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Fyn and ZAP-70 Are Required for Vav Phosphorylation in T Cells Stimulated by Antigen-presenting Cells*

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In T cells, triggering of the T cell antigen receptor or of the co-stimulatory receptor CD28 can direct tyrosine phosphorylation of the signaling protein Vav. We investigated the role played by the protein tyrosine kinases Fyn, Lck, and ZAP-70 in these processes in a T cell hybridoma after physiological stimulation of the T cell receptor (TCR) and CD28. A dominant-negative mutant approach based on overexpression of catalytically inactive alleles of these kinases showed that CD28-induced Vav phosphorylation preferentially requires Fyn, whereas ZAP-70 had no role. Consistently, Vav was strongly phosphorylated in Lck-deficient JCAM-1 cells after CD28 ligation. In contrast, ZAP-70 appeared to control TCR-directed Vav phosphorylation. However, overexpression of ZAP-70 carrying a mutated Tyr^{315}, contained within a motif previously suggested to be a Vav Src homology 2 domain binding site, had little or no effect. Immunoprecipitation assays showed that phosphorylated Vav associated with Fyn after CD28 triggering and that this interaction, likely to involve binding of Fyn Src homology 2 domain to Vav, was more strongly detectable after concomitant CD28 and TCR stimulation. These data suggest that Fyn plays a major role in controlling Vav phosphorylation upon T cell activation and that the mechanism implicating ZAP-70 in this process may be more complex than previously anticipated.

Vav is a signaling protein expressed almost exclusively in cells of hematopoietic origin and is phosphorylated on tyrosine residues in response to a wide variety of stimuli (1). Vav contains two SH3[1] domains spaced by an SH2 domain, a cysteine-rich zinc-binding domain, a pleckstrin homology domain, and a Dbl homology region characteristic of guanine nucleotide exchange factors for the small GTPases of the Rho family (1, 2). Recent studies have provided some clues as to the function of Vav. Thus, its role in positive regulation of lymphocyte activation is inferred from the severe defect in TCR and B cells antigen receptor-mediated activation in Vav-null mice (3–6) and from studies showing that Vav synergizes with TCR stimulation for IL-2 gene transcription (7). Moreover, Vav has been reported to associate with PTKs, adapter proteins such as Grb2, SLP-76, Nck, Crk, as well as cytoskeletal proteins (1) suggesting its potential implication in different signaling pathways. Tyrosine phosphorylation of Vav results in an augmentation of its GDP/GTP exchange activity for the Rho family GTPases, Rac, CDC42, and RhoA (8, 9). These proteins regulate signaling leading to actin cytoskeleton changes (10), and interestingly, T cells lacking Vav are defective in actin cap formation induced by TCR triggering (11, 12). In addition, Vav may be implicated, via the Rho family of GTPases, in the activation of c-Jun N-terminal kinase (8, 13).

In T cells, Vav becomes tyrosine-phosphorylated upon stimulation of the TCR by antibodies (14) or of the co-stimulatory receptor CD28 by its natural ligands B7-1 (CD80) or B7-2 (CD86) and by specific antibodies (15). Why Vav is one of the few PTK substrates whose phosphorylation is directed by the TCR and CD28 (15) is unclear. However, this dual control on Vav may reflect an important regulatory mechanism in lymphocyte activation. Indeed, TCR ligation alone by antigen-major histocompatibility complexes is usually insufficient to trigger full T cell activation and requires co-engagement of CD28 (16). Moreover, also in B cells, Vav phosphorylation is controlled by both the antigen receptor and co-stimulatory molecules (1, 17). Thus, establishing how these receptors control Vav activation is important for understanding its specific function in the context of lymphocyte activation.

The identity of the PTKs that phosphorylate Vav during T cell activation, that is the PTKs connecting the TCR and CD28 to Vav, remains unclear as little effort has been made to address this question in a physiological setting. Co-expression experiments in heterologous cell systems and *in vitro* kinase assays with recombinant proteins indicated that either Lck, Fyn, Syk, or ZAP-70 could phosphorylate Vav (8, 18–20). Moreover, Vav was reported to form a complex with Lck (21) or with ZAP-70 in anti-TCR-stimulated Jurkat T cells (19, 22, 23). However in other works (24, 25), the association of Vav with ZAP-70 was not clearly detected.

In this work, we investigated which PTKs control Vav phosphorylation in T cells upon physiological engagement of CD28 and TCR by their respective ligands. Toward this end, we expressed catalytically inactive mutants of Lck, Fyn, and ZAP-70 in a T cell hybridoma and monitored the effect on Vav phosphorylation. This approach provided evidence for a preferential role of Fyn in the phosphorylation of Vav after CD28 ligation. Moreover, following CD28 stimulation, Vav physically associated with Fyn, and this interaction was more strongly detected upon concomitant engagement of CD28 and TCR. We also show that ZAP-70 influences Vav phosphorylation after TCR engagement by antigen-major histocompatibility complex, but mutation of Tyr^{315} of ZAP-70, a site previously suggested to

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[1] The abbreviations used are: SH, Src homology; Ab, antibodies; Ag, antigen; APC, antigen-presenting cell; KD, kinase-defective; IL-2, interleukin 2; mAb, monoclonal antibody; PTK, protein tyrosine kinase; DMEM, Dulbecco's modified Eagle's medium; TCR, T cell antigen receptor; WT, wild-type.

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be a Vav-binding site, has little or no influence on phosphorylation of the latter.

