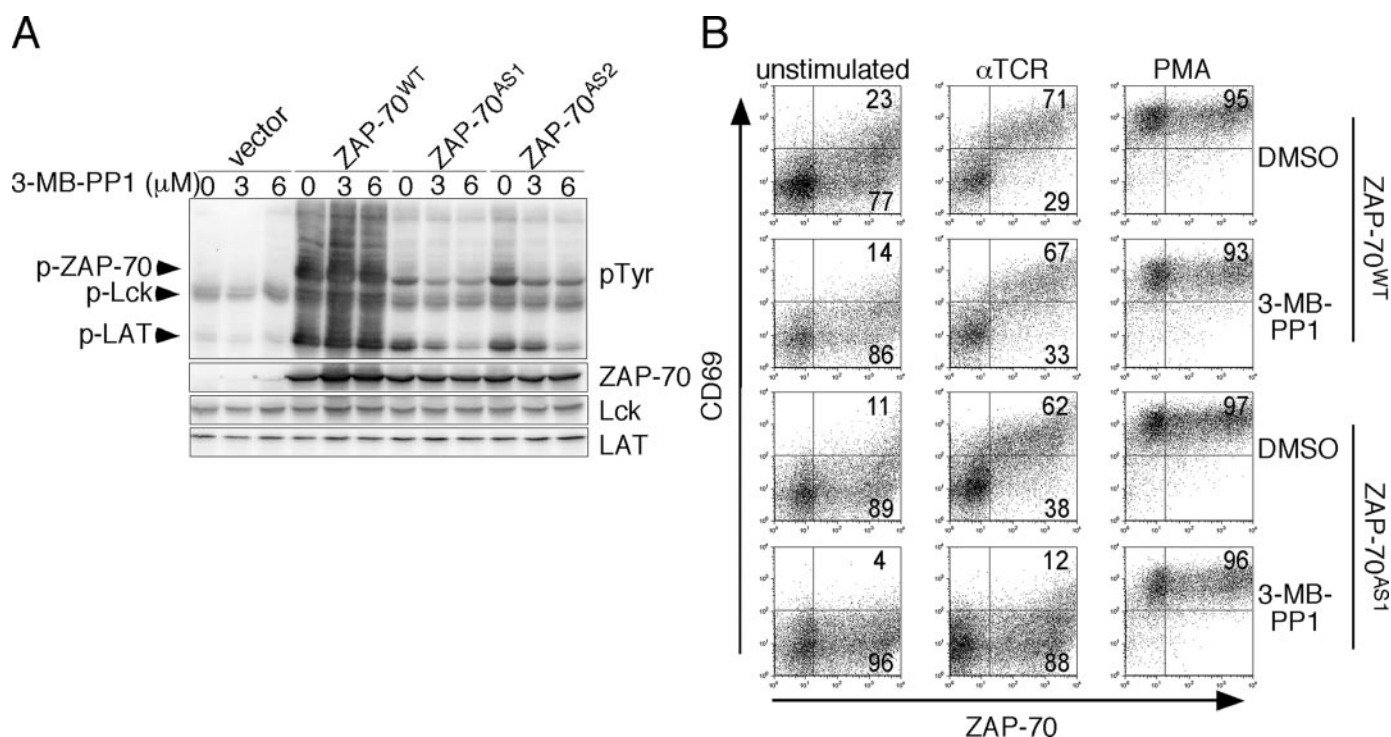
regulated any surface CD69. No 3-MB-PP1-mediated effect was seen with either of the ZAP-70 alleles after PMA stimulation, which bypasses proximal TCR signaling. Thus, the ZAP-70<sup>AS</sup> is functional and is inhibited by 3-MB-PP1 in a T cell system. Moreover, although Lck and many downstream kinases are required for CD69 induction, only the cells expressing the ZAP-70<sup>AS</sup> alleles are sensitive to inhibition by 3-MB-PP1.

One of the principal challenges to developing inhibitor systems is avoiding off-target effects. Therefore, we further tested the specificity of 3-MB-PP1 inhibition by analyzing its effect on other relatively upstream kinase/substrate pairs implicated in TCR signaling in 293 transient transfection assays (Fig. 3). Lck activity was monitored by cotransfecting a CD8- $\zeta$  chimera, which contains the cytoplasmic domain of  $\zeta$  and the extracellular domain of CD8, as a substrate. Tec kinase activity was measured by observing PLC $\gamma$ 1 phosphorylation. Both Lck and Tec kinase activity were only minimally affected even after incubation with high doses (9–10  $\mu$ M) of 3-MB-PP1.

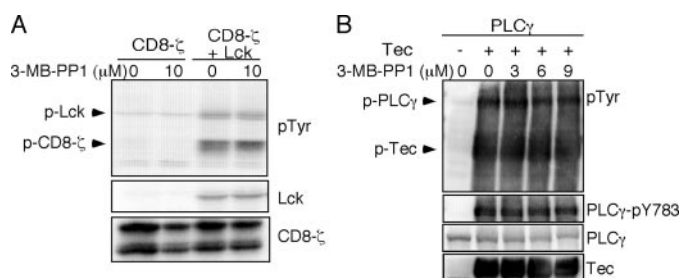
*ZAP-70 Is Required to Both Initiate and Maintain TCR-mediated Increases in Cytoplasmic Free Calcium ([Ca<sup>2+</sup>]<sub>i</sub>)*—ZAP-70-deficient P116 cells fail to increase [Ca<sup>2+</sup>]<sub>i</sub> after TCR stimulation. Therefore, we wanted to test the effect of 3-MB-PP1 on [Ca<sup>2+</sup>]<sub>i</sub>. We first generated P116 clones that stably expressed ZAP-70<sup>WT</sup>, ZAP-70<sup>AS1</sup>, or ZAP-70<sup>AS2</sup> to reconstitute TCR signaling. All clones had similar surface levels of CD3 (supplemental Fig. 1). We focused on four stable lines for these studies. Both of the ZAP-70<sup>WT</sup> clones expressed more ZAP-70 than the parental Jurkat line (supplemental Fig. 1). Therefore, we selected ZAP-70<sup>AS2</sup> and ZAP-70<sup>AS1a</sup>, which express comparable amounts of ZAP-70 to the wild-type clones. In addition, we utilized ZAP-70<sup>AS1b</sup> because it expresses ZAP-70 equivalently to parental Jurkat cells and, therefore, allowed us to rule out any potential artifact of ZAP-70 overexpression.

