The Src Family Kinase Fyn Mediates Signals Induced by TCR Antagonists

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FcR nonbinding anti-CD3 mAbs elicit partial TCR signaling that leads to T cell unresponsiveness and tolerance in vivo. In this study, the membrane-proximal events that promote T cell inactivation by FcR nonbinding anti-CD3 mAbs were examined. In the context of FcR nonbinding anti-CD3, TCR complexes did not aggregate and failed to translocate into glycolipid-enriched membrane microdomains. Furthermore, FcR nonbinding anti-CD3 mAbs induced tyrosine phosphorylation of the Fyn substrate Cbl, but not the ZAP-70 substrate linker for activation of T cells. Overexpression of Fyn, but not Lck, restored the mitogenicity of FcR nonbinding anti-CD3 in primary T cells. Taken together, these results suggest that Fyn mediates the partial signaling induced by TCR antagonists. The Journal of Immunology, 2002, 168: 4480–4487.

Activation of T cells requires multivalent engagement and aggregation of the TCR/CD3 complex. Under physiological conditions, this is accomplished by multivalent interactions of the TCR with MHC/peptide complexes expressed on APCs. These conditions can be mimicked by anti-TCR/CD3 mAbs that mediate cross-linking of the TCR at the interface of FcR-bearing cells. TCR cross-linking leads to a cascade of intracellular biochemical events that promote cell division and cytokine production.

In vitro, bivalent, FcR nonbinding forms of anti-CD3 mAbs fail to promote TCR aggregation or activate naive and primary cells. The altered anti-CD3 mAbs induce unresponsiveness of proinflammatory Th1 cells and deviation of undifferentiated T cells toward an IL-4-producing Th2 phenotype (1–3). Moreover, in vivo, FcR nonbinding anti-CD3 therapy prolongs graft survival and effectively blocks autoimmunity in murine models (4, 5). Additionally, in a recent clinical trial, humanized forms of FcR nonbinding anti-CD3 mAbs have shown promise in treating transplant rejection without eliciting systemic cytokine release and first dose toxicity often observed with conventional FcR-binding Abs (6). Thus, FcR nonbinding anti-CD3 mAbs not only are a useful reagent in studying T cell activation, anergy induction, and differentiation, but are superior immunosuppressive drugs that have potential in treating autoimmune diseases and transplant rejection.

One of the earliest signaling events induced after multivalent cross-linking of the TCR/CD3 complex by Ag or anti-CD3 mAbs is the triggering of a signaling cascade resulting in the phosphorylation of the CD3ε, -γ, -δ and -ζ chains and the recruitment and activation of cytoplasmic signaling molecules such as tyrosine kinase ζ-associated protein 70 kDa (ZAP-70).3 These membrane-proximal changes lead to an increase in intracellular calcium, activation of mitogen-activated protein kinase cascades, and transcriptional activation of proteins essential for cellular expansion (7). Previous studies have shown that the treatment of cells with FcR nonbinding anti-CD3 mAbs results in a partial TCR signaling pattern similar to that observed in T cells activated with altered peptide ligands (APL) (1). The partial signaling pattern was characterized by incomplete phosphorylation of TCR ζ, recruitment of ZAP-70 without its subsequent activation by phosphorylation, attenuated intracellular calcium response, and lack of mitogen-activated protein kinase activation.

Lck and Fyn are the two most abundantly expressed Src family kinases in T cells and share a high degree of sequence homology (8, 9). Both kinases can mediate phosphorylation of TCR/CD3 subunits and ZAP-70 when expressed in heterologous systems. Therefore, it is widely accepted that the two kinases provide critical functions in TCR-mediated signal transduction. Both Lck and Fyn are enriched within the glycolipid-enriched membrane microdomains (GEMs). The GEMs have been reported to be integral to TCR signal transduction (10–13). Upon T cell activation, TCR/CD3, ZAP-70, SLP-76, and phospholipase Cγ1 are found in the GEMs in their highly phosphorylated, active forms. The contribution of GEMs to T cell activation is further supported by the observation that chemical disruption of GEMs resulted in a block of TCR-induced calcium influx. Thus, the outcome of TCR-mediated signal transduction most likely depends on the spatial relationship of the TCR/CD3 complex with the GEMs and Src family kinases. However, the functions of Lck and Fyn are distinct. Lck-deficient mice display a profound defect in thymocyte development, which leads to almost complete absence of peripheral T cells (14), whereas Fyn-deficient mice have a relatively normal peripheral T cell compartment despite an early signaling defect in their immature thymocytes (15, 16). Additionally, the two kinases localize to distinct subcellular compartments. Lck is found mostly at the plasma membrane, and Fyn is associated with microtubules and the cytoplasmic membrane (17). Finally, Lck associates with co-receptors CD4 or CD8 (18–20) and is recruited to the TCR/CD3 complex after T cell activation. In contrast, a small fraction of Fyn...
is constitutively associated with the TCR/CD3 complex in resting T cells (21, 22).

