T-cell receptor antagonists induce Vav phosphorylation by selective activation of Fyn kinase

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T cell receptor (TCR) antagonists inhibit antigen-induced T cell activation and by themselves fail to induce phenotypic changes associated with T cell activation. However, we have recently shown that TCR antagonists are inducers of antigen-presenting cell (APC)–T cell conjugates. The signaling pathway associated with this cytoskeleton-dependent event appears to involve tyrosine phosphorylation and activation of Vav. In this study, we investigated the role played by the protein tyrosine kinases Fyn, Lck, and ZAP-70 in antagonist-induced signaling pathway. Antagonist stimulation increased tyrosine phosphorylation and kinase activity of Fyn severalfold, whereas little or no increase in Lck and ZAP-70 activity was observed. Second, TCR stimulation of Lck$^-$, Fyn^{hi} Jurkat cells induced strong tyrosine phosphorylation of Vav. In contrast, minimal increase in tyrosine phosphorylation of Vav was observed in Lck^{hi}, Fyn^{lo} Jurkat cells. Finally, study of T cells from a Fyn-deficient TCR transgenic mouse also showed that Fyn was required for tyrosine phosphorylation and activation of Vav induced by both antagonist and agonist peptides. The deficiency in Vav phosphorylation in Fyn-deficient T cells was associated with a defect in the formation of APC–T cell conjugates when T cells were stimulated with either agonist or antagonist peptide. We conclude from these results that Vav is a selective substrate for Fyn, especially under conditions of low-affinity TCR-mediated signaling, and that this signaling pathway involving Fyn, Vav, and Rac-1 is required for the cytoskeletal reorganization that leads to T cell–APC conjugates and the formation of the immunologic synapse.

The earliest documented events following engagement of TCRs by their specific ligands, peptide/MHC complexes, involves the activity of Src and Syk/ZAP-70 family protein tyrosine kinases and the phosphorylation of a number of their cellular substrates (1, 2). These include the immunoreceptor, tyrosine-based activation motifs (ITAM) of the TCRξ and CD3 chains (3), the adaptor proteins LAT (4) and SLP-76 (5), and the proto-oncogene product, Vav (6), as well as proteins of less-defined function, such as SLAP-130/FYB (7, 8). Vav, a guanine nucleotide exchange factor for Rho-like small GTPases such as Rac, plays an important role in T cell activation (9). One of its functions is related to the reorganization of the T cell actin cytoskeleton after TCR stimulation, and studies with Vav$^{-/-}$ T cells have established the requirement for Vav in actin-dependent TCR/CD3 cap formation after TCR crosslinking (10). The upstream protein tyrosine kinase(s) responsible for the phosphorylation and activation of Vav have not been well delineated, since previous studies have shown that Vav can serve as a substrate for all four of the proximal tyrosine kinases: Lck, Fyn, Syk, and ZAP-70 (11–14). In contrast, individual PTKs appear to preferentially phosphorylate some of the other important downstream substrates, e.g., LAT is a specific substrate for ZAP-70 (4), ZAP-70 itself is a substrate for Lck (15), and SLP-130/FYB and PYK-2 are substrates for Fyn (16, 17).

Some of these activation-induced tyrosine phosphorylation events appear to be exquisitely sensitive to the affinity of interaction between the TCR and its MHC/peptide ligand. Studies on single amino acid-substituted antigenic peptide variants, so-called altered peptide ligands (APL), have indicated that low-affinity interactions often lead to an incomplete pattern of tyrosine phosphorylation. Initial studies indicated that stimulation by APL led to a preponderance of an incompletely phosphorylated ζ chain, with the resulting accumulation of a low molecular weight mass TCRζ. Furthermore, antagonist APL failed to phosphorylate and activate ZAP-70 (18–20). More recently, we have demonstrated that antagonist peptides are capable of inducing the signaling pathway that is required for cytoskeletal reorganization in T cells, the formation of stable T cell/APC conjugates, and the localization of certain T cell proteins to the areas of contact between APC and T cell. This signaling pathway is characterized by the phosphorylation and activation of Vav guanine nucleotide exchange factor activity and subsequent activation of the small G protein, Rac (21). This finding indicated the presence of a hierarchy of T cell signal transduction that is sensitive to the affinity of TCR/ligand interaction. To characterize further this signaling pathway, we wished to determine which PTK(s) is activated by antagonist peptide/MHC engagement of the TCR and is responsible for initiating the signaling cascade that leads to the partial activation of T cells and the subsequent formation of APC/T cell conjugates. In this report, we provide evidence that under conditions of both agonist and antagonist stimulation of T cells, Vav is preferentially phosphorylated by the Src family kinase, Fyn.

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Materials and Methods

Animals. Cytochrome c-specific AD10 TCR transgenic mice were bred on a B10.A background (H2K) (22, 23) in our animal facility. B10A and Fyn$^{-/-}$ mice were purchased from the Jackson Laboratory. The Fyn$^{-/-}$ AD10 mice were generated in our animal facility.