To test the requirement of ZAP-70 for initiating [Ca<sup>2+</sup>]<sub>i</sub> increases, cells were pretreated with a 6  $\mu$ M dose of 3-MB-PP1 or vehicle for 75 s and then stimulated with anti-TCR antibody. In both ZAP-70<sup>AS1</sup> and ZAP-70<sup>AS2</sup> stably transfected cells, 3-MB-PP1 treatment blocked TCR-mediated signaling, and there was only a negligible effect on ZAP-70<sup>WT</sup> cells (data not shown; Fig. 4*A*). In addition, Src family kinases such as Lck in Jurkat T cells are also required for inducing [Ca<sup>2+</sup>]<sub>i</sub> increases because of their ability to mediate ITAM phosphorylation and ZAP-70 activation. Treatment with the Src family kinase inhibitor PP2 had a comparable effect to that of inhibiting ZAP-70 in both wild-type and mutant stables, whereas only ZAP-70<sup>AS2</sup> was sensitive to blockade by 3-MB-PP1, suggesting a high degree of specificity. There was also no effect of 3-MB-PP1 treatment on ionomycin-dependent calcium release in ZAP-70<sup>WT</sup> or ZAP-70<sup>AS</sup> cells (data not shown).

This block in Ca<sup>2+</sup> signaling was interesting but was anticipated from studies of the parental P116 cells. However, we were more interested in determining the requirement of the ZAP-70 catalytic function for maintaining [Ca<sup>2+</sup>]<sub>i</sub> elevation. The inhibitor system provides a powerful tool for answering such questions because 3-MB-PP1 can be added post-stimulation. To test this idea directly, we treated the stably transfected cells with 3-MB-PP1 after the maximal Ca<sup>2+</sup> response was achieved (75 s



**FIGURE 2. ZAP-70<sup>AS</sup> cellular activity is selectively inhibited by 3-MB-PP1.** *A*, 293 transient transfection of ZAP-70 constructs in the presence or absence of 3-MB-PP1. Cells were lysed in 2× concentrated SDS-PAGE sample buffer and analyzed by immunoblotting with antibody against phosphotyrosine (top panel). Important phospho-species are denoted by arrowheads. Total ZAP-70, Lck, and LAT levels were determined by blotting with specific antibodies. Unless noted otherwise, all experiments shown in Figs. 2–8 are representative of at least three independent experiments. *B*, FACS analysis of ZAP-70 and CD69 expression after stimulation of P116 cells transiently transfected with either ZAP-70<sup>WT</sup> or ZAP-70<sup>AS1</sup>. Six h after transfection, cells were left unstimulated or stimulated with the anti-TCR antibody C305 or with PMA (25 ng/ml) for 16 h in the presence of vehicle (DMSO) or 3-MB-PP1. Cells were stained for surface CD69 and then fixed, permeabilized, and stained for intracellular ZAP-70. The numbers represent the percentage of ZAP-70<sup>+</sup>CD69<sup>+</sup> (upper right) and ZAP-70<sup>+</sup>CD69<sup>−</sup> (lower right). Although ZAP-70 staining was used in the experiment shown here, future experiments confirmed this result using cotransfected green fluorescent protein as a surrogate marker of ZAP-70.



**FIGURE 3. 3-MB-PP1 does not affect Lck or Tec kinase activity.** *A*, 293 transient transfection of Lck and CD8-ζ, serving as the kinase substrate, in the presence or absence of 3-MB-PP1. Cells were lysed as described in the legend for Fig. 2A. *B*, 293 transient transfection of Tec and PLCγ1. Important phospho-species identified by blotting for total phosphotyrosine are denoted by arrowheads. PLCγ1-pY783 was blotted for with a phosphospecific antibody. Total Lck, CD8-ζ, Tec, and PLCγ1 were determined by blotting with specific antibodies. Each panel is representative of two independent experiments.

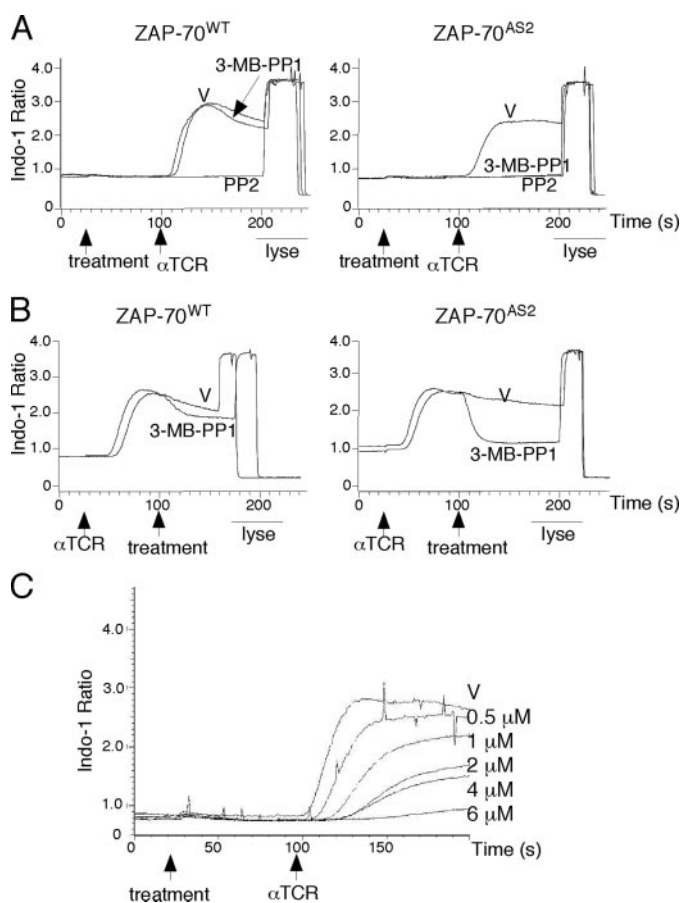
after stimulation). Interestingly, under these conditions, 3-MB-PP1 treatment completely abrogated TCR-mediated  $[Ca^{2+}]_i$  increase in analog-sensitive stables, returning  $[Ca^{2+}]_i$  to base line within 20 s (Fig. 4B). This suggests an ongoing requirement for ZAP-70 catalytic function beyond signal initiation by the TCR. The striking inhibition of calcium flux was also noted in a dose-response analysis to 3-MB-PP1. As shown in Fig. 4C, it is clear that the calcium flux of ZAP-70<sup>AS2</sup> cells is sensitive to even very low doses (including 0.5 and 1.0 μM) of 3-MB-PP1.