In this study, we examined the effects of bivalent anti-CD3 mAb-mediated TCR signal transduction, GEM formation, and the activation of Lck and Fyn. The results demonstrate that FcR nonbinding anti-CD3 mAbs fail to maximally activate Fyn and Lck or to induce the translocation of the TCR/CD3 complex into the GEMs. However, tyrosine phosphorylation of an in vivo Fyn substrate, Cbl, is induced by FcR nonbinding anti-CD3, suggesting that Fyn is responsible for the partial signal transduction induced by FcR nonbinding anti-CD3 mAbs. In support of this hypothesis, overexpression of Fyn but not Lck reconstituted proliferation of primary T cells to FcR nonbinding anti-CD3. Based on these findings, we propose that TCR antagonists induce a tolerogenic signal as a consequence of Fyn-dependent partial phosphorylation of the TCR/CD3 complex in the absence of recruitment into the kinase-rich GEMs.

Materials and Methods
Mice
Six- to 8-wk-old BALB/c and C57BL/6 mice were purchased from Frederick Cancer Research Institute Laboratories (Frederick, MD) and maintained at the University of Chicago (Chicago, IL) and the University of California (San Francisco, CA). Fyn-deficient mice backcrossed four generations to C57BL/6 were provided by Dr. A. DeFranco at the University of California, San Francisco.

Reagents
Abs used in this study were as follows: anti-CD1 (145-2C11), goat anti-mouse IgG3 (Sigma-Aldrich, St. Louis, MO), goat anti-hamster IgG (Cappel, West Chester, PA), and anti-CD28 (PV-1; Dr. C. June, University of Pennsylvania, Philadelphia, PA). The FcR nonbinding anti-CD3-fos F(ab’), and the anti-CD3-fos × anti-CD4-jun bisspecific F(ab’-Zipper) Ab were developed and provided by Dr. H. Palmer, Basel Institute of Immunology, Basel, Switzerland, mouse anti-enhanced green fluorescent protein (eGFP) mAb (clone F56-6A1.2.6), anti-MHC I (clone 145-2C11), has identical functional activities as its FcR nonbinding counterpart (145-2C11; 1).

T cells were generated by stimulating C57BL/6 T cells in vitro with anti-CD3 mAb (clone F56-6A1.2.6), anti-CD28 (PV-1; 1), and 10 U/ml recombinant human IL-2. After 4 days of expansion, cells were harvested and resuspended at 2 × 10^8 cells/ml for biochemical analysis.

T cell stimulation for biochemical analysis
Naive or primary CD4^+ T cells from BALB/c, C57BL/6, or Fyn-deficient mice were used in all experiments except for Fig. 1, in which the OVA-IgG3 was conjugated cholera toxin. Mice were used in all experiments except for Fig. 1, in which the OVA-activated with anti-CD3 mAb, 145-2C11, with or without a secondary conjugated cholera toxin