Antibodies and Reagents. The following antibodies were used in this study: monoclonal antiphosphotyrosine antibody (4G10) and polyclonal antibodies against LAT and Fyn (Upstate Biotechnology, Lake Placid, NY); anti-ZAP-70 and Fyn monoclonal antibodies (Transduction Laboratories, Lexington, KY); Fyn polyclonal antibodies (kindly provided by T. Kawakami, La Jolla Institute for Allergy and Immunology, San Diego, CA); monoclonal anti-Lck and polyclonal antibodies against Vav and ZAP-70 and Lck (Santa Cruz Biotechnology); rabbit anti-mouse IgG antibody (Cappel); hybridomas producing antibody against mouse CD3ε (2C11) were purchased from the American Type Culture Collection (Rockville, MD); polyclonal antibodies against SLAP-130/FYB were kindly provided by G. Koretzky

Abbreviations: TCR, T cell receptor; APC, antigen-presenting cell.

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(University of Pennsylvania, Philadelphia, PA) and C. Rudd (Harvard Medical School, Boston, MA); anti-human CD3 antibody (OKT3) was kindly provided by H. Umehara (Osaka Dental University, Osaka, Japan); the enhanced chemiluminescence technique immunodetection system, peroxidase-conjugated sheep anti-mouse and anti-rabbit IgG, Texas red-conjugated donkey anti-rabbit Ig antibody (Amersham Life Science); biotinylated anti-mouse Vβ3 TCR (KJ25) (PharMingen); lipophilic green fluorochrome $DiOC_{18}$ and the red fluorochrome $DiIC_{18}$ (Molecular Probes); and FluoroGuard Antifade Reagent (Bio-Rad). [γ-^{32}P] ATP was purchased from ICN. The cdb3/T7–7 expression vector containing the cytoplasmic domain (residues 1–379) of human erythrocyte band 3 (cdb3) gene was a gift of P. S. Low (Purdue University, IN). This construct was used to transform *Escherichia coli* Bl21 (DE3) (pLysS) (Novagen), and expression and purification of cdb3 was performed essentially as described (24).

Peptide Synthesis. Peptides were synthesized on a Rainin Symphony synthesizer (Rainin Instruments) and purified as previously described (20). Routinely, purity was >95% after high-pressure liquid chromatography. Analogs of moth cytochrome *c* 88–103 containing single amino acid substitutions are named according to the substitution and its position, e.g., T102G has glycine at position 102.

Cells. The T cell clone AD10, B lymphoma CH27, and the I-E^k-expressing fibroblast line DCEK.ICAM ($CD54^+$ $CD80^+$) cells were cultured as previously described (21). Fyn-deficient AD10 $CD4^+$ T cells were purified by antibody and complement treatment as described (25). In brief, the cells from lymph nodes of AD10 $Fyn^{-/-}$ mice were incubated with antibodies to CD8 (3.155), heat-stable antigen (J11d), class II MHC (M5/114 and CA-4.A12), B cells (RA3.6B2), macrophages (M1/70), natural killer cells (PK136), and dendritic cells (33D1) and then crosslinked with mouse anti-rat κ (MAR18.5). Residual APCs and *in vivo*-activated T cells were removed by isolating high-density cells obtained after Percoll (Sigma) step-gradient (45%, 53%, 62%, 80%) centrifugation. The resultant cells were >95% $CD4^+$. More than 95% of these cells possessed a phenotype associated with naïve CD4 cells ($CD45RB^+$, $CD62L^+$, CD44low), along with expression of the Vβ3/Vα11 TCR. For generation of Fyn-deficient AD10 T cell lines, 2×10^5 naïve $CD4^+$ T cells were incubated with 1×10^6 irradiated (3,000 rad) B10.A splenocytes in the presence of 30 μg/ml pigeon cytochrome *c* (PCC) for 4 days. They were cultured in the presence of IL-2 (30 units/ml) containing RPMI-1640 medium with 10% FCS and restimulated with antigen and irradiated APCs every 2 weeks. JCaMl cells transfected with the genes for Lck (JCl/Lck) or Fyn (JCl/Fyn) were kindly provided by David Straus (University of Chicago, Chicago, IL) (26) and were grown in RPMI-1640 medium with 10% FCS, L-glutamine, and antibiotics supplemented with G418 and hygromycin. JCaMl cells were obtained from T. Mustelin (The Burnham Institute, San Diego, CA). To stimulate AD10 cells, 1×10^7 T cells were incubated with either antagonist (100 μg/ml) or agonist (1 μg/ml) pulsed APC (1×10^7) at 37°C for 1 min. These concentrations were used in all experiments involving ligand-mediated stimulation of AD10 cells and were chosen because they were the optimal concentrations for induction of proliferation (antigen) or antagonism of proliferation (antagonist).

Immunoprecipitation and Immunoblotting. Detection of tyrosine phosphorylation of proteins was performed as described (21, 27). In brief, cells were lysed in ice-cold TNE buffer (20 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Nonidet P-40/5 mM EDTA/1 mM Na_3VO_4/10 μg/ml aprotinin and leupeptin/1 mM PMSF). Lysates were incubated for 30 min on ice. Insoluble material was removed by centrifugation and the supernatants subjected to immunoprecipitation with Protein G or Protein A beads precoated with Fyn, ZAP-70, Vav, SLAP-130, LAT or CD3 antibodies, as indicated. The resulting immunoprecipitates were fractionated by SDS/PAGE (8% gels), transferred onto Immobilon-P membrane, and immunoblotted with antiphosphotyrosine antibody. The blots were developed by enhanced ECL. The same membrane was stripped and immunoblotted with antibodies against Fyn, ZAP-70, Vav, SLAP-130, or LAT, as indicated. All experiments were performed three to five times.