EXPERIMENTAL PROCEDURES

Antibodies and Peptides—Goat anti-hamster IgG (Southern Biotechnology, Inc.) was used to cross-link anti-CD3 mAb. Polyclonal rabbit antisera used were as follows: anti-Fyn N-terminal sc-16 (Santa Cruz Biotechnology); anti-Fyn serum 428 generated against a trpE fusion protein containing amino acids 25–141 of murine Fyn (26) (a gift from A. Veillette, McGill Cancer Center, Montreal, Canada); anti-Lck N-terminal 3810 directed against a synthetic peptide corresponding to amino acids 39–64 (27) (provided by S. Fischer, Hopital Cochin, Paris), anti-Lck C-terminal 2102 (Santa Cruz Biotechnology), anti-Vav (Upstate Biotechnology Inc.), and anti-ZAP-70 antiserum 4.07 (28). The mAb used were as follows: anti-Lck 3A5 (Santa Cruz Biotechnology); anti-Vav (Vav 30, kindly given by J. Griffin, Dana Farber Cancer Institute, Boston, MA); and anti-phosphotyrosine 4G10 (Upstate Biotechnology). Anti-CD3ϵ mAb 145–2C11 (29) was purified from ascites by protein A. The tetanus toxin peptide tt830-843 was purchased from Neosystem (Strasbourg, France). Unphosphorylated and tyrosine-phosphorylated peptide EPQYEEIPI (27) was obtained from the Organic Chemistry Unit (Institut Pasteur, Paris).

Plasmids and Constructs—The mammalian expression vector pSRα-puro containing the gene coding for the puromycin resistance and ZAP-70-VSV-G (ZAP-70 WT) or kinase-defective ZAP-70 KD obtained by mutation of Asp^{461} to Asn were previously described (30). The ZAP-70-Y315F mutant,[2] containing a phenylalanine substitution at Tyr^{315}, was derived from the ZAP-70WT construct bearing a C-terminal VSV G-protein tag, previously described (30); a 5′ primer (713–734 base pairs) encompasses the *Mlu*I unique site; the 3′ primer, encoding the Y315F mutation, included 1147–1184 base pairs and contained a *Sac*I site. The *Mlu*I-*Sac*I-digested polymerase chain reaction products were ligated with both a *Sac*I-*Nsi*I fragment (1179–1736 base pairs) and a 3.8-kilobase pair ZAP-70WT pBS fragment restricted with *Mlu*I and *Nsi*I. ZAP-70 constructs were subcloned into the *Eco*RI-*Xba*I sites of the pSRα-puro expression vector (pSRα-puro) (30). Mutations were verified by nucleotide sequencing. Kinase-defective Fyn, FynT-MF, was provided from D. Davidson (26) (McGill Cancer Center, Montreal, Canada). This cDNA was then subcloned into pSRα-puro and is referred to as Fyn-KD. The Lck cDNA carrying the mutation at the ATP-binding site (K273A) (a gift of D. Littman, Skirball Institute, New York, NY) was subcloned as an *Eco*R*I*-*Xba*I fragment into pSRα-puro and is referred to as Lck-KD.

Cell Lines—The murine T cell hybridoma T8.1 was previously described and referred to as T.AL.8.1 (31). Murine L transfectant cells were as follows: L625.7 expressing murine B7-1 (CD80) and HLA-DR*1102 (32); Dap-3 cells expressing or not murine B7-1 (a gift from G. Lombardi, Hammersmith Hospital, London, UK); 5-3.1 cells expressing HLA-DRB1*0101 and human B7-1 or not (33). Expression of CD28 and B7-1 was assessed by immunofluorescence by flow cytometry. T8.1 was maintained in DMEM with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (complete medium) supplemented with 400 nM methotrexate, 1 mg/ml G418, 10 mM Hepes, 50 μM β-mercaptoethanol. Media for L cells were as follows: complete minimum Eagle's medium with 250 μg/ml G418 for L625.7 cells; complete DMEM for B7-1$^-$ L cells and complete DMEM supplemented with 50 μg/ml hygromycin for B7-1$^+$ 5-3.1 and Dap-3 cells. Lck-deficient JCAM-1 human T cells (ATCC) were grown in RPMI 1640 supplemented with 10% fetal calf serum, antibiotics, and L-glutamine.

Cell Transfections—Stable transfectants expressing the above constructs were obtained as follows. T8.1 cells (1×10^7 in DMEM, 20% fetal calf serum) were electroporated in a Gene Pulse cuvette (Bio-Rad) with 30 μg of plasmid at 960 microfarads, 0.25 V. After 48 h in normal medium, cells were placed in 96-well tissue culture plates in DMEM containing metothrexate (400 nM), G418 (1 mg/ml), and puromycin (1 μg/ml). Puromycin-resistant transfectants were analyzed for T cell marker expression of CD3, CD4, CD28, CD11a/CD18, CD102 by fluorescence-activated cell sorter (Becton Dickinson) and screened by Western blot for overexpression of the corresponding PTK. Quantification of the proteins was performed by immunoblotting cell lysates with primary antibodies followed by ^{125}I-labeled protein A (Amersham, France) and by using ImageQuant software after scanning in a PhosphorImager (Molecular Dynamics).

T Cell Stimulation and Immunoprecipitation—-T8.1 and derived transfectants were stimulated with B7-1$^+$L625.7 prepulsed or not for 3 h with tt830-843 peptide at the indicated concentrations and as previously described (25). Ab activation was as follows: cells (1 x 10^8/ml) were incubated with 10 μg/ml anti-CD3ϵ 145-2C11 mAb at 4 °C for 30 min and then washed and further incubated after a short prewarming with goat anti-hamster IgG (10 μg/ml) for 1–2 min at 37 °C. Cells were rapidly pelleted and lysed for 20 min on ice in 1% Nonidet P-40 lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA in the presence of inhibitors of proteases and phosphatases (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Pefabloc-sc, 50 mM NaF, 10 mM $Na4P_2O_7$, and 1 mM $NaVO_4$). Postnuclear lysates were precleared for 1 h at 4 °C with protein A-Sepharose (Pharmacia Biotech Inc., Uppsala, Sweden) and then incubated for 2–3 h with antibodies preadsorbed to protein A. Immunoprecipitates were washed twice in 1% Nonidet P-40, twice in 0.05% Nonidet P-40 lysis buffer, and boiled in 2× SDS-polyacrylamide gel electrophoresis reducing sample buffer before electrophoresis on 8% SDS-polyacrylamide gels. Re-immunoprecipitation experiments, immunoblotting, and detection of proteins were performed by enhanced chemiluminescence as previously described (34).