Fraction of cell lysate by step density sucrose gradient
An aliquot of 2 × 10^6 cells was used for each stimulation condition. The cells were activated as described above, pelleted, and lysed with 500 μl of ice-cold lysis buffer (final concentration: 0.2% Triton X-100, 50 mM Tris (pH 7.6), 100 mM NaCl, 5 mM EDTA, 1 mM NaVO_4, 10 μg/ml each aprotinin and leupeptin, 25 μM nitrophenyl guanidinobenzoate, and 1 mM benzamidine) for 30 min on ice. The lysates were disrupted with 10 strokes of a Dounce cell homogenizer and mixed in a Beckman ultracentrifuge tube (Beckman Coulter, Fullerton, CA) containing an equal volume of 80% sucrose in TNE buffer (20 mM Tris (pH 8), 150 mM NaCl, 50 mM EDTA) to form the bottom layer of 40% sucrose plus lysis mixture. This layer was overlaid with 3 ml of 30% sucrose in TNE and 1 ml of 5% sucrose in TNE. The tube was then filled to the top with TNE buffer. These gradients were spun at 150,000 g for 40,000 min at 3 °C for 4 °C in a Beckman ultracentrifuge. The gradients were harvested into ten 0.5-ml fractions from top to bottom. The GEMs were identified by dot blot analysis of the cholera toxin β subunit as described (12). Briefly, nitrocellulose strips, spotted with 2 μl of each fraction, were incubated with HRP-conjugated β subunit of cholera toxin (8 ng/ml) for 2 h and then developed using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL) as per the manufacturer’s instructions. The separation of GEMs from the rest of the cellular components was confirmed by a strong cholera toxin signal in top fractions and no signal in heavy fractions after prolonged exposure. The individual samples were subjected to further analysis by immunoprecipitation and Western blotting. In some experiments, the cholera toxin-positive GEM fractions and the bottom 1-ml heavy fractions were pooled before immunoprecipitation and Western blot analysis.

Immunoprecipitation and Western blot
TCR distribution in GEM and heavy fractions was determined as follows. An aliquot of lysate from the GEM and heavy fractions equivalent to 40 × 10^6 cells was immunoprecipitated with 50 μl of 50% protein A-agarose bead slurry (Pharmacia, Peapack, NJ) precoated with 2 μg of anti-CD3 (145-2C11) for 2 h at 4 °C. For analysis of tyrosine-phosphorylated proteins in GEM and heavy fractions, 20 × 10^6 cell equivalents of whole cell lysates were separated. The immunoprecipitates and the whole cell lysate fractions were resolved on 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) for Western blot analysis. Blots were developed using SuperSignal Substrate (Pierce) as described above. Bands of phosphorylated LAT, Lck, Fyn, and ZAP-70 were initially identified by Western blot with specific Abs, and subsequently by their molecular mass in some later experiments.

To assess tyrosine phosphorylation of Cbl and LAT, naive and primary T cells were stimulated and lysed as described above, except a 1% solution of the detergent Nonidet P-40 were used in the lysis buffer. Cbl or LAT was immunoprecipitated from 20 × 10^6 cell equivalent of cell lysates with 30 μg of protein A beads precoated with 1 μg anti-Cbl or 1 μl anti-LAT Abs. Four-fifths of each immunoprecipitate sample was resolved on 10% SDS-PAGE. Western blot analysis of tyrosine phosphorylation. The remaining one-fifth of the sample was separated on a 10% SDS-PAGE to determine the level of Cbl or LAT protein present in each sample. The blots were developed using SuperSignal West Fermo Maximum Sensitivity Substrate (Pierce), and images were captured with a Kodak Image Station 440CF (Eastman Kodak, Rochester, NY) and quantified using Kodak Digital Science ID Image Analysis software 3.0. The level of tyrosine phosphorylation was normalized against variations in Cbl or LAT protein in each sample.

Retroviral DNA constructs
The C-terminal regulatory tyrosines in Lck and Fyn were mutated to phenylalanine to create constitutively active kinases. The constructs were cloned into a murine Moloney leukemia virus-based retroviral vector LXIE (a gift from Dr. H. Spits, University Hospital Free University, Amsterdam, The Netherlands) (23, 24) upstream of eGFP. The eGFP encoding Lck or Fyn was then amplified by PCR and introduced into a retroviral DNA transfectant Phoenix Eco cells (gift from Dr. Gary Nolan, Stanford University, Stanford, CA) were added to each well. These Phoenix cells were used 2 days after transfection with LXIE vector or LXIE containing Lck or Fyn. The
cells were treated with 50 μg/ml mitomycin C and washed extensively before addition to the T cell cultures. The T cells and Phoenix cells were cocultured for 3 days to allow infection of the T cells. The transduced T cells were sorted using a FACStar® Plus FACS based on CD4 and eGFP expression. The sorted cells were rested for 3 days in medium containing 20 U/ml IL-2 and 0.1 mg/ml gentamicin. To access the level of Lck and Fyn expression in the transduced cells, lysate from 0.5 × 10^6 Lck, Fyn, or vector transduced cells were separated on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The top halves of the blots were analyzed for Lck or Fyn expression, and the bottom halves were analyzed for eGFP expression.