Analysis of Conjugate Formation and Immunofluorescence Microscopy. These procedures were performed as previously described (21). In brief, for conjugate formation, CH27 APCs were membrane stained with the lipophilic green fluorochrome $DiOC_{18}$, and AD10 or Fyn-deficient AD10 T cells were stained with the red fluorochrome $DiIC_{18}$. APCs were subsequently pulsed with peptides at various concentrations for 2 h at 37°, distributed in round-bottomed microtiter plates (2.0×10^5 cells/well), and mixed with T cells at a 1:1 ratio. After 30-min incubation at 37°C, cells were vigorously pipetted to disrupt nonspecific conjugates and immediately analyzed by flow cytometry (FACScan, Becton Dickinson). Ten thousand cells were analyzed, and the percentage of total APCs that formed conjugates with T cells was calculated. For immunofluorescence microscopy, peptide-pulsed APCs (1 μg/ml PCC 88–104 or 100 μg/ml T102G) and T cells were mixed at 1:1 ratio and incubated at 37°C for 15 min. Cells were placed on glass slides (Superfrost Plus, Fisher) and fixed in 3% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton-X for 2 min, washed, and incubated with anti-Fyn and biotinylated anti-mouse TCR Vβ3 antibody. The cells were washed and stained with Texas red-conjugated anti-rabbit Ig antibody and FITC-conjugated streptavidin. After washing, the cells were mounted in FluoroGuard antifade reagent. Capping of protein was then analyzed visually by using a confocal microscope (Bio-Rad). At least 200 conjugates were analyzed for cap formation in each experiment.

Kinase Assay. *In vitro* kinase assays were performed on Fyn and Lck, as described (28, 29). In brief, the immunoprecipitates were washed twice with ice-cold TNE buffer and once in kinase buffer (20 mM Hepes, pH 7.2/5 mM $MgCl_2$/5 mM $MnCl_2$). The samples were then suspended in 40 μl kinase buffer containing 10 μCi of γ-[^{32}P] ATP and incubated at room temperature for 15 min. Reactions were terminated by the addition of 10 μl of 5× SDS sample buffer. The samples were heated for 5 min at 95°C, separated by SDS/PAGE, and transferred to Immobilon-P. The membranes were treated with 1 M KOH for 1 h at 50°C to remove alkali-labile phosphate groups from serine- and threonine-phosphorylated proteins. Radiolabeled tyrosine-phosphorylated proteins were detected by autoradiography. For ZAP-70, *in vitro* kinase assays were performed by a similar procedure, but reactions were started by the addition of 30 μl kinase buffer containing 50 μM ATP 10 μCi[γ^{32}P] ATP and 3 μg cdb3 as exogenous substrate. The samples were incubated for 10 min at room temperature, and the reactions were terminated with 30 μl of ×2 SDS sample buffer. The samples were analyzed by SDS/PAGE, Immobilon-P transfer, and autoradiography, as described above.

Results

Stimulation with TCR Antagonist Peptides Selectively Induces Activation of Fyn Kinase. In previous studies, evidence had been presented that both Src and Syk family PTKs are capable of phosphorylating Vav (31). To investigate which of these PTKs might be responsible for Vav phosphorylation after TCR antag-

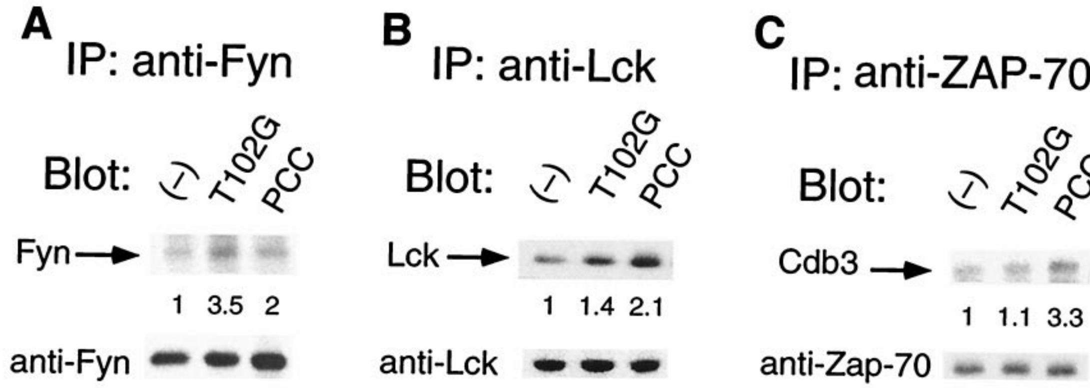

Fig. 1. Capacity of antagonist peptides to induce protein tyrosine kinase activity. AD10 cells (1×10^7) were stimulated for 1 min at 37°C with antagonist (T102G) pulsed, agonist (PCC 88–104) pulsed, or unpulsed DCEK.ICAM APCs (1×10^7). Anti-Fyn (*A*), Lck (*B*), or ZAP-70 (*C*) immunoprecipitates were subjected to *in vitro* kinase assays and analyzed by SDS/PAGE. Proteins were transferred to membranes and incubated with 1 M KOH for 1 h at 50°C. The tyrosine-phosphorylated proteins were analyzed by autoradiography (*Upper*). The numbers under the autoradiograph indicate the relative radioactivity normalized to the amount observed in unstimulated cells. The same membranes were immunoblotted with anti-Fyn, Lck, or ZAP-70 (*Lower*). Results are representative of three similar experiments.