RESULTS

CD28 Engagement by B7-1 Induces Tyrosine Phosphorylation of Vav in the T Cell Hybridoma T8.1—T8.1 is a murine T cell hybridoma expressing CD28 as well as a chimeric human-mouse TCR specific for a tetanus toxin peptide (tt830-843) presented by DR*1102 (31, 35). Fig. 1*A* shows that a substantial increase in tyrosine phosphorylation of Vav occurred in T8.1 cells following incubation for 1–2 min with the DR*1102$^+$ L625.7 fibroblast line (APC) in the absence of antigen, confirming previous results (25). Under the same conditions, addition of an optimal concentration (10 μg/ml) of tt830–843 peptide antigen (Ag) inducing maximal IL-2 production (not shown) resulted in further phosphorylation of Vav (Fig. 1*A*) which could be detected associated with the adapter protein SLP-76 (25). Antigen-independent tyrosine phosphorylation of Vav has been described in the human Jurkat T cell line to depend on CD28 stimulation by its natural ligands B7-1 (CD80) or B7-2 (CD86) (36). This was also the case for the T8.1 hybridoma as incubation with B7-1$^+$ L625.7 cells in the presence of murine CTLA-4Ig, which inhibits the interaction of CD28 with B7 family proteins, resulted in a strong reduction of Vav phosphorylation (Fig. 1*B*), whereas IgG1 control had no effect. These data were further substantiated by demonstrating that only Dap-3 fibroblasts expressing B7-1, but not control Dap-3 cells, were able to induce Vav tyrosine phosphorylation in T8.1 cells (Fig. 1*C*). The *lower panels* of Fig. 1, *B* and *C,* show that similar amounts of Vav, which is expressed only in T8.1 cells (25), were immunoprecipitated. Thus, as for human T cells (36), in T8.1 murine hybridoma CD28 ligation by B7-1 induced tyrosine phosphorylation of Vav independently of antigen-mediated TCR stimulation.

Tyrosine Phosphorylation of Vav Induced by CD28/B7-1 Interaction in T8.1 Cells Is Predominantly Controlled by the PTK Fyn—Previous studies have indicated that the Src-like PTKs Lck, Fyn, and the Syk family PTK ZAP-70 are able to phosphorylate Vav when co-expressed in a heterologous cell system (20). T8.1 hybridoma stimulated by B7-1$^+$ APC provided a more physiological setting to investigate the possible involvement of these PTKs in the phosphorylation of Vav induced by CD28 or TCR stimulation. To this end, a dominant-negative mutant approach was utilized. Thus, kinase-defective (KD) mutants of Fyn (Fyn-KD), Lck (Lck-KD), and ZAP-70 (ZAP-70-KD) were overexpressed in T8.1 to test their relative ability to interfere with the phosphorylation of Vav by the corresponding endogenous wild-type PTKs. Fig. 2, *B* and *D,* shows the detection of Fyn-KD, Lck-KD, or ZAP-70-KD in a number of transfectants and the calculated fold of expression over the wild-type endogenous counterparts (KD/WT ratio) by comparing anti-PTKs immunoblotting of cell lysates from the transfectants and

[2] V. Di Bartolo, D. Mège, V. Germain, M. Pelosi, E. Dufour, J.-M. Pascussi, F. Michel, G. Magistrelli, A. Isacchi, and O. Acuto, manuscript in preparation.

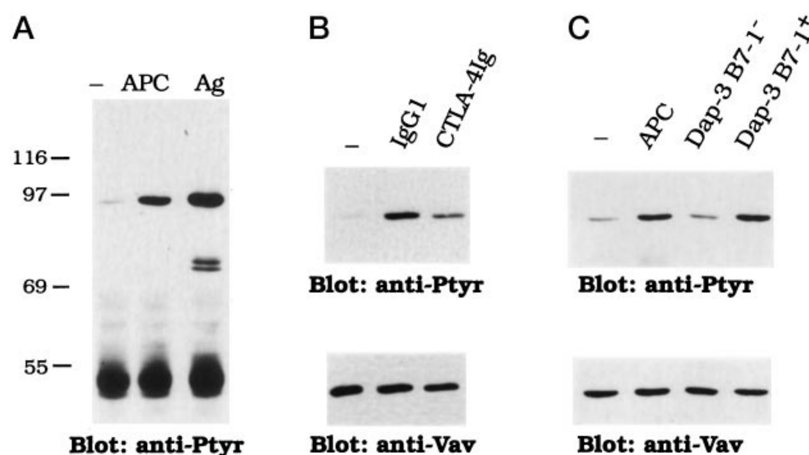

FIG. 1. **Tyrosine phosphorylation of Vav is induced by the CD28 engagement by B7-1 in T8.1 cells.** *A,* T8.1 cells (10×10^6) were incubated for 1.5 min at 37 °C alone (−), with L625.7 cells B7-1$^+$ (3.3×10^6) (APC), or L625.7 B7-1$^+$ cells prepulsed with 10 μg/ml tt830–843 peptide antigen (Ag). Vav was immunoprecipitated from cell lysates with purified polyclonal antiserum and was analyzed after 8% SDS-polyacrylamide gel by anti-phosphotyrosine (*anti-Ptyr*) immunoblotting. *B*, T8.1 cells (6×10^6) were stimulated for 1.5 min at 37 °C with L625.7 B7-1$^+$ (1.2×10^6) in the presence of 50 μg/ml murine CTLA-4Ig or control IgG1. *C*, T8.1 cells (6×10^6) were incubated alone (−) or with L625.7 B7-1$^+$ (*APC*), Dap-3 B7-1$^-$, or Dap-3 B7-1$^+$ cells (2×10^6) for 1.5 min at 37 °C. Dap-3 cells do not express B7–2 (data not shown). *Bottom panels* in *B* and *C*, blots were stripped and reprobed with mAb against Vav.