[44x728] /H9262 methyl-3[H]Thymidine incorporation

After a 3-day rest, the sorted T cells were washed and placed in 96-well U-bottom plates. Each well contained 10,000–15,000 T cells, 50,000–75,000 gamma-irradiated BALB/c splenocytes, and varying dose of Abs or peptides in a final volume of 200 μl of complete medium. During the last 8 h of a 48-h culture period, 1 μCi of [methyl-3][H]thymidine was added to each well. Thymidine incorporation was assessed as previously described (2) and was used as an index of mitogenic activity.

**Results**

Parental anti-CD3 (145-2C11) mAb induced a membrane-proximal signaling pattern similar to FcR nonbinding anti-CD3 (2C11-IgG3) using purified T cells

Previous studies have shown that activation of T cells with FcR nonbinding anti-CD3, even in the presence of APCs, results in a proximal signaling pattern characterized by selective TCR ζ phosphorylation (reversal of p23:p21) followed by limited ZAP-70 and LAT phosphorylation when compared with 145-2C11-mediated stimulation. We hypothesized that the partial activation was a consequence of bivalent vs multivalent TCR/CD3 cross-linking. To directly examine this, purified T cells, prepared from a T cell clone (pGL10), were stimulated with FcR nonbinding anti-CD3, 2C11-IgG3 (Fig. 1A), or its parental anti-CD3 counterpart, 145-2C11 (Fig. 1B), with or without secondary cross-linking Abs. Cell lysates were prepared, followed by anti-TCR ζ immunoprecipitation, followed by anti-phosphotyrosine immunoblotting. As shown in Fig. 1, both anti-CD3 reagents induced identical phosphoprotein patterns in the absence of cross-linking reagent, confirming the need for effective multimerization of the TCR/CD3 complex to effectively activate the T cells.

Engagement of TCR/CD3 complex without multivalent cross-linking fails to recruit TCR into GEMs

Previous studies have shown that TCR cross-linking leads to the translocation of the TCR/CD3 complex into the GEMs (10, 11). Moreover, after TCR triggering, the GEM-associated TCR ζ-chains are fully phosphorylated, migrating with an apparent molecular mass of 23 kDa (p23) in a reducing SDS-PAGE gel. This suggests that the reorientation of the TCR complex into the GEMs correlates with T cell activation. We hypothesized that the partial activation induced by FcR nonbinding anti-CD3 mAbs may be

**FIGURE 1.** FcR nonbinding anti-CD3 induces a membrane-proximal signaling pattern similar to parental anti-CD3 145-2C11 on purified T cells. T cell clone pGL10 was stimulated with FcR nonbinding anti-CD3 (2C11-IgG3) (A), or its parental anti-CD3 counterpart, 145-2C11 (B) with or without secondary cross-linking Abs. The lysates of the stimulated cells were subjected to anti-TCR ζ immunoprecipitation and Western blot for tyrosine-phosphorylated proteins.