The requirement of ZAP-70 for  $[Ca^{2+}]_i$  elevation was striking, but these measurements were made during short

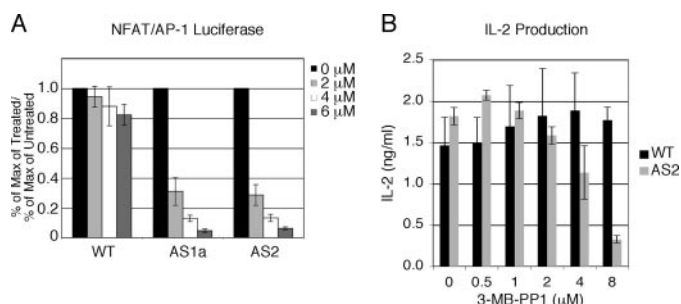
time intervals following the initiation of TCR signaling. Therefore, it was important to monitor NFAT transcriptional events in order to determine the long-term effect of this failure to mobilize intracellular calcium. We transfected a NFAT/AP-1 luciferase reporter into our stable lines and monitored the activity after 6 h of TCR stimulation. As expected from the calcium data, NFAT activity was severely and equivalently diminished by 3-MB-PP1 treatment in both the ZAP-70<sup>AS1a</sup> and ZAP-70<sup>AS2</sup> lines after anti-TCR antibody stimulation (Fig. 5A). In addition, the response was dose-dependent. The highest dose at 6 μM, which effectively blocked calcium responses, inhibited almost all NFAT-driven luciferase activity (~5% remaining activity compared with vehicle treated).

**ZAP-70 Inhibition Suppresses Superantigen-mediated IL-2 Induction**—The inhibition of calcium increases and NFAT-transcription in 3-MB-PP1-treated cells demonstrated the utility of this analog approach and exposed the requirement of ZAP-70 to both initiate and maintain the calcium response after TCR antibody stimulation. However, we also wanted to test the system using a more physiological approach. Therefore, we decided to measure IL-2 production following stimulation with staphylococcus enterotoxin E superantigen-loaded antigen-presenting cells. This stimulation method is more physiological than antibody treatment because it requires antigen-presenting cells and also because humans T cells respond to superantigens in pathologic situations such as toxic shock syn-





**FIGURE 4. ZAP-70 is required for initiating and maintaining TCR-mediated  $\text{Ca}^{2+}$  flux.** Cells were loaded with Indo-1 dye and stimulated with anti-TCR antibody and either pretreated (A) or treated post-maximal  $\text{Ca}^{2+}$  flux (B) with 6  $\mu\text{M}$  3-MB-PP1 or vehicle (V; DMSO). In A, PP2 was added at a final concentration of 20  $\mu\text{M}$ . The experiment in A is representative of multiple independent experiments, but PP2 was added as a control in two experiments. C, ZAP-70<sup>AS2</sup> cells were pretreated with a range of 3-MB-PP1 concentrations as indicated. Data are representative of two experiments.



**FIGURE 5. Inhibition of NFAT transcription and IL-2 production.** A, stable lines were transiently transfected with NFAT/AP-1-luciferase. The cells were then left unstimulated, stimulated with anti-TCR antibody, or stimulated with 50 ng/ml PMA plus 1  $\mu\text{M}$  ionomycin for 6 h at 37 °C and then assayed for luciferase activity. The NFAT response was first calculated as a percentage of the maximum as determined by PMA plus ionomycin treatment. Then the activity was determined for each 3-MB-PP1 concentration relative to untreated (no 3-MB-PP1 or 0  $\mu\text{M}$  3-MB-PP1). Error bars represent the standard deviation of three independent experiments each done in triplicate. B, ZAP-70<sup>WT</sup> and ZAP-70<sup>AS2</sup> cells were incubated with staphylococcus enterotoxin E-loaded antigen-presenting cells for 18 h in the presence of a range of 3-MB-PP1 concentrations before IL-2 concentration was determined by enzyme-linked immunosorbent assay. Error bars represent the standard deviation of triplicate wells in one experiment. Data are representative of two experiments.

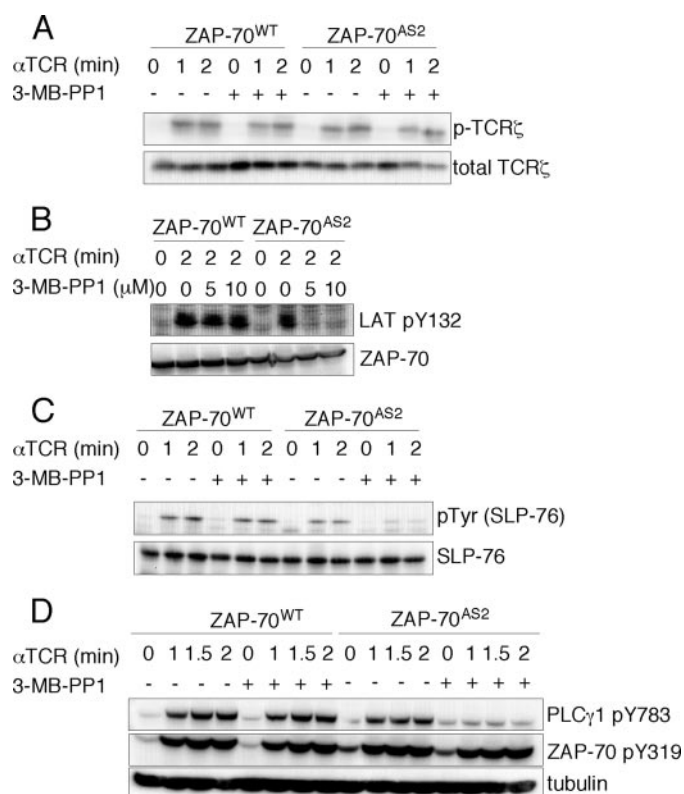
drome or food poisoning. As shown in Fig. 5B, IL-2 production was specifically inhibited in ZAP-70<sup>AS2</sup> inhibitor-treated cells. Thus, our findings show that this analog approach works in a physiologic context and that ZAP-70 is required to mediate a superantigen response to an enterotoxin. Interestingly, the ZAP-70<sup>AS2</sup> cells were less sensitive to 3-MB-PP1 than in the NFAT-transcriptional assay shown in Fig. 5A. IL-2 production is a more integrated response and therefore may be less sensitive to lower levels of ZAP-70 inhibition.

**Decreased Phosphorylation of the LAT-SLP-76-PLC $\gamma$ 1 Signaling upon ZAP-70 Inhibition**—ZAP-70 has been thought to have at least two direct downstream targets, the adaptor proteins LAT and SLP-76. Both LAT and SLP-76 are key signaling adaptors, and cells that lack expression of either protein fail to propagate many of the downstream TCR signals. In ZAP-70-deficient P116, neither LAT nor SLP-76 is efficiently phosphorylated; and, the same cells overexpressing a kinase-inactive form of ZAP-70 also fail to exhibit these phosphorylated substrates (1, 28).

LAT and SLP-76 interact indirectly and are responsible for forming a complex of signaling molecules downstream of the TCR. Phosphorylation of LAT is absolutely required for formation of the complex (30). SLP-76 is recruited to LAT via the Grb2-related adaptor protein, GADS. SLP-76 then recruits a number of important molecules including PLC $\gamma$ 1, guanine nucleotide exchange factor Vav, and the Tec family kinase, interleukin-2-inducible T cell kinase (ITK), the latter two of which appear to require SLP-76 phosphorylation (31). In addition to its indirect interaction with SLP-76 via GADS, LAT also interacts with numerous signaling molecules through its phosphorylated tyrosines, including the adaptor Grb2 (growth factor receptor-bound protein 2) as well as PLC $\gamma$ 1. The formation of a LAT- and SLP-76-containing signalosome serves as a nucleation point for TCR signaling events. Phosphorylation of these adaptors, mediated by ZAP-70, plays a critical role in the formation and functional activity of this signaling complex.