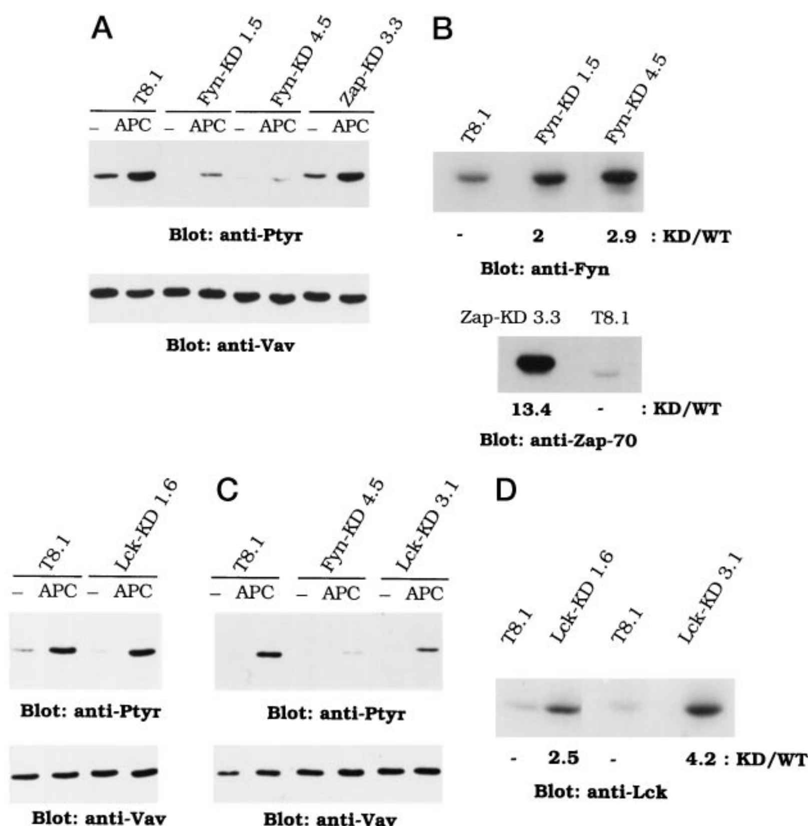

FIG. 2. **Preferential involvement of Fyn in CD28-induced tyrosine phosphorylation of Vav.** *A* and *C*, comparison of CD28-induced Vav phosphorylation between T8.1 cells and transfectants expressing Fyn-KD (*A* and *C*), Lck-KD (*C*), and ZAP-70-KD (*A*). T8.1 cells and the indicated transfectants (7×10^6 in *A* and 5×10^6 in *C*) were incubated at 37 °C for 1.5 min alone (−) or in the presence of L625.7 (*APC*). Vav was immunoprecipitated from cell lysates with polyclonal antiserum and analyzed for tyrosine phosphorylation. *Bottom panels*, immunoblots were stripped and reprobed with mAb anti-Vav. *B* and *D*, quantitation of Fyn-KD (*B*), ZAP-70-KD (*B*) and Lck-KD (*D*) in T8.1 transfectants. Equivalent cell lysates from transfectants and T8.1 cells ($0.6–1.2 \times 10^6$) were loaded on gels, immunoblotted with the indicated antibodies followed by ^{125}I-protein A, and analyzed with a PhosphorImager as described under "Experimental Procedures." *KD/WT* indicates the ratios of KD mutant over endogenous WT protein expression and was determined from band volume values as follows: (KD − WT)/WT. *Numbers* indicated are the means of three different quantification assays. *anti-Ptyr,* anti-phosphotyrosine.

T8.1 parental cells. All the transfectants expressed CD28 and TCR/CD3 at levels similar to those detected in T8.1 cells, as determined by flow cytometry (not shown). Moreover, they exhibited a decrease in IL-2 production in response to Ag stimulation which correlated with increasing levels of KD mutants expression (not shown).

Fig. 2*A* shows that basal (−) and CD28-mediated (APC) tyrosine phosphorylation of Vav were considerably inhibited in the transfectant Fyn-KD 1.5 (KD/WT: 2), and a greater effect was observed in the transfectant Fyn-KD 4.5 expressing a higher level of the mutant (KD/WT: 2.9). Similar inhibitions were observed with two other transfectants expressing comparable amounts of Fyn-KD (not shown). In contrast, no inhibition of CD28-induced phosphorylation of Vav was obtained in the transfectant Lck-KD 1.6 expressing the mutant protein 2.5-fold more than the endogenous Lck (Fig. 2*C*). The difference in the effect of Fyn-KD *versus* Lck-KD was also evident when comparing directly transfectants expressing higher levels of the mutants such as Fyn-KD 4.5 and Lck-KD 3.1, respectively (Fig. 2*D*). Although CD28-mediated Vav phosphorylation began to decrease in the Lck-KD 3.1 cells (KD/WT: 4.2), this effect was less pronounced than in Fyn-KD 4.5 which had a lower KD/WT ratio (2.9). Other transfectants with KD/WT ratios of ~10 for the two Src PTKs were also analyzed. However, in these cells the inhibition of Vav phosphorylation was complete irrespective of the mutant overexpressed (data not shown). The

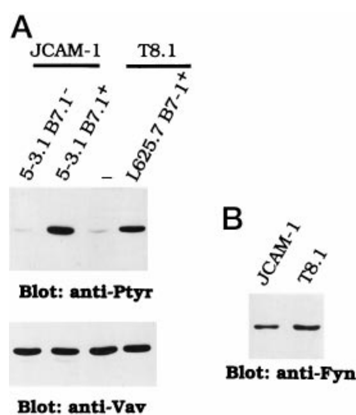

FIG. 3. **CD28-mediated tyrosine phosphorylation of Vav in the Lck-deficient human JCAM-1.** *A*, JCAM-1 (6×10^6) was incubated for 1.5 min at 37 °C with 5–3.1 L cells (2×10^6) expressing or not human B7-1. In parallel, T8.1 cells were kept unstimulated (−) or incubated in the same conditions with L625.7 cells expressing murine B7-1. Vav was immunoprecipitated with polyclonal antiserum and analyzed for tyrosine phosphorylation by immunoblotting. *Bottom panel*, immunoblots were stripped and reprobed with mAb anti-Vav. *B*, equivalent amounts of protein determined by Bradford colorimetric assay were immunoblotted with polyclonal antiserum against Fyn. *anti-Ptyr,* anti-phosphotyrosine.

strong dominant-negative effect produced by only a 2–3-fold excess of Fyn-KD over endogenous wild-type was somewhat surprising but may be explained in part by a threshold effect. These data suggested that Fyn has a role in controlling the phosphorylation of Vav upon CD28 physiological ligation and may be dominant with respect to Lck. In contrast to Src PTKs, a ZAP-70 kinase-defective mutant expressed at much higher levels over endogenous wild-type in the T8.1 transfectant Zap-KD 3.3 (KD/WT: 13.4, see Fig. 2*B*) caused no detectable change in CD28-mediated Vav phosphorylation (Fig. 2*A*). This same mutant behaves as a dominant-negative to inhibit TCR-mediated signaling in Jurkat (30) and in T8.1 cells[2] (and see below).