**FIGURE 2.** Engaging TCR/CD3 without aggregation induces partial TCR signaling outside GEMs. Lysates from stimulated primary lymph node cells from BALB/c mice were separated on sucrose step density gradients and analyzed for the presence of TCR components within the GEMs. The fractions containing the GEMs were confirmed by dot blot analysis with β-subunits of cholera toxin. In A and C, cholera toxin-positive GEM fractions (G) and the heavy fraction (H) at 40% were pooled before immunoprecipitation and Western blot analysis, whereas in B individual fractions were analyzed directly. A, Cell equivalents (4 × 10^6) of raft and heavy density fractions were analyzed for CD3ε by Western blot. B, Cell equivalents (12 × 10^6) of each fraction were analyzed for tyrosine-phosphorylated proteins. Regions between 20 and 30 kDa containing phosphorylated TCR/CD3 components are shown. C, Cell equivalents (12 × 10^6) of each fraction were analyzed for tyrosine-phosphorylated proteins. Regions >30 kDa are shown. Results are representative of eight independent experiments.
related to an inability of the partially activated TCR complexes to translocate into the GEMs. To test this hypothesis, we analyzed TCR distribution in low-density GEM and heavy fractions of the cell lysates of primary BALB/c mouse lymph node T cells activated with anti-CD3 mAb with or without cross-linking. The amount of CD3ε and tyrosine-phosphorylated proteins in each fraction was determined by Western blot analysis of anti-CD3ε immunoprecipitates. Before activation, the CD3ε chains were observed almost exclusively in the heavy fractions. In contrast, a significant portion of CD3ε translocated into the GEM fraction upon anti-CD3 multivalent cross-linking (Fig. 2A). In fact, phosphorylated CD3ε protein was found exclusively in the GEM fraction. Furthermore, the TCR ε-chains in the GEMs were also enriched for the fully phosphorylated p23 form (Fig. 2B, lower panel). A phosphotyrosine blot of whole cell lysate fractions of multivalent anti-CD3 activated T cells showed increased tyrosine phosphorylation of many proteins, most notably proteins at 36 and 70 kDa, and higher than 100 kDa. The 36- and 70-kDa bands represent LAT and ZAP-70, respectively (Fig. 2C). A significant fraction of these proteins were observed in the lysate fractions containing the GEMs. This result is consistent with previous observations suggesting that GEMs are the cell signaling hot spots. By comparison, when cells were incubated with the bivalent anti-CD3 mAb, the majority of the CD3ε protein remained in the heavy fraction (Fig. 2A). Moreover, although an increase in the phosphorylation of the CD3ε and p21 ε was observed, all the CD3ε and p21 ε protein was observed in the heavy fractions (Fig. 2B, middle panel). Furthermore, no p23 ε or other proteins were detected in either the GEM or heavy fractions. Collectively, these results suggest that the partial signaling induced by bivalent anti-CD3 engagement occurred outside GEMs.

**Phosphorylation of Fyn substrate, Cbl, in the absence of TCR cross-linking**

Because Src family kinases, including Lck and Fyn, have been shown to be critical for robust T cell activation, we investigated their activity following partial TCR signaling by analyzing tyrosine phosphorylation of substrates specific to each kinase. Previous studies have shown that Fyn phosphorylates Cbl during T cell activation (25–28). Therefore, we examined the effect of bivalent vs multivalent engagement of TCR/CD3 complex by anti-CD3 Abs on Cbl activation. As seen in Fig. 3A, incubation of T cells with bivalent anti-CD3 mAbs induced a marked increase of tyrosine phosphorylation of Cbl (Fig. 3, A and B). In contrast, tyrosine phosphorylation of LAT, which is downstream of the Lck-ZAP-70 pathway, is only observed in cells activated by multivalent cross-linking of the TCR/CD3 complex (Fig. 3, C and D). The level of induction of Cbl phosphorylation by bivalent anti-CD3 varied in repeated experiments, due to differences in detection sensitivity and responsiveness of the cells used in each experiment. Nonetheless, in every experiment, Cbl phosphorylation was increased only an additional 2- to 3-fold upon multivalent cross-linking, demonstrating that bivalent anti-CD3 was quite effective at activating Cbl phosphorylation. Moreover, Fyn-deficient T cells did not show any increase in Cbl phosphorylation after bivalent anti-CD3 treatment (Fig. 3, A and B), suggesting that partial activation of Cbl phosphorylation in wild-type T cells is mediated by Fyn. It is notable that Fyn-deficient cells also showed reduction in LAT phosphorylation upon TCR cross-linking. Together, these results are consistent with the hypothesis that bivalent engagement of the TCR/CD3 complex induces a partial signal through Fyn.

**FIGURE 3.** Fyn-dependent activation of Cbl tyrosine phosphorylation in T cells activated with bivalent anti-CD3. Aliquots of 10–20 × 10⁶ naive or primary C57BL/6 or Fyn-deficient CD4⁺ T cells/sample were stimulated as indicated. Cbl or LAT was immunoprecipitated from the cell lysates. 80% of the immunoprecipitate was used to analyze tyrosine phosphorylation, and the remaining 20% was used to determine the level of immunoprecipitated proteins in each samples. A, Immunoprecipitation and Western blot analysis of Cbl tyrosine phosphorylation. B, Quantification of the level of Cbl phosphorylation. C, Immunoprecipitation and Western blot analysis of LAT tyrosine phosphorylation. D, Quantification of the level of LAT phosphorylation. In both graphs, open bars, filled bars, and gray bars represent resting, bivalent anti-CD3-activated, and multivalent anti-CD3-activated samples, respectively. Results represent six independent experiments, two of which included cells from Fyn-deficient mice.
Overexpression of Fyn, but not Lck, overcomes the proliferative defect in primary T cells activated with FcR nonbinding anti-CD3 mAbs