Because phosphorylation of LAT and SLP-76 by ZAP-70 is so critical for initiation of many of the downstream signaling events, we wanted to examine the phosphorylation status of LAT and SLP-76 after treatment with 3-MB-PP1 in the analog-sensitive clones. However, to first control for more proximal events that are not ZAP-70-dependent, we monitored Lck-dependent TCR $\zeta$  phosphorylation in ZAP-70<sup>WT</sup> and ZAP-70<sup>AS2</sup> cells. As seen in Fig. 6A, TCR $\zeta$  phosphorylation was not significantly altered after incubation with the inhibitor in either cell line, thereby reinforcing the idea that 3-MB-PP1 specifically targets ZAP-70<sup>AS</sup>-dependent events. It is important to note that the increase in total TCR $\zeta$  immunoprecipitated from 3-MB-PP1-treated ZAP-70<sup>WT</sup> cells was not reproducible.

We then examined LAT Tyr<sup>132</sup> phosphorylation, because PLC $\gamma$ 1 has been shown to bind to this tyrosine when phosphorylated (32). Once activated, PLC $\gamma$ 1 cleaves phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into diacylglycerol and inositol 1,4,5-trisphosphate (IP<sub>3</sub>), where IP<sub>3</sub> binds receptors on the endoplasmic reticulum that lead to release of  $\text{Ca}^{2+}$  stores. Mutation of Tyr<sup>132</sup> to phenylalanine has been shown to decrease PLC $\gamma$ 1 phosphorylation and its binding to LAT, in addition to diminishing the overall [ $\text{Ca}^{2+}$ ]<sub>i</sub> increase (30, 32). As



**FIGURE 6. Effect of ZAP-70 inhibition on TCR $\zeta$ , LAT, SLP-76, and PLC $\gamma$ 1.** P116 stable lines were incubated with vehicle or 5  $\mu$ M 3-MB-PP1 (unless indicated otherwise) and treated with either anti-TCR antibody or left unstimulated. Inhibitor and stimulus were added concurrently. **A**, after stimulation,  $10 \times 10^6$  cells were lysed in 1% Nonidet P-40 lysis buffer, and TCR $\zeta$  was immunoprecipitated. Immunoprecipitated lysates were immunoblotted for total phosphotyrosine and then stripped and blotted for total TCR $\zeta$ . **B**, cells were lysed in 2 $\times$  concentrated SDS-PAGE sample buffer and analyzed by immunoblotting with phosphotyrosine-specific LAT pY132 and total ZAP-70 antibodies. Approximately  $0.4 \times 10^6$  cell equivalents were loaded onto a SDS-polyacrylamide gel. **C**, after stimulation, SLP-76 was immunoprecipitated as described in **A**. Immunoprecipitated lysates were immunoblotted for total phosphotyrosine and SLP-76. Results are representative of three independent experiments, which were carried out using either ZAP-70<sup>AS2</sup> or ZAP-70<sup>AS1a</sup>. **D**, lysates were prepared as described in **B** and immunoblotted with antibodies specific for PLC $\gamma$ 1 pY783, ZAP-70 pY319, and tubulin.

shown in Fig. 6B, induced phosphorylation of LAT Tyr<sup>132</sup> is markedly inhibited after cotreatment with anti-TCR antibody and 5–10  $\mu$ M 3-MB-PP1. In addition to LAT phosphorylation, total SLP-76 phosphorylation was reduced over a 2-min time course (Fig. 6C). Vehicle-treated ZAP-70<sup>AS2</sup> mutant cell samples often exhibited a somewhat decreased phosphorylation relative to ZAP-70<sup>WT</sup> cells, which we hypothesized was due to the decreased catalytic activity of the analog-sensitive mutant. Despite the decreased magnitude of phosphorylation in the untreated ZAP-70<sup>AS2</sup> cells, the response is still sufficient to generate substantial biochemical and transcriptional responses. Moreover, in very preliminary studies, we have been able to reconstitute T cell development of ZAP-70 null mice with the ZAP-70<sup>AS</sup> clone expressed as a transgene, suggesting that the reduced catalytic activity of the mutant ZAP-70 is not substantially functionally impaired.

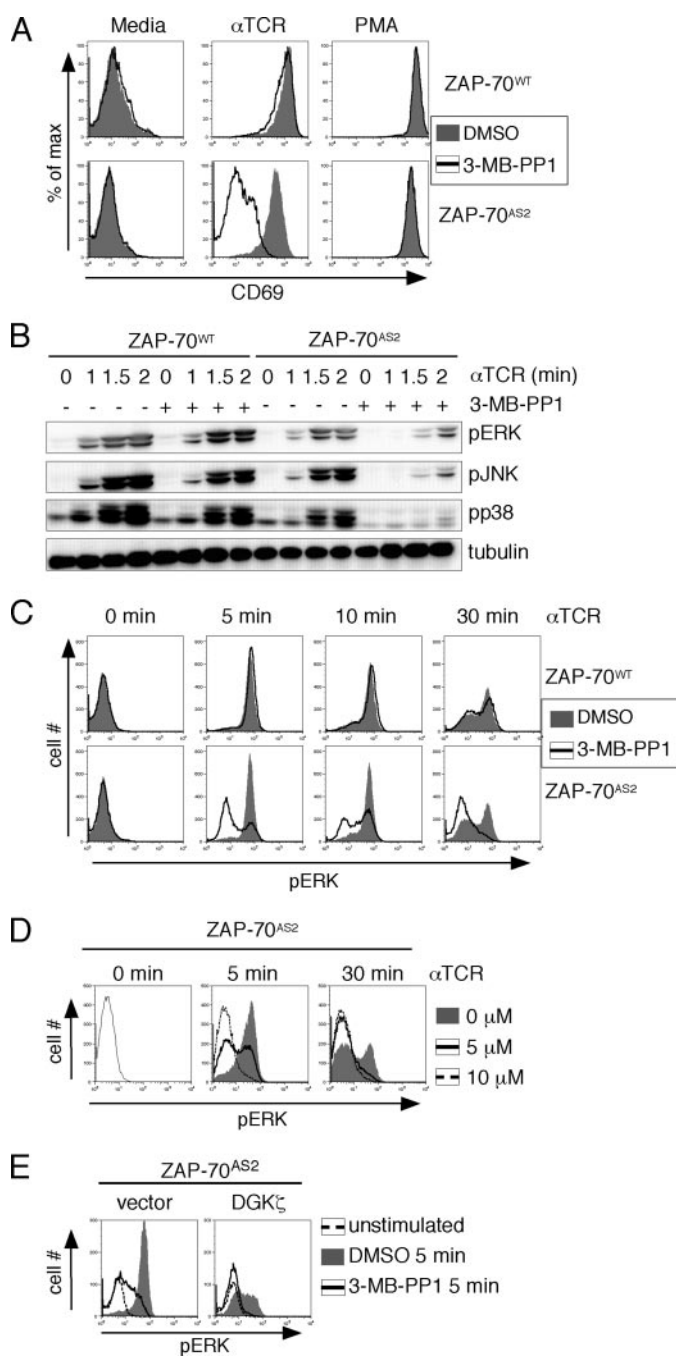
Phosphorylation of PLC $\gamma$ 1 is required for activation of the enzyme. In particular, phosphorylation of Tyr<sup>783</sup> has been shown to facilitate the interaction between Tyr<sup>783</sup> and the C-terminal SH2 domain of PLC $\gamma$ 1, which leads to enzyme acti-