To provide additional evidence for a role of Fyn in Vav phosphorylation, we made use of the Jurkat-derived T cell line JCAM-1 that lacks Lck but still retains Fyn expression (37) and asked whether CD28 engagement could still induce Vav phosphorylation. Previous studies have indicated that TCR-mediated signaling is largely inhibited in JCAM-1 cells (37). However, similarly to T8.1 stimulated with L625.7 cells, tyrosine phosphorylation of Vav was strongly induced in JCAM-1 following incubation with murine fibroblasts expressing human B7-1 (5–3.1 B7-1$^+$ cells) but not with the same cells lacking B7-1 (Fig. 3*A*) or in the presence of human CTLA4Ig (not shown). Immunoblots with anti-Fyn and anti-Vav Abs revealed that both proteins were comparably detectable in JCAM-1 and T8.1 cells (Fig. 3*B*).

CD28 Ligation by B7-1 Induces an Association of Fyn with Vav That Augments after Concomitant TCR Engagement—Src kinases have often been found to associate with their substrates via their regulatory SH2 and/or SH3 domains (38). In view of the above results (Fig. 2), we assessed whether Fyn was associated with Vav after T cell stimulation. As illustrated in Fig. 4*A*, when T8.1 hybridoma was stimulated with L625.7 cells (APC), lysed and subjected to immunoprecipitation with anti-Fyn Ab, a tyrosine-phosphorylated 95-kDa molecular species was detected that co-migrated with tyrosine-phosphorylated Vav (*cf. lanes 2* and *4*). Moreover, the intensity of the 95-kDa phosphoprotein detected in anti-Fyn immunoprecipitate increased when L625.7 cells were prepulsed with tt830-841 peptide (Ag). Similar results were obtained with another anti-Fyn antiserum (not shown). Re-immunoprecipitation of denatured

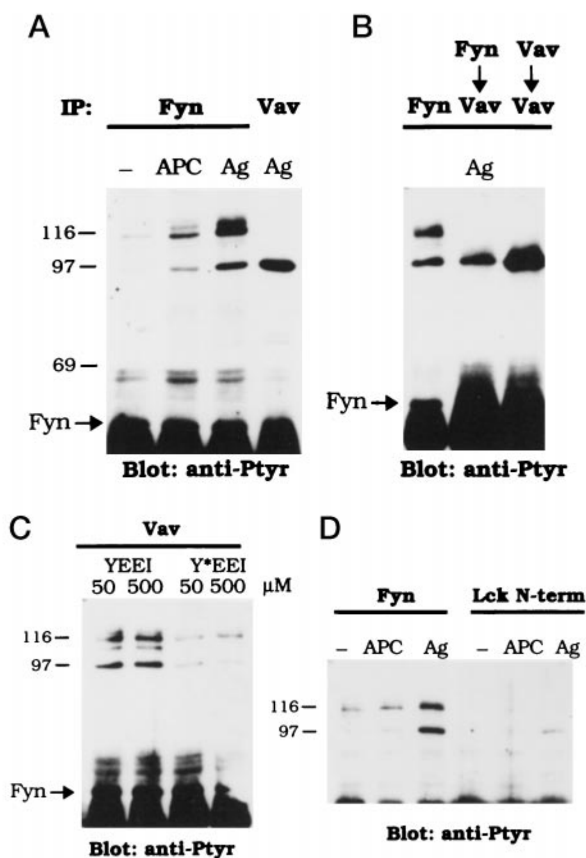

FIG. 4. **Coprecipitation of Vav with Fyn after B7-1 and antigenic stimulation of T8.1 cells.** *A*, T8.1 cells (20×10^6) were incubated for 2 min at 37 °C alone (−), with L625.7 cells (7×10^6) (*APC*), or L625.7 cells prepulsed with tt830–843 (10 µg/ml) (*Ag*). Cell lysates were immunoprecipitated with anti-Fyn polyclonal antiserum. In parallel, T8.1 cells (5×10^6) were stimulated with antigen-pulsed L.625.7 cells as above, and Vav was immunoprecipitated from cell lysates with polyclonal antiserum. *B*, T8.1 cells (30×10^6) were stimulated with antigen-pulsed L625.7 cells as in *A*. Cell lysate was divided into three aliquots; one-third (Fyn) was precipitated with anti-Fyn polyclonal antiserum; one-third (Fyn → Vav) was first immunoprecipitated with anti-Fyn antiserum, subjected to denaturation in SDS to elute bound material, quenched with Nonidet P-40 as described (34), and re-immunoprecipitated with polyclonal anti-Vav antiserum; one-third (Vav → Vav) was first immunoprecipitated with anti-Vav polyclonal antiserum, subjected to a denaturation/quenching cycle as above, and re-immunoprecipitated with anti-Vav antiserum. This sample served as a control for total Vav recovered. *C*, T8.1 cells (40×10^6) were stimulated with antigen as in A. Lysates corresponding to 10×10^6 cells were immunoprecipitated with polyclonal anti-Fyn antiserum in the presence of the phosphorylated QY*EEIPI or unphosphorylated peptide at the indicated concentrations. *D*, T8.1 cells (20×10^6) were incubated for 1.5 min at 37 °C alone (−), with L625.7 cells (7×10^6) (*APC*), or peptide-pulsed L625.7 cells (*Ag*). One-half of each lysate was immunoprecipitated with polyclonal anti-Fyn antiserum and the second half with antiserum against Lck N-terminus (*N-term*) (3810). Immunoprecipitates were immunoblotted with anti-phosphotyrosine mAb. *anti-Ptyr,* anti-phosphotyrosine.