To further delineate the contribution of Fyn and Lck to T cell activation by FcR nonbinding anti-CD3 mAbs, we examined the functional effect of overexpressing constitutively active Lck or Fyn in primary CD4⁺ T cells. Primary lymph node T cells were transduced with a retroviral vector encoding a constitutively active form of Lck or Fyn, which have their C-terminal regulatory tyrosine residue mutated to phenylalanine. The retroviral vector also encoded a gene for eGFP, which was separated from the genes of interest (e.g., Lck or Fyn) by an internal ribosomal entry site. The simultaneous expression of the genes of interest and eGFP allowed tracking and separation of transduced T cells by FACS (Fig. 4A). Overexpression of Lck or Fyn in transduced cells was confirmed by Western blot analysis, where cells transduced with Lck or Fyn showed a comparable 50–100% increase of Lck or Fyn expression when compared with the vector-transduced cells (Fig. 4B).

This modest increase in Lck or Fyn, but not control vector, expression resulted in a marked increase of T cell proliferative response to multivalent anti-CD3 mAb stimulation, especially at suboptimal doses (Fig. 5A), indicating that both of the overexpressed proteins were functionally active. In contrast, only the overexpression of Fyn, but not Lck, partially restored T cell proliferation to FcR nonbinding bivalent anti-CD3 (2C11-fos) in a dose-dependent manner (Fig. 5B). Similar results were observed when another form of FcR nonbinding anti-CD3, 2C11-IgG3, was used in the assay (Ref. 1 and data not shown). Overexpression of wild-type Fyn (50–100%) resulted in proliferation of the transduced T cells to FcR nonbinding anti-CD3, demonstrating that the increase in Fyn expression level, not its constitutive activation state, was critical for overcoming the signaling defect (data not shown). Taken together, these results suggest that bivalent anti-CD3 can preferentially activate Fyn, but the level of Fyn in normal cells is a limiting factor in driving cell proliferation. It is important to note that the differential effect of Lck and Fyn was observed with only a very moderate level of overexpression (<2-fold), suggesting that the results are physiologic as compared with the effects of high levels of overexpression in other systems.

The inability of Lck overexpression to restore proliferation to FcR nonbinding anti-CD3 was further explored. One notable difference between Lck and Fyn is their subcellular localization. Fyn associates with the TCR complex constitutively, whereas Lck is associated with the coreceptor CD4 outside the TCR complex. Lck can be artificially recruited to the TCR/CD3 complex by a bispecific Ab anti-CD3-fos x anti-CD4-jun (1). This Ab lacks FcR and does not induce TCR capping and T cell proliferation in naive (data not shown) and primary (Fig. 5C) T cells even in the presence of splenic APC. Recruitment of Lck to the TCR complex using this bispecific Ab induced vigorous proliferation in Lck-transduced cells (Fig. 5C), suggesting that the failure of these cells to respond to FcR nonbinding anti-CD3 mAb can be overcome by

![FIGURE 4](image_url)

**FIGURE 4.** Overexpression of Lck Y505F or Fyn Y528F in BALB/c lymph node T cells by retroviral transduction. A, Dot plot of FACS separation of retroviral transduced CD4⁺ cells. B, Level of Lck, Fyn, and eGFP expression in transduced cells.

![FIGURE 5](image_url)

**FIGURE 5.** Overexpression of Fyn restores T cell proliferation to FcR nonbinding anti-CD3. Aliquots of 1 x 10⁴ BALB/c lymph node CD4⁺ T cells transduced with Lck Y505F, Fyn Y528F, or vector alone were mixed with 5 x 10⁴ irradiated BALB/c splenocytes and varying dose of conventional anti-CD3 (A), FcR nonbinding anti-CD3-fos (B), or FcR nonbinding anti-CD3-fos x anti-CD4-jun bispecific mAb (C) in U-bottom 96-well plates. Proliferation was determined by measuring incorporation of [³H]thymidine between 40 and 48 h of culture. Results are representative of eight independent experiments.
artificially recruiting Lck into the TCR complex under the bivalent cross-linking conditions.