vation (33). Recently, it was demonstrated that SLP-76-bound ITK phosphorylates PLC $\gamma$ 1 on this key residue (34). As shown in Fig. 6D, phosphorylation of Tyr<sup>783</sup> on PLC $\gamma$ 1 was markedly reduced in 3-MB-PP1-treated ZAP-70<sup>AS2</sup> cells. Similar results were observed in ZAP-70<sup>AS1a</sup> and ZAP-70<sup>AS1b</sup> cells (data not shown). The specificity of the 3-MB-PP1 toward ZAP-70<sup>AS</sup> was further emphasized in this experiment by the finding that ZAP-70 Tyr<sup>319</sup> phosphorylation, mediated by Lck, was not affected by the addition of the inhibitor (Fig. 6D). Recent crystallographic studies have shown that the Tyr<sup>319</sup> residue, which is phosphorylated by Lck, is involved in the autoinhibition of ZAP-70 (35). Together, the loss of LAT, SLP-76, and PLC $\gamma$ 1 phosphorylation can account for the marked inhibition we observed in calcium mobilization. We noticed only a modest decrease in total ITK phosphorylation in 3-MB-PP1-treated ZAP-70<sup>AS1b</sup> and ZAP-70<sup>AS2</sup> cells (data not shown). This is in agreement with the fact that Src kinases regulate Tec kinase phosphorylation (36); however, ITK phosphorylation has recently been shown to be defective in SLP-76-deficient cells (34). The remaining SLP-76 phosphorylation in our 3-MB-PP1 cells may be sufficient to support ITK phosphorylation. Thus, these studies indicate that phosphorylation of two of the most important downstream ZAP-70 substrates is impaired upon 3-MB-PP1 treatment of ZAP-70<sup>AS</sup>-expressing cells but that other substrates such as the TCR $\zeta$  chain, ITK, and ZAP-70 itself, which are all phosphorylated by Lck, are not substantially affected.

**Maximal and Persistent Ras/MAPK Phosphorylation Requires ZAP-70 Catalytic Activity**—Given the substantial 3-MB-PP1-mediated inhibition of calcium and PLC $\gamma$ 1 phosphorylation in ZAP-70<sup>AS</sup>-expressing cells, we wanted to examine other PLC $\gamma$ 1-mediated signaling events, particularly activation of the Ras/MAPK pathway via the generation of diacylglycerol. We had already demonstrated during the initial screening of 3-MB-PP1 that inhibitor treatment diminishes CD69 up-regulation, a downstream transcriptional target of Ras activation, in cells transiently transfected with ZAP-70<sup>AS</sup> (Fig. 2B). We confirmed this result in stable lines (Fig. 7A). In the presence of 3-MB-PP1, all ZAP-70<sup>AS</sup> lines failed to maximally up-regulate CD69 after 16 h of stimulation with anti-TCR antibody. However, there was no effect on PMA-mediated activation, which bypasses proximal TCR signaling (Fig. 7A and data not shown).

The failure to express surface CD69 in 3-MB-PP1-treated analog-sensitive cells was expected to correlate with the lack of activation of the MAPK, ERK. We used MAPK phosphorylation as an indirect marker of activation. However, we repeatedly noticed that we had substantial, albeit diminished and delayed, ERK phosphorylation at 2 min in 3-MB-PP1-treated ZAP-70<sup>AS</sup> cells. It became clear, both by Western blotting and intracellular staining of pERK using flow cytometry, that ERK phosphorylation was delayed and not maintainable in the inhibitor-treated ZAP-70<sup>AS</sup> cells (Fig. 7, B and C). Analysis of ERK phosphorylation by the use of a phosphospecific ERK antibody on whole cell lysates examined by SDS-PAGE showed clear induction of ERK phosphorylation by 1 min in untreated ZAP-70<sup>AS2</sup> cells and ZAP-70<sup>WT</sup> cells, but there was no detectable pERK phosphorylation above basal levels in inhibitor-treated





**FIGURE 7. ZAP-70 catalytic activity is required for complete and persistent Ras/MAPK phosphorylation.** *A*, FACS analysis of CD69 expression after stimulating stable lines for 16 h with or without anti-TCR antibody or PMA (25 ng/ml) in the presence or absence of 10  $\mu$ M 3-MB-PP1. *B*, P116 stable lines were incubated for a 2-min time course with either vehicle or 5  $\mu$ M 3-MB-PP1 and treated with anti-TCR antibody or left unstimulated. Inhibitor and stimulus were added concurrently. Cells were lysed in 2 $\times$  concentrated SDS sample buffer and blotted for pERK, pJNK, pp38, and tubulin. *C*, P116 stable lines were incubated with anti-TCR antibody over a 30-min time course with vehicle (filled histogram) or 5  $\mu$ M 3-MB-PP1 (open histogram). At the appropriate time points, cells were fixed, permeabilized, and stained intracellularly with phosphospecific ERK antibody. *D*, same as in *C* except cells were treated with either vehicle (filled), 5  $\mu$ M 3-MB-PP1 (solid line), or 10  $\mu$ M 3-MB-PP1 (dotted line). *E*, ZAP-70<sup>AS2</sup> was transiently transfected with 20  $\mu$ g of green fluorescent protein vector and either empty vector (left) or 20  $\mu$ g of DGK $\zeta$  (right) and stimulated and stained as described in *C*. Transfected cells were identified by gating on GFP<sup>+</sup> cells. The histograms are labeled as follows: unstimulated (dotted line); 5 min C305 + DMSO (filled); 5 min C305 + 5  $\mu$ M 3-MB-PP1 (solid line). Data are representative of two experiments.