onist stimulation, *in vitro* kinase assays were performed after agonist and antagonist stimulation of the AD10 T cell clone. As shown in Fig. 1*A*, Fyn tyrosine kinase activity was significantly increased after stimulation by both agonist and antagonist peptides, with the antagonist ligand inducing almost twice the activity induced after agonist stimulation. In contrast, under the same conditions of stimulation, antagonist peptides were very inefficient at inducing any increase in Lck activity (Fig. 1*B*) or ZAP-70 activity (Fig. 1*C*), whereas agonist stimulation induced 2- to 3-fold increases in the activity of both enzymes. Western blotting confirmed that similar amounts of Fyn, Lck, and ZAP-70 had been precipitated after each stimulation (Fig. 1 *A–C Lower*). It was not possible to perform experiments with Syk, because no detectable Syk was present in this T cell clone.

To explore further the kinase activity of the three detectable PTKs in AD10 T cells (Lck, Fyn, and ZAP-70) after antagonist stimulation, the tyrosine phosphorylation state of some of their substrates was examined. For this purpose, we analyzed SLAP-130/FYB (Fyn substrate), ZAP-70 (Lck substrate), and LAT (ZAP-70 substrate), as shown in Fig. 2. Whereas all three substrates were efficiently tyrosine phosphorylated after agonist stimulation, only the Fyn substrate, SLAP-130/FYB, was tyrosine phosphorylated after antagonist stimulation (Fig. 2*A*).

In summary, examination of *in vitro* kinase activity and phosphorylation of natural substrates indicates that of the four Src and Syk family members under consideration, Fyn kinase activity was most strikingly induced after stimulation with a TCR antagonist peptide.

To analyze further the effect of antagonist stimulation on Fyn, we examined the degree to which Fyn itself was tyrosine phosphorylated after antagonist- and agonist-mediated signaling, and we also analyzed the association of Fyn with the CD3-TCR complex after agonist and antagonist stimulation. The data on tyrosine phosphorylation of Fyn, shown in Fig. 3*A*, indicates that both agonist and antagonist stimulation leads to a notable increase in the tyrosine phosphorylation of Fyn itself, and similar to the *in vitro* kinase assay, antagonist was somewhat more efficient than agonist in stimulating this activity. With respect to Fyn association with the TCR, although both agonist and antagonist led to an increased coprecipitation of Fyn with TCR/CD3, antagonist stimulation resulted in a 3-fold increase, whereas agonist stimulation resulted in only a 60% increase over that found in unstimulated cells.

Localization of Fyn to the Immunologic Synapse After Agonist and Antagonist-Induced Conjugate Formation. Previous reports have shown that many of the molecules involved in TCR-mediated signaling localize to the area of the T cell that interacts with the APC, the so-called immunologic synapse (32–34). The molecules that have thus far been shown to localize to the immunologic synapse after stimulation with agonist- and antagonist-pulsed APC include LFA-1, actin, talin, Rac-1, and

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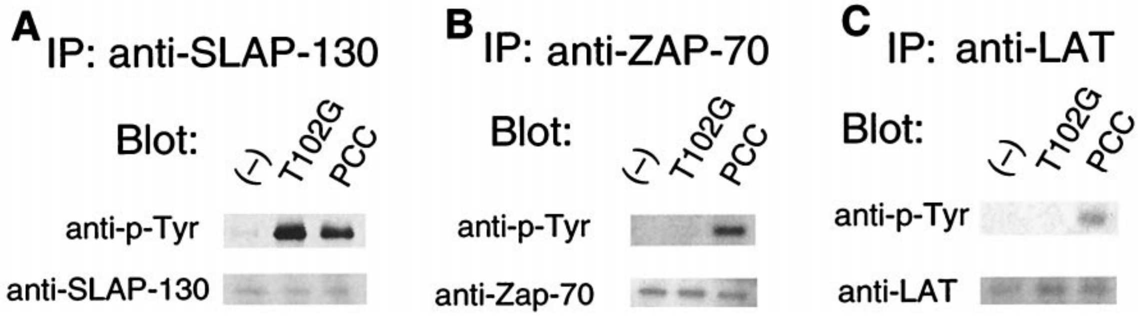

Fig. 2. Antagonist peptides induce phosphorylation of SLAP-130/FYB. AD10 cells (1×10^7) were stimulated with antagonist (T102G) pulsed, agonist (PCC 88–104) pulsed, or unpulsed DCEK.ICAM APCs (1×10^7). After 1 min of stimulation, cells were lysed, immunoprecipitated with anti-SLAP-130, ZAP-70, or LAT antibodies and analyzed by SDS/PAGE. Proteins were transferred to membranes and immunoblotted with antiphosphotyrosine antibody (*Upper*). Membranes were subsequently stripped and analyzed for SLAP-130, ZAP-70, or LAT protein (*Lower*). Results are representative of five similar experiments.