Overexpression of Src family kinases overcomes APL-induced proliferative defect
Because FcR nonbinding anti-CD3 and altered peptide ligand (APL) induce similar intracellular signals and cellular functions, we were interested in determining whether Fyn overexpression could reconstitute T cell proliferation to APL. T cells from TCR-transgenic DO.11.10 mice were transduced with constitutively active Lck, Fyn, or control vector as described above, and their proliferation to wild-type OVA peptide or antagonist OVA peptide H331A (23) was examined. Both Lck- and Fyn-overexpressing cells exhibited enhanced proliferation to wild-type OVA peptide when compared with cells transduced with vector alone (Fig. 6A). By comparison, Fyn- but not Lck-overexpressing cells proliferated to OVA APL (H331A) in a dose-dependent manner similar to the response observed with FcR nonbinding anti-CD3 (Fig. 6B). These results demonstrate that Fyn possesses the unique ability to overcome signaling defects induced by TCR antagonists.

Discussion
In this study, we addressed the molecular basis of the inability of bivalent anti-CD3 to induce primary T cell activation. We report that FcR nonbinding anti-CD3 mAbs failed to promote TCR translocation to GEMs and were unable to enhance Lck and Fyn activity (data not shown). Overexpression of Fyn but not Lck restored T cell proliferation to FcR nonbinding anti-CD3 mAb. The effect is due to the ability of overexpressed Fyn to at least partially compensate for the signaling deficiency induced by TCR antagonists. These data suggest that the inability of antagonist anti-CD3 mAbs to activate T cells results from, at least in part, the lack of Lck and Fyn activation and the inability of the engaged TCR/CD3 complexes to redistribute to the GEMs.

We reasoned that the difference between the ability of overexpressed Lck and Fyn to restore T cell proliferation is likely due to their distinct subcellular localization pattern. Because FcR nonbinding anti-CD3 mAbs are unable to recruit and activate Lck (1), the overexpressed Lck, as endogenous Lck, are not targeted to the TCR complex to participate in TCR activation. In contrast, Fyn associates with the TCR complex constitutively and is basally active in naïve and primary cells (data not shown). Therefore, overexpression of Fyn may lead to higher TCR-Fyn association and promotion of partial signaling by FcR nonbinding anti-CD3 to full T cell activation. This interpretation is supported by the finding that overexpression of Lck can restore proliferation to FcR nonbinding anti-CD3 when Lck is recruited to the TCR complex by bispecific anti-CD3 × anti-CD4 mAbs (Fig. 5C). In the current study, anti-CD3-fos × anti-CD4-jun bispecific Ab was not mitogenic for naïve and primary T cells. This differs from our previous work and most likely reflects a higher amount of aggregated Ab present in the preparation used in those earlier experiments (1).

Our finding of differential use of Src kinases in TCR antagonist signaling is consistent with several recent reports suggesting a unique role for Fyn in signaling by TCR antagonists. Lck-deficient JCaM1 cells reconstituted with Fyn exhibited an altered tyrosine phosphorylation pattern resembling that induced by APL or FcR nonbinding anti-CD3 (29). In addition, an antagonist cytochrome c peptide, T102G, induced activation of Fyn signal transduction in T cells from AD10 TCR-transgenic mice, which correlated with increased tyrosine phosphorylation of vav and T cell APC conjugate formation (30). However, our findings with the FcR nonbinding anti-CD3 mAb differ from those observed by Huang et al. (30) using antagonist peptide in that we did not detect significant augmentation of Fyn kinase activity after partial T cell activation. This difference may be because FcR nonbinding anti-CD3 mAbs are unable to induce TCR redistribution to form TCR caps (1), whereas antagonist peptides are able to induce loosely organized immunological synapses (31). Nonetheless, in our system, FcR nonbinding anti-CD3 did induce tyrosine phosphorylation of Cbl in a Fyn-dependent manner. In addition, the partial signals elicited by FcR nonbinding anti-CD3 were sufficient to induce anergy in Th1 cells (1) and Th2 deviation of undifferentiated Th0 cells (2), suggesting maximal activation of Fyn activity is not necessary for these cellular functions.