ZAP-70<sup>AS2</sup> cells until 1.5–2 min after anti-TCR antibody stimulation (Fig. 7*B*). This pattern of ERK phosphorylation held true for other MAPK members, p38 and JNK, albeit basal levels of pp38 were also reduced. We were especially interested in p38 induction given the direct role that ZAP-70 is thought to play in activating p38 in T cells (37). Analysis of pERK by intracellular flow cytometry showed similar results at the early time points, with only a small percentage of the cells containing phosphorylated ERK 5 min post-stimulation (Fig. 7*C*). This minimal phosphorylation was not sustained. By 30 min, ERK phosphorylation in the analog-sensitive cells returned to base line, whereas untreated cells were still substantially positive for pERK (Fig. 7*C*). There was no difference in ERK phosphorylation between ZAP-70<sup>WT</sup> and ZAP-70<sup>AS2</sup> cells treated with vehicle.

Although we were able effectively to eliminate increases in  $[Ca^{2+}]_i$  with 3-MB-PP1, treatment with the same inhibitor still generated a substantial, yet abbreviated, pattern of ERK phosphorylation. This discrepancy between the  $Ca^{2+}$  data and the ERK phosphorylation was interesting because it either suggested that the level of PLC $\gamma$ 1 activity post-treatment was sufficient for some ERK phosphorylation but not  $Ca^{2+}$  mobilization or that the residual ERK phosphorylation was PLC $\gamma$ 1-independent. Such a PLC $\gamma$ 1-independent pathway could be mediated by Grb2-SOS recruitment to the phosphorylated TCR  $\zeta$ -chain, as had been suggested previously (38), although Grb2-SOS is also known to be recruited to phosphorylated LAT (39, 40). Interestingly, the existence of a ZAP-70-independent pathway leading to ERK phosphorylation has been reported. In these studies, P116 cells repeatedly exhibited delayed and transient ERK phosphorylation; however, this required the use of high levels of CD3 cross-linking (40, 41).

We used two approaches to test whether residual PLC $\gamma$ 1 activity was the mediator of the ERK activation. First, we increased the concentration of 3-MB-PP1 to 10  $\mu$ M and found that this eliminated the remaining ERK phosphorylation (Fig. 7*D*). Second, we transiently overexpressed diacylglycerol kinase  $\zeta$  (DGK $\zeta$ ), which converts diacylglycerol into phosphatidic acid by phosphorylation of the free hydroxyl group. We hypothesized that if the remaining ERK phosphorylation was PLC $\gamma$ 1-dependent, then overexpression of DGK $\zeta$  would eliminate the remaining ERK in the 3-MB-PP1-treated ZAP-70<sup>AS</sup> cells. Overexpression of DGK $\zeta$  did, in fact, eliminate the remaining ERK phosphorylation at 5 min post-stimulation in the presence of 3-MB-PP1, thus providing additional evidence that the ERK phosphorylation appears to be PLC $\gamma$ 1-dependent (Fig. 7*E*). Therefore, although  $[Ca^{2+}]_i$  increases were severely inhibited by 3-MB-PP1 treatment, similar doses of the inhibitor still allowed for enough PLC $\gamma$ 1 activity to initiate the MAPK cascade to some extent. However, this level of residual ERK phosphorylation was not sufficient to maintain long-term phosphorylation or promote maximal transcriptional changes. These data are consistent with the notion that the mechanisms to amplify or produce positive feedback on ERK activation are more sensitive to low levels of PLC $\gamma$ 1 activity than the mechanisms that regulate the calcium pathway.

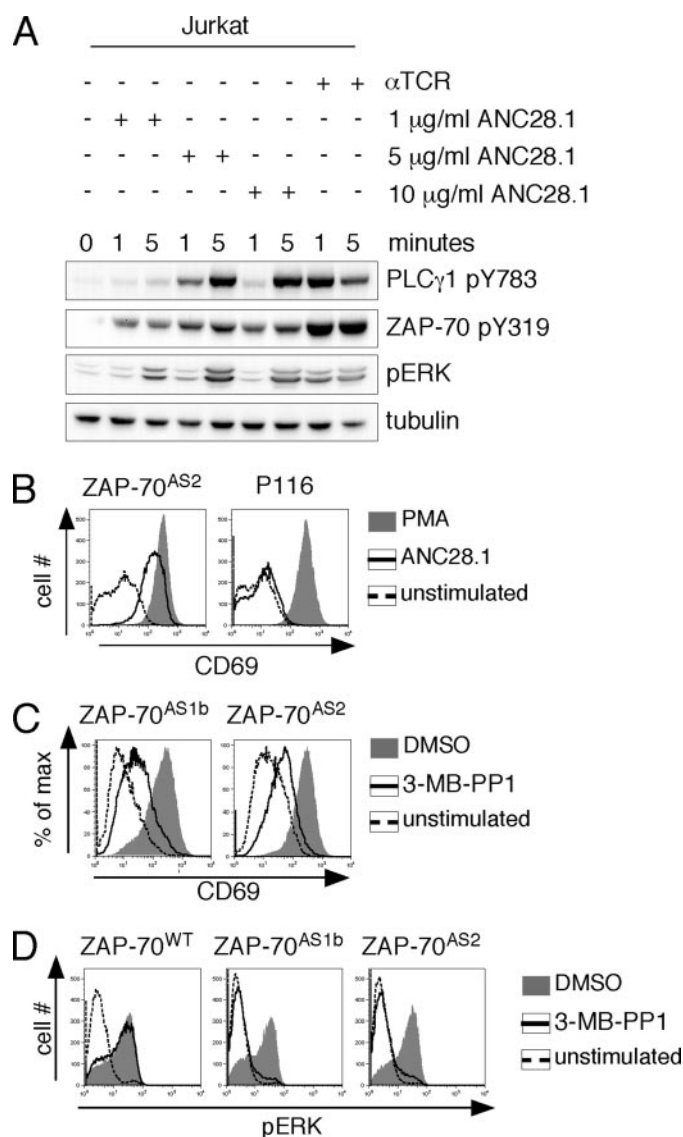
**Superagonist CD28 Antibody Is ZAP-70-dependent**—One of the benefits of utilizing a small molecule inhibitor system is to



be able to test the role of the given protein in a variety of pathways where its function is controversial or not easily determined. For instance, ZAP-70 is well known for its critical role in proximal TCR signaling, but its function in CD28 superagonist signaling has been unclear. Conventional anti-CD28 antibodies require TCR coengagement to induce proliferation, but there is a class of anti-CD28 antibodies, termed stimulating or superagonists, that can stimulate T cell IL-2 production and proliferation in the absence of TCR antibodies. One human CD28 superagonist was recently utilized in a clinical trial because it had been shown to induce development of regulatory T cells, but when administered to the healthy volunteers in a phase I clinical trial, the superagonist induced an unanticipated cytokine storm followed by multiorgan failure in volunteer subjects participating in this trial (42). Thus, understanding the biochemical mechanisms by which CD28 superagonist antibodies function might help to prevent such unforeseen, disastrous complications with immunologically active agents in the future.