anti-Fyn immunoprecipitates with anti-Vav Ab from antigen-stimulated cells confirmed that the 95-kDa species was Vav (Fig. 4*B*). These latter experiments also showed that a substantial fraction of tyrosine-phosphorylated Vav was found associated with Fyn especially after Ag stimulation (*cf*. Fig. 4*B, lanes 2* and *3*). Co-immunoprecipitation of Fyn with Vav was also seen after anti-CD3 stimulation (data not shown). The reverse experiment, namely the immunoprecipitation with anti-Vav Ab and the detection of Fyn by immunoblotting or by re-immunoprecipitation, was attempted. However, because of the high background caused by the immunoglobulin heavy chains, it was impossible to visualize Fyn. Other proteins of 110–130 kDa coprecipitated with Fyn of which the major one is likely to be p116 Cbl, previously reported to form a complex with Fyn

(39–41). The association of Fyn with Vav could be mediated by the SH2 domain of Fyn. Indeed, Vav possesses in its acidic region a phosphorylatable tyrosine (Tyr^{174}) in a YEDL motif (19) similar to the YEEI optimal binding motif for Src-PTK SH2 domains (42), and Vav SH2 domain does not bind to Fyn (23). If Fyn bound via its SH2 domain to Vav, a phosphorylated YEEI peptide, but not its unphosphorylated form, should be able to dissociate their complex, as we have previously shown for other SH2-mediated protein-protein interactions (33, 34). Thus, lysates of T8.1 cells stimulated with antigen presented by L625.7 cells were preincubated with different concentrations of phosphorylated YEEI (Y*EEI) or with its unphosphorylated analog and then subjected to immunoprecipitation with anti-Fyn Ab. Fig. 4*C* shows that Y*EEI was able to dissociate Fyn from Vav, in agreement with the hypothesis that their interaction is SH2-mediated. Of note is that Fyn was also dissociated from the 110–130-kDa phosphoproteins.

To test whether the association of Vav with Fyn is preferential or whether it can also involve Lck, the capacity of these PTKs to coprecipitate with Vav was compared. As shown in Fig. 4*D*, coprecipitation of Vav with Lck was barely detectable when using an Ab directed at the N terminus of Lck. Similar data were obtained with another antiserum directed at the C terminus of Lck (data not shown). These anti-Lck Abs do not seem to sterically interfere with the SH2 domain of Lck as they have been shown to reveal an SH2-mediated association of Lck with ZAP-70 (30, 34). The other proteins of 110–130 kDa usually present in anti-Fyn precipitates were also not seen associated with Lck. These results indicated that upon stimulation of T8.1 cells via CD28 or CD28 plus TCR, Vav associates preferentially with Fyn and reinforces the notion that Fyn may be implicated in Vav phosphorylation.

ZAP-70-KD Mutant Inhibits Tyrosine Phosphorylation of Vav by TCR Triggering, Whereas ZAP-70-Y315F Mutant Has Little or No Effect—Stimulation of the T8.1 hybridoma with Ag-pulsed $B7\text{-}1^{+}$ APC leads to an increase in Vav phosphorylation above the level induced by CD28 engagement alone (Fig. 1). Studies in Jurkat cells stimulated with agonistic anti-TCR Abs and in heterologous cell systems co-expressing Vav and Syk family PTKs have suggested that the phosphorylation of Vav can be mediated by Syk and/or ZAP-70 (19, 20, 43). Although in our T cell hybridoma we could detect Vav association with Fyn after Ag stimulation (Figs. 3 and 4) but not with ZAP-70 (25),[3] the involvement of ZAP-70 in Vav phosphorylation was investigated using the transfectant Zap-KD 3.3 expressing high levels of ZAP-70-KD (Fig. 2*A*). Fig. 5*A* shows that phosphorylation of Vav induced by anti-CD3 was nearly abolished in the transfectant Zap-KD 3.3 as well as detection of coprecipitating SLP-76 and is in agreement with similar data obtained by overexpressing in Jurkat cells a ZAP-70 mutant lacking the catalytic domain (43). When Zap-KD 3.3 was stimulated by Ag (Fig. 5*B*), Vav phosphorylation also decreased but returned approximately to the level induced by the CD28/B7-1 interaction (APC) and SLP-76 detection was lost. The same observations were reproduced in another transfectant, Zap-KD 1.1, expressing similar high levels of ZAP-70-KD (data not shown). These results are consistent with ZAP-70 playing a role in the additional phosphorylation of Vav due to TCR engagement. In contrast, SLP-76 appears to be a PTK substrate exclusively controlled through the TCR, most likely via ZAP-70 (20, 44). The reason why Vav coprecipitated only with the faster migrating band of the SLP-76 doublet when using anti-CD3 while both bands were seen after Ag stimulation is unclear. However, this observation appears to be in accordance