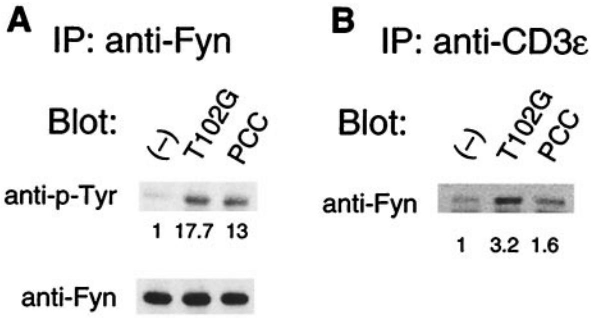

Fig. 3. Increased Fyn tyrosine phosphorylation and association with TCR/CD3 complex after antagonist stimulation. AD10 cells (1×10^7) were stimulated with antagonist (T102G) pulsed, agonist (PCC 88–104) pulsed, or unpulsed DCEK.ICAM APCs (1×10^7). After 1 min of stimulation, cells were lysed, immunoprecipitated with anti-Fyn or CD3ε antibody, and analyzed by SDS/PAGE. Proteins were transferred to membranes and immunoblotted with: (*A*) antiphosphotyrosine antibody (*Upper*) or (*B*) anti-Fyn. Membranes were subsequently stripped and analyzed for Fyn protein (*A Lower*). The numbers under *Upper* indicate the intensity of staining relative to that observed in the unstimulated controls.

TCR. On the other hand, some molecules, such as CD28 and PKCθ, are localized to this area only after agonist stimulation, and other molecules, such as CD4, are preferentially localized to the synapse after agonist stimulation (21). To determine whether Fyn is localized to the APC-T cell synapse after antagonist stimulation, the conjugates were doubly labeled with anti-TCR (Vβ3) and anti-Fyn antibodies. Immunofluorescent analysis (Fig. 4) showed that TCR and Fyn colocalized to the APC-T cell contact zone after both agonist (Fig. 4 *D–F*) and antagonist stimulation (Fig. 4 *G–I*). Of the conjugates that contained TCR caps after antagonist stimulation, greater than 90% also contained cocapped Fyn.

Vav Phosphorylation in Transfected JCaMI Cells. To substantiate further the role of Fyn in tyrosine phosphorylation of Vav, we investigated Vav phosphorylation in JCaMI (JCI) mutant Jurkat

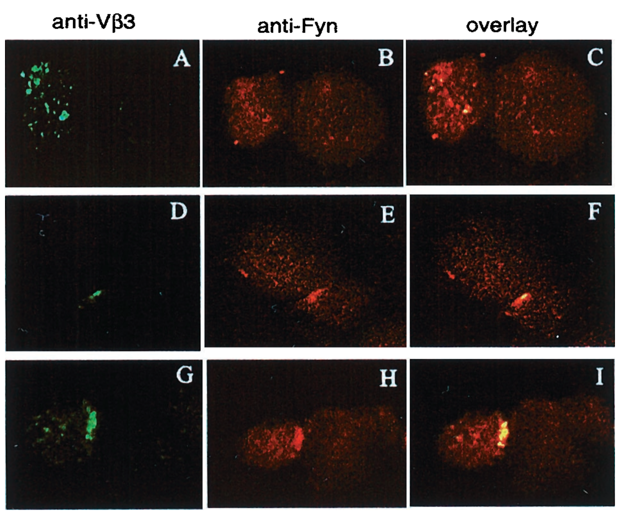

Fig. 4. Fyn localizes to the immunologic synapse after agonist- and antagonist-induced conjugate formation. Conjugates were formed between AD10 and CH27 cells that were pulsed with no peptide (*A–C*), agonist peptide (*D–F*), or antagonist peptide (*G–I*). Representative T cell–APC pairs depicting the distribution of the following proteins are illustrated: TCR staining by anti-Vβ3 antibody (*A*, *D*, *G*); Fyn staining by anti-Fyn antibody (*B*, *E*, *H*); double immunofluorescence to determine colocalization of Fyn and TCR (*C*, *F*, *I*).