Although the use of stimulating anti-CD28 antibody has been shown to activate some common events downstream of the TCR, including SLP-76 and Vav phosphorylation, all TCR-mediated pathways are not induced (43). Moreover, the role of ZAP-70 in this process has been controversial. Initially, it was assumed that ZAP-70 did not function in superagonist stimulation because ZAP-70 did not appear to be phosphorylated after stimulation in primary rat cells (44). However, although a more recent study also did not observe ZAP-70 phosphorylation, the same group found that overexpression of a dominant negative ZAP-70 construct resulted in inhibition of the anti-CD28-induced IL-2 production, thus suggesting an important yet unappreciated role for ZAP-70 in this signaling process (43). Therefore, given the controversial role for ZAP-70 signaling in this signaling cascade and the importance of understanding how superagonists function, we decided to utilize our inhibitor system to dissect the role of this kinase in superagonist stimulation.

Previous researchers had not observed ZAP-70 phosphorylation following CD28 superagonist stimulation, but the stimulating CD28 AN28.1/5D10 antibody induced detectable ZAP-70 Tyr<sup>319</sup> phosphorylation after a 1-min stimulation of Jurkat T cells, albeit at a substantially lower level than anti-TCR-stimulated cells (Fig. 8A). Interestingly, as has been reported previously, the same cells induced substantial PLC $\gamma$ 1 Tyr<sup>783</sup> phosphorylation, with delayed kinetics when compared with TCR stimulation. ERK phosphorylation was also evident in these cells. Therefore, the presence of ZAP-70 phosphorylation in these cells suggests that ZAP-70 could play a role in the signaling cascade. To explore this idea further, we first used P116 to see if the cells could be activated by ANC28.1 stimulation. Fig. 8A demonstrates that superagonist stimulation induces pERK, so we decided to monitor CD69 as a readout for ANC28.1-mediated Ras activation, which has been reported previously to be induced in superagonist-treated cells (44). Although ZAP-70<sup>AS2</sup> cells clearly up-regulated CD69 after overnight stimulation with soluble ANC28.1 at 1  $\mu$ g/ml, P116 failed to induce surface CD69 expression (Fig. 8B). Therefore, ZAP-70 appears to be required for ANC28.1 stimulation.



**FIGURE 8. ZAP-70 is required for T cell activation mediated by a stimulating CD28 antibody.** A, Jurkat cells were stimulated for 1 or 5 min with either anti-TCR antibody or a final concentration of 1, 5, or 10  $\mu$ g/ml ANC28.1/5D10 antibody at 37 °C. Cells were lysed in 2 $\times$  concentrated SDS sample buffer and analyzed by immunoblotting for PLC $\gamma$ 1 pTyr<sup>783</sup>, ZAP-70 pTyr<sup>319</sup>, pERK, and tubulin. Data are representative of two independent experiments. B, FACS analysis of CD69 expression after stimulating ZAP-70<sup>AS2</sup> or P116 for 16 h with ANC28.1 antibody (1  $\mu$ g/ml) or PMA (25 ng/ml). The histograms are labeled as follows: unstimulated (dotted line); ANC28.1 (solid line); PMA (filled). Data are representative of two independent experiments. C, CD69 analysis of ZAP-70<sup>AS1b</sup> and ZAP-70<sup>AS2</sup> after ANC28.1 antibody (1  $\mu$ g/ml) stimulation with or without 5  $\mu$ M 3-MB-PP1. The histograms are labeled as follows: unstimulated (dotted line); ANC28.1 + vehicle (filled); ANC28.1 + 3-MB-PP1 (solid line). D, pERK analysis similar to that in Fig. 7C, except cells were stimulated with ANC28.1 antibody (1  $\mu$ g/ml) for 5 min with or without 5  $\mu$ M 3-MB-PP1. Histograms are labeled as described in C. Data are representative of two independent experiments.

The induction of ZAP-70 phosphorylation in Jurkat and the failure of P116 to be activated by ANC28.1 clearly suggest that stimulating CD28 antibody treatment requires ZAP-70. However, previous research by Dennehy *et al.* (43) suggests that only tonic signaling via ZAP-70 might be required to prime the system. This hypothesis cannot be tested in P116 cells, which do not express ZAP-70 and thus would not exhibit ZAP-70-dependent tonic signals. The best way to test this hypothesis is

with a small molecule inhibitor; therefore, we treated our ZAP-70<sup>AS</sup> cells with 3-MB-PP1 concurrently with 1  $\mu$ g/ml ANC28.1 antibody. This treatment inhibited CD69 up-regulation and pERK phosphorylation in two analog-sensitive lines but had no effect on ZAP-70<sup>WT</sup> cells (Fig. 8, C and D). Thus, the use of 3-MB-PP1 and the ZAP-70<sup>AS</sup>-expressing cells demonstrates the requirement of ZAP-70 in CD28 superagonist-mediated signaling as well as the utility of having an inhibitor system to study the role of ZAP-70.

### DISCUSSION

We successfully generated two ZAP-70 alleles that retain kinase activity but are sensitive to inhibition by the PP1 analog 3-MB-PP1. In both 293 and P116 cells, ZAP-70<sup>AS</sup> reconstitutes ZAP-70 functions but is inhibitable by the addition of 3-MB-PP1 in a dose-dependent fashion. This inhibition of ZAP-70 function ablates  $[Ca^{2+}]_i$  increases and NFAT/AP-1 activity. Failure to initiate or maintain calcium mobilization was associated with and a likely consequence of the inhibition of phosphorylation of the key ZAP-70 adaptor targets, LAT and SLP-76. Without the phosphorylation of those important adaptor molecules, PLC $\gamma$ 1 cannot be recruited to the membrane, phosphorylated, or activated properly to generate the IP<sub>3</sub> required for  $[Ca^{2+}]_i$  increase.

The physiological relevance of this approach was, in part, verified by the ability of 3-MB-PP1 to inhibit IL-2 production in superantigen-stimulated ZAP-70<sup>AS</sup> cells. Interestingly, although IL-2 production was inhibited, it was relatively insensitive to lower concentrations of 3-MB-PP1 as compared with other readouts such as calcium flux. This differential sensitivity in the various assays might be due to the difference in stimulations but also may be the result of the former being a more integrated response. Overall, we found that the half-maximal inhibitory concentration (IC<sub>50</sub>) varied among different readouts used throughout this study, although it was consistently within the range of 1 to 10  $\mu$ M. Such differences in the IC<sub>50</sub> between different functional readouts have been reported for other kinases (45).