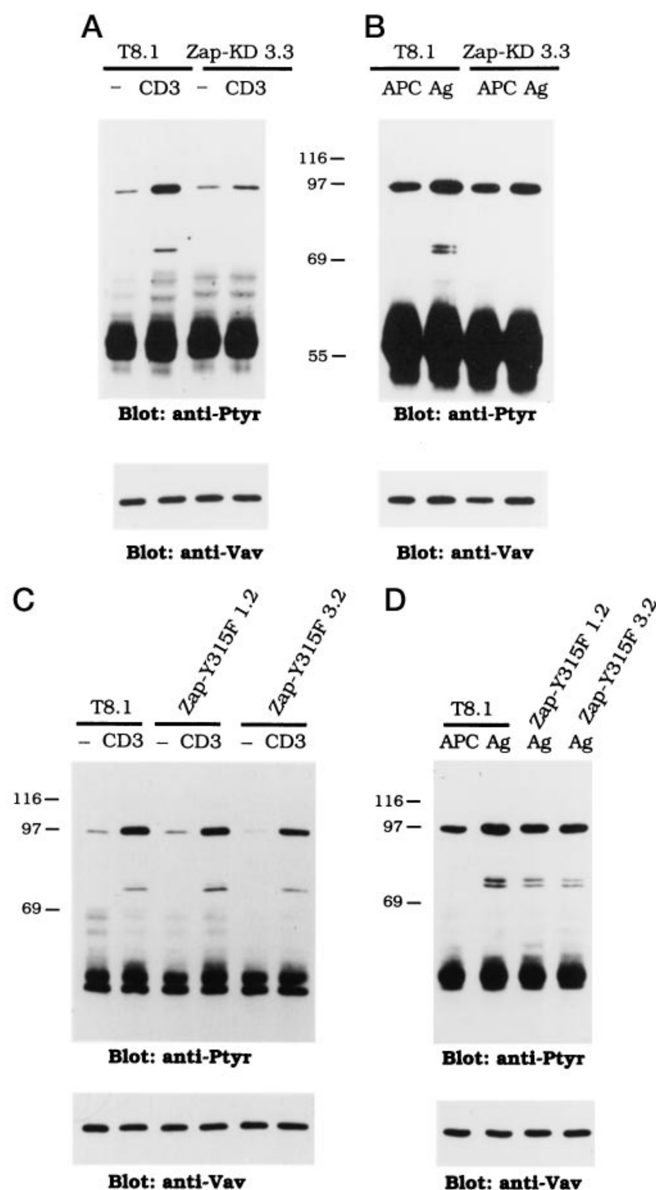

FIG. 5. **TCR-mediated Vav tyrosine phosphorylation is dependent on ZAP-70 kinase activity but not or only weakly so on the Tyr^{315}.** *A*, T8.1 cells and the transfectant Zap-KD 3.3 expressing catalytically inactive ZAP-70 (10×10^6) were stimulated or not with anti-CD3ϵ 145–2C11 for 2 min at 37 °C. *B*, the same cells were stimulated for 2 min at 37 °C with L625.7 (3.3×10^6) prepulsed with peptide (10 μg/ml) (*Ag*) or not (*APC*). *C*, T8.1 cells and the indicated Zap-Y315F transfectants were stimulated with anti-CD3ϵ as in *A*. *D*, hybridomas were stimulated by antigen as in *B*. Vav was immunoprecipitated from cell lysates with polyclonal Ab and immunoblotted with anti-phosphotyrosine mAb. *Bottom panels*, immunoblots were stripped and reprobed with mAb anti-Vav. *anti-Ptyr,* anti-phosphotyrosine.

with a previous work (45) showing that concomitant engagement of CD28 and TCR favors TCR-dependent substrate phosphorylation.

It has been proposed that Vav binds through its SH2 domain to a conserved Tyr^{315} ESP motif present in the linker region of Syk and ZAP-70, and as a consequence, Vav is phosphorylated by these PTKs (19, 46). Therefore, to provide a more precise basis for the control of Vav phosphorylation by ZAP-70, we tested in our system the effect of mutating Tyr^{315} of ZAP-70. T8.1 transfectants were generated expressing levels of a ZAP-70-Y315F mutant comparable to those attained with ZAP-70-KD. The results for two of them, Zap-Y315F 1.2 and Zap-Y315F 3.2 (KD/WT of 15 and 16, respectively), are shown. In contrast

[3] F. Michel, L. Tuosto, and O. Acuto, unpublished observations.

to ZAP-70-KD mutant, overexpression of ZAP-70-Y315F did not appreciably modify Vav phosphorylation induced by anti-CD3 stimulation compared with T8.1 parental cells (Fig. 5*C*). Fig. 5*D* shows also that in the same transfectants Ag-mediated Vav phosphorylation (and coincidentally SLP-76 association) was only slightly decreased, and in repeated experiments, we could never observe a return to the phosphorylation levels induced by APC alone in these transfectants. Moreover, in two other transfectants tested that expressed comparable levels of ZAP-70-Y315F little or no change in Ag-induced Vav phosphorylation was observed (data not shown). In ZAP-70-Y315F-overexpressing cells, Ag-induced IL-2 production was not or only weakly decreased compared with T8.1 cells, whereas it was markedly altered in Zap-KD 3.3 and Zap-KD 1.1 transfectants.[2] Taken together, these data suggest that TCR-mediated phosphorylation of Vav induced by antigenic stimulation is dependent on ZAP-70 activity but that mutation of the previously described Vav-binding site on ZAP-70 had little or no effect on Vav phosphorylation.

DISCUSSION

In this work we investigated which of the PTKs, Lck, Fyn, and ZAP-70 that play a major role in T cell activation, control Vav phosphorylation upon TCR and CD28 engagement with Ag/major histocompatibility complex and B7-1, respectively. By employing a dominant-negative mutant approach, we show that Vav phosphorylation due to CD28 ligation is dependent on the Src PTKs, in particular on Fyn. In contrast, ZAP-70 did not appear to play any role in CD28-induced Vav phosphorylation. We show also that co-engagement of CD28 and TCR leads to additional accumulation of phosphorylated Vav. We have recently demonstrated that under conditions that mimic physiological stimulation (*e.g.* Ag presented by APC), the absence of CD28 engagement results in a dramatic reduction of TCR-proximal signaling capacity (*e.g.* tyrosine phosphorylation of ζ and ZAP-70) (45), suggesting that some CD28 signals may directly feed into the TCR. Thus, the sole contribution of the TCR to Vav phosphorylation could not be formally analyzed under the stimulatory conditions used here (*e.g.* antigen presentation). However, our data strongly suggest that ZAP-70 is implicated in this pathway, as the increment of Vav phosphorylation due to TCR engagement was abolished by overexpression of the ZAP-70-KD mutant. The observation that a sizable fraction of phosphorylated Vav was co-immunoprecipitated with Fyn after Ag stimulation (Fig. 4) supports the idea that Fyn is also implicated in controlling Vav activation via the TCR. Although Fyn-KD inhibited also TCR-mediated Vav phosphorylation (data not shown), this latter result cannot be unambiguously interpreted as this mutant may interfere with immunoreceptor tyrosine-based activation motif phosphorylation and therefore with ZAP-70 activation. The substantial levels of phosphorylated Vav already attained with CD28 alone and the additive accumulation after TCR triggering suggest that the co-stimulatory signal plays a major role in Vav activation. Thus, it is possible that when a T cell encounters a professional APC (*e.g.* a dendridic cell expressing high levels of B7 family proteins) Vav phosphorylation is primarily directed by CD28 due to the higher density of its ligands compared with the density of TCR ligands (*e.g.* orders of magnitude lower) and have a critical role in helping to sustain TCR signaling (45). Interestingly, Klasen *et al.* (47) reported that CD28-induced Vav phosphorylation is more persistent than the one stimulated via the TCR.