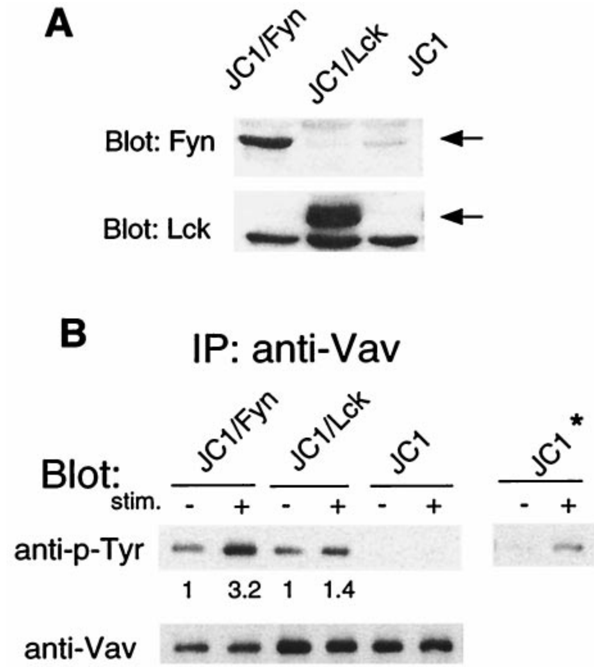

Fig. 5. Vav tyrosine phosphorylation in transfected JCaMI (JCI) cells. (*A*) An equal number (2.5×10^5) of JCI/Fyn (Fyn$^+$, Lck$^-$) JCI/Lck (Lck$^+$, Fyn^{lo}), or JCI cells were lysed in 1% Nonidet P-40 and analyzed by immunoblotting with either anti-Fyn (*Upper*) or anti-Lck antibodies (*Lower*). (*B*) JCI/Fyn, JCI/Lck, or JCI cells (1×10^7) were incubated with or without 10 μg/ml anti-CD3 antibodies for 30 min on ice. After washing, cells were incubated with rabbit anti-mouse IgG antibody (20 μg/ml) at 37°C for 3 min. After washing with cold PBS, cells were lysed, immunoprecipitated with anti-Vav, and analyzed by SDS/PAGE. Proteins were transferred to membranes and immunoblotted with antiphosphotyrosine antibody. Films were exposed for 5 sec (*Left Upper*) or for 2 min (*Right Upper* JCI*); membranes were subsequently stripped and analyzed for Vav protein (*Lower*). Numbers indicate the fold increase in Vav phosphorylation after TCR crosslinking relative to the Vav phosphorylation in unstimulated cells.

cells that were stably transfected with Fyn (JCI/Fyn) or Lck (JCI/Lck) genes. These transfected cell lines have been recently generated by Straus and colleagues and characterized with respect to their signaling capabilities (26). These investigators have analyzed the expression levels of Lck and Fyn before and after transfection and have reported that, whereas untransfected JCI cells express no Lck (35) and about 5,000 Fyn molecules/cell, JCI/Lck cells express the same low amount of Fyn and similar quantities of Lck to the parental Jurkat cells (≈180,000 molecules/cell), and that JCI/Fyn cells express no Lck and 100,000–180,000 Fyn molecules/cell. This analysis is consistent with the data we have obtained with these cells (Fig. 5*A*); that is, both Lck and Fyn are highly expressed in their respective transfectants, and the parental JCI cells lack Lck and express a small amount of Fyn. These cells were stimulated by crosslinking of CD3 and analyzed for Vav tyrosine phosphorylation after immunoprecipitation with anti-Vav antibodies (Fig. 5*B*). After anti-CD3 stimulation, JCI/Fyn cells increased their Vav tyrosine phosphorylation by 3-fold over the unstimulated cells, whereas Vav tyrosine phosphorylation of stimulated JCI/Lck cells increased by only 40% compared with unstimulated cells. By increasing the exposure time, a small amount of Vav phosphorylation in stimulated JCI cells could also be detected (Fig. 5*B Far Right*), consistent with the low-level expression of Fyn in these cells.

In summary, these experiments support the data obtained with AD10 cells and add further support to the idea that Vav is

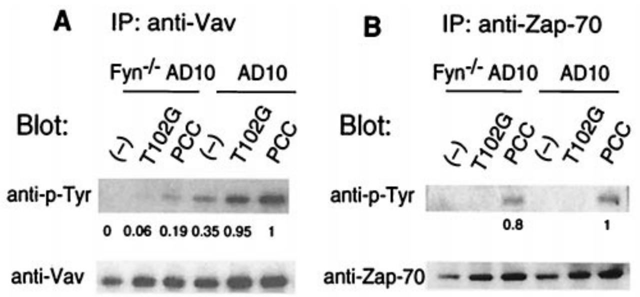

Fig. 6. Fyn requirement for tyrosine phosphorylation of Vav induced by antagonist and agonist peptides. AD10 or Fyn-deficient AD10 T cells (1×10^7) were stimulated with antagonist (T102G) pulsed, agonist (PCC 88–104) pulsed, or unpulsed DCEK.ICAM APCs (1×10^7). After 1 min of stimulation, cells were lysed, immunoprecipitated with anti-Vav (*A*) or anti-ZAP-70 (*B*), and analyzed by SDS/PAGE. Proteins were transferred to membranes and immunoblotted with antiphosphotyrosine antibody (*Upper*). Membranes were subsequently stripped and analyzed for Vav or ZAP-70 protein (*Lower*). The numbers indicate the amount of phosphorylation of Vav (*A*) or ZAP-70 (*B*) relative to agonist-stimulated AD10 cells. Results are representative of three similar experiments.