Although phosphorylation of Tyr<sup>783</sup> on PLC $\gamma$ 1 was not readily inducible nor were elevations of  $[Ca^{2+}]_i$  detectable following 3-MB-PP1 treatment of ZAP-70<sup>AS</sup> cells, we consistently observed evidence of ERK phosphorylation in a percentage of these cells. The level of ERK phosphorylation was delayed, reduced in magnitude, and not sustained when compared with ZAP-70<sup>WT</sup> or untreated ZAP-70<sup>AS</sup> cells. Nonetheless, this diminished response was observed reproducibly. It had been reported previously that ZAP-70-deficient cells, P116, are capable of activating ERK, thus providing support for a ZAP-70-independent mechanism (40, 41). However, such ZAP-70-independent activation required high levels of TCR stimulation. It was also plausible to consider a possible PLC $\gamma$ 1-independent mechanism, as Grb2-SOS have been reported to interact with the phosphorylated TCR $\zeta$  chain (38) and also with phosphorylated LAT (40). Thus, there could be separate ZAP-70- and PLC $\gamma$ 1-independent mechanisms leading to the activation of the Ras/MAPK pathway. However, when we increased the dose of 3-MB-PP1 or, more importantly, transiently transfected DGK $\zeta$  to convert the remaining diacylglycerol into phosphatidic acid, pERK was no longer induced. This suggests that the remaining ERK phosphorylation is still PLC $\gamma$ 1- and ZAP-70-dependent. It remains unclear why 3-MB-PP1 completely inhibited calcium mobilization but not ERK phosphorylation. The experiments were performed under different conditions, with the cells being suspended in a larger volume in the  $Ca^{2+}$  assay than in the pERK experiment. This larger volume increases the ratio of drug to cell even though similar concentrations of 3-MB-PP1 were used in the two different experiments. In addition, given the delay in ERK phosphorylation in 3-MB-PP1-treated ZAP-70<sup>AS</sup> cells, we hypothesized that a positive feedback loop over ERK is strong enough to support transient ERK phosphorylation in these cells. This positive feedback could involve the functional amplification loop of the two guanine nucleotide exchange factors, RasGRP and SOS, that we have described previously (46). Thus, our studies herein support the notion that Grb2-SOS plays a less important role than RasGRP in TCR signaling leading to Ras activation.

ZAP-70 not only has kinase function but also has been shown to serve as an adaptor for many proteins, including Lck, Vav, c-Cbl, and Crk, via its phosphorylated tyrosine residues. One of the outstanding questions has been: what downstream TCR signaling functions is ZAP-70 able to fulfill as an adaptor in the absence of its catalytic function? Following the addition of 3-MB-PP1 to ZAP-70<sup>AS</sup> stable cell lines, we did not observe that any substantial TCR signaling functions were maintained. However, we did not determine whether the association of ZAP-70 with the other proteins was still inducible. Our laboratory had previously reported that the deletion of the region between the second SH2 domain and kinase domain of ZAP-70, termed interdomain B, which contains the key tyrosines that are known to interact with other proteins when phosphorylated, does not eliminate kinase activity (47). In addition, point mutations of tyrosines 315 and 319 to alanine or glutamic acid do not ablate kinase activity (1). The recently solved crystal structure of ZAP-70 supports a role for the interdomain B tyrosines in maintaining ZAP-70 in an inactive and closed conformation when unphosphorylated (35). Upon phosphorylation of ZAP-70 by Lck, the former kinase can adopt an active and open conformation. Thus, although biochemical evidence has shown that the ZAP-70 tyrosines promote binding with other important signaling molecules, these events are clearly not sufficient for substantial downstream signaling in the absence of ZAP-70 catalytic activity. Overall, these findings allow for the possibility that the role of ZAP-70 as an adapter might be significant only in the context of a catalytically active protein kinase.

One of the benefits of using a small molecule inhibitor is to be able to study an enzymatic role in a process where having a genetically deficient system is undesirable. We have demonstrated here a critical role for ZAP-70 in superagonist CD28 signaling. Although previous researchers did not observe ZAP-70 phosphorylation upon superagonist stimulation, we were able to detect induction of ZAP-70 phosphorylation, albeit weaker than after TCR antibody stimulation. Recently, a role for ZAP-70 has been implicated in superagonist stimulation, but it was hypothesized that only tonic signaling is required (43). Our data using 3-MB-PP1 and analog-sensitive

tidic acid, pERK was no longer induced. This suggests that the remaining ERK phosphorylation is still PLC $\gamma$ 1- and ZAP-70-dependent. It remains unclear why 3-MB-PP1 completely inhibited calcium mobilization but not ERK phosphorylation. The experiments were performed under different conditions, with the cells being suspended in a larger volume in the  $Ca^{2+}$  assay than in the pERK experiment. This larger volume increases the ratio of drug to cell even though similar concentrations of 3-MB-PP1 were used in the two different experiments. In addition, given the delay in ERK phosphorylation in 3-MB-PP1-treated ZAP-70<sup>AS</sup> cells, we hypothesized that a positive feedback loop over ERK is strong enough to support transient ERK phosphorylation in these cells. This positive feedback could involve the functional amplification loop of the two guanine nucleotide exchange factors, RasGRP and SOS, that we have described previously (46). Thus, our studies herein support the notion that Grb2-SOS plays a less important role than RasGRP in TCR signaling leading to Ras activation.



ZAP-70 alleles suggest that ZAP-70 is actively required in this signaling cascade. Although the level of ZAP-70 phosphorylation is lower following stimulation with the CD28 superagonist, the induced signaling events appear to be ZAP-70-dependent, suggesting the presence of an amplification loop downstream of ZAP-70. It is unclear why we observed phosphorylation of ZAP-70, when Dennehy *et al.* (43, 44) did not observe it in either rat primary T cells or Jurkat cells. The differences in stimulation conditions and blotting antibodies could play a role. The stimulating CD28 antibody used in our current experiment was also different than the one used in those previous studies. Overall, these data support the general utility of this ZAP-70 inhibitor system and the general benefit of using multiple biological methods to dissect protein function.

Protein kinases are important targets of drug development. However, broad expression of many of these enzymes makes them unappealing targets because inhibition of the kinase, when outside of the disease context, can lead to undesirable side effects. ZAP-70 is an attractive target because it is expressed predominantly in T cells. In situations where overactive T cells are a substantial component of disease, such as in autoimmune diseases or transplantation, targeting ZAP-70 could provide a means to target the T cells while avoiding adverse effects on other cells. The system that we have generated for analyzing the effect of ZAP-70 inhibition will allow us to identify the utility of a ZAP-70 inhibitor in preclinical models in mice that we have recently reconstituted with the analog-sensitive allele of ZAP-70. Importantly, in preliminary studies, we have confirmed that peripheral T cells isolated *ex vivo* from the analog-sensitive transgenic animals are uniquely susceptible to inhibition by 3-MB-PP1, thus validating an *in vivo* system for our future studies. These studies together with crystal structures of the intact autoinhibited ZAP-70 protein and the activated isolated kinase domain may help in the development of a clinical ZAP-70 inhibitor (35, 48).

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