Three lines of evidence suggest that Fyn may play a major role in CD28-driven phosphorylation of Vav. First, when expressed at comparably low mutant/wild-type ratios in T8.1 cells, Fyn-KD was more effective than Lck-KD as a dominant-negative mutant to inhibit Vav phosphorylation. Second, upon CD28 ligation Fyn was found associated with Vav, an interaction that may promote efficient Vav phosphorylation (discussed below). We found no gross difference between Fyn and Lck expression in T8.1 cells with the antisera used in this study, whereas in Jurkat cells the same reagents evidenced higher levels of Lck over Fyn (data not shown). It is therefore unlikely that our results were biased by an abnormally higher expression of Fyn over Lck. Third, Vav phosphorylation occurred efficiently in CD28-stimulated JCAM-1 cells which lack Lck but express Fyn. This result is of relevance since in this cell line TCR-driven tyrosine phosphorylation of cellular substrates is dramatically inhibited (37). Fyn (and Lck) can phosphorylate Tyr^{173} (48) in the intracellular portion of CD28. However, the inhibition of Vav phosphorylation produced by Fyn-KD (and to a minor extent by Lck-KD) cannot be due to an indirect effect on Tyr^{173} as it was recently shown that a Y173F mutation does not modify CD28-induced Vav phosphorylation (47). Nevertheless, the neighboring sequence C-terminal to Tyr^{173} was found to be critical for Vav phosphorylation (47, 49), but the underlying mechanism remains to be understood. Alternatively, Itk, a PTK reported to interact with, and be activated by, CD28 could be responsible for Vav phosphorylation. Since Itk is activated by Src PTKs, including Lck (50–52), the effects of Fyn-KD on Vav could be explained by an inhibition of Itk activation. However, Gibson *et al.* (52) have reported that Itk is not activated via CD28 in JCAM-1 and requires expression of Lck, whereas Vav phosphorylation occurs in this mutant cell line, as shown here. Moreover, mutation of Tyr^{173} considerably reduced CD28-induced Itk activation (53) but not Vav phosphorylation (47). These data argue against Itk being implicated in Vav phosphorylation. Thus, in light of our results, Fyn is the most likely candidate to phosphorylate Vav directly.

We found that ZAP-70 catalytic activity was critical for directing phosphorylation of Vav after physiological engagement of the TCR. However, Tyr^{315} present in the linker region of ZAP-70, thought to be important for Vav phosphorylation (23, 46), appears to be essentially dispensable for this function (Fig. 5) and also for IL-2 production.[2] Our results contrast with those of Wu *et al.* (46) who found that the same mutation could not complement a Syk PTK-deficient chicken B cell lymphoma for a defective phosphorylation of several signaling proteins including Vav and for nuclear factor of activated T cell activation. This discrepancy may be due to the different cellular systems utilized. However, we also found that, compared with ZAP-70-KD, ZAP-70Y315F exerted a very weak effect on TCR-induced nuclear factor of activated T cell activation when overexpressed in Jurkat cells.[2] Thus, additional experiments are needed to definitively establish whether Tyr^{315} of ZAP-70 has a role in Vav phosphorylation, leaving open the question of how ZAP-70 exerts this function during TCR ligation. In this context, it is intriguing that, despite the inhibiting effect of ZAP-70-KD on TCR-directed Vav phosphorylation, we found that Fyn, but not ZAP-70 (this work and Refs. 24 and 25), was detected associated with Vav. In line with this observation, others (21) have previously reported that in Jurkat cells Vav forms a complex with Lck after TCR stimulation. The difference with our results in the T8.1 cells can be explained by the non-physiological high levels of Lck expressed in Jurkat cells compared with T cell hybridomas (Ref. 54 and data not shown) and by the largely, although not completely, redundant roles of Lck and Fyn in T cell activation (55–57). Our result indicating that Vav-Fyn interaction requires tyrosine phosphorylation is consistent with the observation that recombinant SH2 domain of Fyn binds to phosphorylated Vav (58). A direct SH2-mediated association is likely to promote an effective catalytic reaction as it

has been suggested for Fyn and Src with some of their known substrates (38, 59, 60). According to a processive mechanism proposed for Src PTKs (59, 61), Fyn could phosphorylate Vav at the very same tyrosine serving as an SH2-docking site to ensure further phosphorylation of Vav. Support for a kinase-substrate relationship between Fyn and Vav comes from *in vitro* data (8, 18, 20) but also from *in vivo* data in T cells overexpressing wild-type or activated Fyn (62) and in CD4/CD8 double negative T cells from *lpr* mice that presented elevated Fyn kinase activity and showed a constitutive tyrosine phosphorylation of Vav (63). ZAP-70 may also contribute to Vav phosphorylation and may be necessary for fine-tuning the Vav function requested during TCR engagement. In this context, it will be interesting to ascertain in our system whether qualitative or quantitative differences exist in the phosphorylation pattern of Vav after CD28 or CD28 plus TCR triggering. If a sequential involvement of Syk and Src PTKs in Vav phosphorylation does exist as suggested by *in vitro* experiments (19), this could apply to the TCR but not the CD28 pathway. Finally, we cannot exclude the possibility that *in vivo* ZAP-70 exerts an indirect effect on Vav phosphorylation by favoring a better access of some substrates to activated Src PTKs during TCR ligation. By showing that Vav interacts with Fyn after physiological stimulation of TCR and CD28, our data may contribute to define better their function during T cell activation.

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