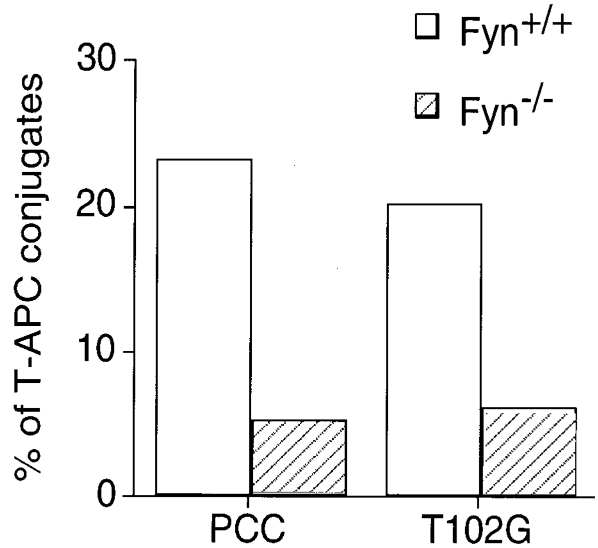

Fig. 7. Fyn is required for the formation of stable APC-T cell conjugates induced by both agonist and antagonist peptides. CH27 APCs labeled with green fluorescent membrane dye were pulsed with antagonist (T102G) or agonist (PCC 88–104) peptide for 2 h at 37°C, washed, and added to AD10 (open bars) or Fyn-deficient AD10 (hatched bars). T cells were labeled with red fluorescent membrane dye. After 30-min incubation at 37°C, conjugate formation was measured by two-color flow cytometry. The percentage of APCs that formed conjugates with T cells, as indicated by double fluorescent positive cell couplets, was quantitated. The background of the percentage of nonspecific conjugates formed in the absence of any added peptide (1.4%) was subtracted from the plotted date.

selectively tyrosine phosphorylated by Fyn after TCR-mediated signaling.

Vav Phosphorylation in Fyn-Deficient TCR Transgenic T Cells. To explore further the requirement for Fyn in the tyrosine phosphorylation observed after antagonist stimulation, we bred AD10 TCR transgenic animals with Fyn-deficient animals to obtain $Fyn^{-/-}$ AD10 TCR transgenic mice. T cell lines generated from these mice were then stimulated with agonist or antagonist peptide and analyzed for tyrosine phosphorylation of Vav (Fig. 6*A*) or as a control for Lck activity, for tyrosine phosphorylation of ZAP-70. Analysis of wild-type AD10 cells was included as a comparison. As shown in Fig. 6*A* and as previously reported (21), in the wild-type AD10 cells, Vav tyrosine phosphorylation was increased about 3-fold after stimulation with either antagonist or agonist peptide. In contrast, antagonist-stimulated $Fyn^{-/-}$ AD10 cells had a barely detectable amount of tyrosine-phosphorylated Vav, only 7% of that observed after antagonist stimulation of wild-type AD10 cells. Agonist stimulation of $Fyn^{-/-}$ AD10 cells led to some increase in tyrosine-phosphorylated Vav, but it was still only about 20% of that obtained after agonist stimulation of wild-type AD10 cells. In contrast to the dramatic effect of Fyn deficiency on Vav phosphorylation, as expected, the tyrosine phosphorylation of ZAP-70 in $Fyn^{-/-}$ AD10 cells was nearly as extensive (80%) as that observed in wild-type AD10 cells (Fig. 6*B*).

Fyn Regulation of APC-T Cell Conjugate Formation. Because previous studies have indicated that Vav functions as a guanine nucleotide exchange factor for Rho family GTPases (especially Rac-1) that regulate cytoskeleton reorganization, it would be expected that failure to phosphorylate and thereby to activate Vav would be associated with a defect in cytoskeletal reorganization. Furthermore, because actin polymerization and reorganization are critical for TCR-mediated up-regulation of LFA-1 avidity (36), which in turn is required for the stable interaction between T cell and APC after agonist or antagonist stimulation, it would be predicted that ligand-induced APC–T cell conjugate formation would be impaired in $Fyn^{-/-}$ T cells. To determine the role of Fyn in APC–T cell conjugate formation, $Fyn^{+/+}$ AD10 or $Fyn^{-/-}$ AD10 T cells were labeled with one fluorochrome, and peptide-pulsed APCs were labeled with another fluorochrome, incubated together for 30 min, and analyzed for the presence of APC–T cell conjugates by two-color flow cytometry. As shown in Fig. 7, conjugate formation induced by either agonist or antagonist peptide was greatly impaired (75–80%) in Fyn-deficient AD10 T cells.

Discussion

This study was carried out with the goal of identifying the upstream protein tyrosine kinase that is responsible for initiation of the signaling pathway triggered by the engagement of the TCR with low-affinity TCR antagonist/MHC complexes. Antagonist-mediated signaling had been previously shown to involve tyrosine phosphorylation and activation of the guanine nucleotide exchange factor, Vav, the activation of the target of Vav guanine nucleotide exchange factor activity, Rac-1, and the reorganization of the actin cytoskeleton that is critical to the formation of stable T cell–APC conjugates (21). Previous studies that have investigated the mechanism of Vav phosphorylation have used either heterologous gene transfection or cell-free systems (11–14). These studies have demonstrated that Vav can serve as a substrate for each of the four candidate upstream protein tyrosine kinases: Lck, Fyn, Syk, and ZAP-70. Of particular interest with respect to our findings, Michel *et al.*, by using an antigen-specific hybridoma transfected with dominant negative mutants of PTK genes, found that CD28-mediated Vav phosphorylation was inhibited by DN Fyn and not DN Lck or ZAP-70, whereas both dominant negative forms of Fyn and ZAP-70 were capable of inhibiting anti-CD3-mediated stimulation of Vav phosphorylation (37).