The basis for the inability of Fyn to mediate a complete tyrosine phosphorylation pattern remains to be determined. It has been proposed that this may be due to the distinct binding orientation of Fyn with the TCR and other molecules, thereby dictating the pattern of substrate phosphorylation (29). This hypothesis implies that Fyn, by complexing with other cytoplasmic proteins, is intrinsically deficient in its ability to induce complete tyrosine phosphorylation and initiation of T cell activation. Our finding that moderate overexpression of Fyn can restore T cell proliferation to FcR...
nonbinding anti-CD3 and antagonist peptides suggests that the defect is largely quantitative, perhaps due to the low stoichiometry of the Fyn-TCR complex in normal cells (21). However, because the Fyn-overexpressing T cells used in this study have normal levels of Lck, it is possible that the increase in Fyn expression restored proliferation through recruiting and activating Lck. Thus, both Fyn directly and Lck indirectly may be involved in the development of the agonist phenotype. A threshold in Fyn recruitment and activation may control Lck activation, which in turn determines the signaling pattern and functional outcome of TCR engagement (32).

The involvement of GEMs may also contribute to the quality of TCR signaling. We demonstrated that activation of both Lck and Fyn through the TCR/CD3 complex requires a high-order aggregation of the receptor complex and is associated with recruitment of the TCR into GEMs. It has been reported recently that a small pool of TCR/CD3 complexes localizes constitutively in the GEMs (33), and that the TCR inside GEMs, but not those outside GEMs, are associated with Fyn. Our preliminary results are consistent with this finding. In addition, we observed that upon engagement of bivalent anti-CD3, Fyn-TCR complexes disappeared from the GEMs and appeared in the heavy fractions (our unpublished observation). Taken together, these data suggest that the lack of TCR in GEMs in FcR nonbinding anti-CD3-treated cells may result from active exclusion.

Results from many experimental systems support the notion that partial signaling by antagonist ligand correlates with deficient Lck activation. T cell activation by agonist peptide induces the formation of a highly organized immunological synapse/supramolecular activation clusters, in which TCR/CD3 and MHC aggregate in the center with a ring of LFA-1 and ICAM-1 in the periphery (31, 34). However, partial agonist and antagonist peptides are unable to induce the organization of the immunological synapse, and the TCR and MHC are diffused loosely at the contact site. Moreover, the addition of a blocking anti-CD4 Ab to T cells before their exposure to an agonist peptide blocks the formation of an organized synapse (31). Recent studies have shown that the formation of these mature immunological synapses is preceded by transient coaggregation of TCR and CD4 at the site of T cell and APC contact and that a partial TCR agonist failed to induce such coaggregation (35). Because Lck is associated with and dependent on CD4 for its cellular localization, the efficiency of CD4 recruitment to the synapse is likely to determine the agonist or partial agonist pattern of the biochemical and functional responses (36). We demonstrated in our previous work that, in Th1 T cell clones, coligation of CD4 can restore the mitogenicity of FcR nonbinding anti-CD3 (1). Collectively, these results suggest that the partial signaling induced by APL or FcR nonbinding anti-CD3 is due to a combination of a lack of Lck recruitment and activation and the partial signals mediated by weak Fyn associations with TCR/CD3 complexes.

The results of our studies and others suggest the following model for agonist vs antagonist signaling (Fig. 7). In resting T cells, a small fraction of the TCR/CD3 complexes are associated with the Fyn inside GEMs, whereas the majority of the TCR/CD3 complexes remain outside GEMs. Upon encounter with an agonistic Ag or multivalent cross-linked anti-CD3 Ab, more TCR/CD3 complexes become associated with GEMs. Because a portion of Lck and Fyn localizes constitutively inside GEMs, recruitment of the TCR into GEMs may be one mechanism by which these kinases and the TCR are brought into close proximity to initiate signal transduction. Enrichment of TCR-Fyn complexes in GEMs may lead to Fyn activation by trans-phosphorylation and further recruitment and activation of Lck. Additional engagement of the costimulatory molecule, CD28, triggers these dispersed GEMs to coalesce at the site of Ag/Ab binding, leading to more intense and sustained activation of T cells (37). In contrast, upon engagement of FcR nonbinding anti-CD3 mAb or altered peptide ligands, the TCR/CD3 complexes move out of GEMs, which prohibits further Fyn activation and Lck recruitment. Consequently, only the basally active TCR-associated Fyn is available to initiate signal transduction, resulting in a partially activated signaling pathway. We further hypothesize that membrane compartmentalization of signaling molecules, such as Lck and Fyn, and TCR trafficking in these compartments set the threshold for T cell activation and control the functional outcome of TCR engagement.

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