The data presented in this report have led to the conclusion that stimulation of Vav tyrosine phosphorylation by TCR antagonists depends on Fyn activity, and furthermore, Fyn is the major kinase responsible for Vav phosphorylation after agonist stimulation of T cells as well. The evidence to support these conclusions comes from three sources:

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(*i*) When an antigen-specific T cell clone was stimulated by an antagonist peptide presented by APCs, Fyn was the only one of the protein tyrosine kinases investigated that was found to significantly increase its kinase activity over the basal state. Lck activity was only marginally increased after antagonist stimulation, with a significant amount of kinase activity being present in unstimulated cells. No detectable Syk was present in the T cells used, and ZAP-70 was not phosphorylated after antagonist stimulation nor was any increase in kinase activity found after stimulation with antagonist peptide. Furthermore, when some of the substrates that are known to be preferential targets of the various PTKs were studied, only the Fyn substrate SLAP-130/FYB was found to be phosphorylated after antagonist stimulation. Also, when anti-CD3 immunoprecipitates were analyzed, Fyn was shown to have an increased association with the TCR after antagonist stimulation. Thus, these studies with AD10 clones suggested that Fyn was preferentially activated by antagonist, although it could not be ruled out that the high basal activity of Lck was responsible for the observed tyrosine phosphorylation of Vav.

(*ii*) The study of JCaMl cells that were transfected with either Fyn or Lck genes indicated that Vav phosphorylation after TCR crosslinking with anti-CD3 antibodies was efficient in Lck^-, Fyn^{hi} JCl/Fyn cells but not in Lck^{hi}, Fyn^{lo} JCl/Lck cells.

(*iii*) The analysis of AD10 TCR transgenic $Fyn^{-/-}$ T cells stimulated by agonist and antagonist peptides indicated that Vav tyrosine phosphorylation after antagonist stimulation was only 7% of that observed with AD10 Fyn-positive T cells. Furthermore, agonist stimulation of $Fyn^{-/-}$ AD10 cells led to Vav phosphorylation that was only 20% of the level obtained in Fyn-positive cells. In contrast, ZAP-70 phosphorylation proceeded normally in agonist-stimulated $Fyn^{-/-}$ cells as would be expected if Lck activity were normal in such cells. APC–T cell conjugate formation in the AD10 Fyn-deficient cells was also greatly diminished, consistent with the critical role that Vav plays in cytoskeletal reorganization in T cells.

Taken together, these experiments strongly indicate that Fyn is required for efficient phosphorylation of Vav after both antagonist and agonist stimulation of T cells. Although the most likely explanation is that Fyn itself is the tyrosine kinase that phosphorylates Vav, a more indirect role of Fyn cannot be ruled out, such as the phosphorylation of a critical adaptor molecule required for the localization of Vav in proximity to the tyrosine kinase that is responsible for its phosphorylation; or the requirement of Fyn for the activation of another tyrosine kinase that directly phosphorylates Vav.

The relative role of the two Src family PTKs, Fyn and Lck, in T cell signaling is not clear. On the one hand, overexpression of either PTK suggests that their function may be redundant because both can phosphorylate some of the same substrates, such as TCRζ and ZAP-70 (2, 3). On the other hand, some substrates appear to be preferentially phosphorylated by one and not the other enzyme, such as SLAP-130/FYB and PYK-2 phosphorylation by Fyn (16, 17), and as shown in this study, Vav by Fyn. Because of the profound effect of Lck deficiency and the minimal effect of Fyn deficiency on T cell development (38, 39), much of the focus on TCR-mediated signaling has been directed toward the study of Lck. However, there are several studies that support our findings that Fyn may have a particularly important role under suboptimal conditions of T cell signaling. First, when T cells are stimulated solely through the TCR without CD28 costimulation, a state of anergy can be induced that is associated with a marked increase in Fyn kinase activity (40, 41) and TCR-associated Fyn (28). Second, a study of the signaling of $Fyn^{-/-}$ TCR transgenic T cells has shown that, whereas high-affinity ligands stimulate $Fyn^{-/-}$ T cells equally as well as $Fyn^{+/+}$ T cells, low-affinity ligands stimulate $Fyn^{-/-}$ T cells relatively inefficiently compared with $Fyn^{+/+}$ T cells, requiring 5- to 10-fold more antigen to achieve a similar degree of proliferation (42). Third, the recent study by Denny *et al.* (26) of the signaling capabilities of Lck-deficient and Fyn-deficient Jurkat cells indicates that TCR-mediated stimulation of Fyn^+, Lck^- T cells leads to a partially activated phenotype characterized by barely detectable ZAP-70 phosphorylation, with no LAT phosphorylation, a strong phosphorylation of SLP-76, and the phosphorylation of ζ chain that results predominantly in the appearance of the lower molecular weight ζ isoforms. Downstream of these PTK-mediated events, although good intracellular Ca^{2+} increases and Ras and Erk activation were observed in Lck^-, Fyn^{hi} T cells, IL-2 production was only 10–15% of that observed in the Lck^+ Jurkat cells. It is particularly striking that the tyrosine phosphorylation pattern described for the JCl/Fyn cells is identical to that described for stimulation of T cells with altered peptide ligands that are partial agonists or antagonists (18–20).

An important unresolved issue is the significance of this Fyn-regulated signaling pathway in T cell function. Is it directly involved in the mechanism of TCR antagonism or anergy induction? Experiments to evaluate these possibilities are in progress. Finally, our studies confirm and extend previous reports with $Vav^{-/-}$ mice that indicate Vav plays a critical role in TCR-mediated actin polymerization with resultant capping of the TCR and other molecules and places Fyn upstream in this process when physiologic stimuli are used for its induction.

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