THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 5, Issue of February 2, pp. 3574–3580, 2001

Printed in U.S.A.

Defective Signaling to Fyn by a T Cell Antigen Receptor Lacking the α-Chain Connecting Peptide Motif *

Received for publication, September 20, 2000, and in revised form, October 31, 2000
Published, JBC Papers in Press, October 31, 2000, DOI 10.1074/jbc.M008588200

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A key role in the communication between the αβTCR and the CD3/ζ complex is played by a specific motif within the connecting peptide domain of the TCR α chain (α-CPM). T cell hybridomas expressing an α-CPM-mutated TCR show a dramatic impairment in antigen-driven interleukin-2 production. This defect can be complemented by a calcium ionophore, indicating that activation of the calcium pathway is impaired. Several lines of evidence implicate Fyn in the regulation of calcium mobilization, at least in part through the activation of phospholipase Cγ. Here we have investigated the potential involvement of Fyn in the TCR α-CPM signaling defect. Using T cell hybridomas expressing either a wild-type TCR or an α-CPM mutant, we show that Fyn fails to be activated by the mutant receptor following SEB binding and fails to generate tyrosine-phosphorylated Pyk2, a member of the focal adhesion kinase family. This defect correlated with an impairment in phospholipase Cγ phosphorylation. Production of interlukin-2 and activation of the transcription factor NF-AT in response to triggering of the TCR α-CPM mutant with SEB were fully restored in the presence of constitutively active Fyn. Hence the signaling defect generated by the TCR α-CPM mutation results at least in part from an impaired coupling of the TCR·CD3 complex to Fyn activation.

Engagement of the T cell antigen receptor (TCR)[1] complex by cognate peptide antigen presented by major histocompatibility complex molecules is a central requirement for T cell development and activation (1, 2). Following interaction with its ligand, the TCR triggers a tyrosine kinase (PTK) cascade orchestrated by Src and Syk family PTKs (2, 3). As opposed to receptor PTKs, the TCR splits the dual function of ligand binding and signal transduction between a recognition module, the αβ heterodimer, which is responsible for ligand binding, and an effector module, the CD3/ζ complex, which interacts with the constant regions of the TCRαβ and is composed of the γε and δε heterodimers and a more loosely bound ζ/ζ or ζ/η dimer (4). The intracellular domains of all the components in the CD3/ζ complex contain specific motifs, termed ITAMs (immunoreceptor tyrosine-based activation motif), which are present as one copy in the γ, δ, and ε chains and as three copies in the ζ chain. Upon TCR engagement these ITAMs become phosphorylated on their dual tyrosine residues by the Src kinases Lck and Fyn and as such become docking sites for SH2 domain containing proteins. This permits recruitment to the activated receptor of a number of signaling proteins, among which the central one is the Syk family PTK, ZAP-70. ZAP-70 preferentially interacts with fully phosphorylated ζ, an event which results in enhancement of its kinase activity and autophosphorylation/transphosphorylation on a number of tyrosine residues, which in turn permits recruitment of the next components in the signaling cascade (2, 3).

The evolution of a signaling strategy based on an effector module composed of multiple elements, each of which can be involved in protein recruitment to the activated receptor, has profound implications in terms of signal output, both in terms of quality and quantity. This is an essential feature for a receptor which must evaluate the quality of the ligand and translate it into a signal which can lead to activation, anergy, or apoptosis. The drawback of a receptor which has distinct ligand binding and signaling modules is the requirement of a mechanism whereby the TCRαβ can inform the CD3/ζ complex that contact with the specific peptide ligand has occurred and that signaling must initiate. An important role in this process has been recently attributed to a conserved motif (α-CPM) within the α-chain connecting peptide domain of the TCRαβ. Mutation of this motif, while not affecting surface expression of the TCR·CD3 complex, results in specific functional deficits (5). Most notably, thymocytes in transgenic mice expressing an α-CPM-mutated TCR fail to undergo positive selection (6). Furthermore, T cell hybridomas expressing this mutated receptor exhibit a dramatic impairment in IL-2 production and activation of the transcription factor NF-AT, despite a normal phosphorylation of the ζ chain. Interestingly, this defect can be complemented by a calcium ionophore, indicating that activation of the calcium pathway is specifically impaired (5).

Several lines of evidence implicate Fyn in the activation and modulation of calcium flux. Overexpression in T cells of wild-type Fyn, or expression of a constitutively active mutant, results in enhanced calcium mobilization and overcomes the requirement for a calcium ionophore in a pharmacological model of NF-AT activation (7, 8). In addition, ζ chain-mediated calcium mobilization can be reconstituted by Fyn coexpression in a heterologous cell system (9). At least part of this effect is achieved, either directly or indirectly, through phosphorylation and subsequent activation of PLCγ (10, 11), which hydrolyses membrane phospholipids, resulting in increased levels of IP3

* The work was supported in part by the Associazione Italiana per la Ricerca sul Canero (AIRC), Telethon Grant E.1161, and the University of Siena (PAR). The Basel Institute for Immunology was founded and is supported by Hoffmann-La Roche Ltd., Basel, Switzerland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a fellowship from the Federazione Italiana per la Ricerca sul Canero (FIRC).

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[1] The abbreviations used are: TCR, T cell antigen receptor; PTK, protein-tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motif; IL-2, interleukin-2; IP_3, inositol 1,4,5-trisphosphate; APC, antigen presenting cell; PLCγ, phospholipase Cγ; mAb, monoclonal antibody; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SEB, staphylococcal enterotoxin B.

and release of calcium ions from intracellular stores. Furthermore, Fyn has been shown regulate the function of the IP3 receptor by phosphorylation, thereby promoting release of calcium from the endoplasmic reticulum (12). Part of the effect of Fyn appears, however, independent of phosphatidylinositol bisphosphate hydrolysis, although the underlying mechanisms are not known (13). Here we have investigated the potential involvement of Fyn in the signaling defect displayed by a TCR harboring a mutant α-CPM.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Plasmids—The T cell hybridomas expressing the SEB-specific TCR 3BBM74 and the TCR α-CPM mutant αIV/βIII have been described previously (5). An Epstein-Barr virus-transformed B cell line (a kind gift of A. Lanzavecchia) was used as antigen presenting cell (APC). Transient transfection experiments were carried out on the TCR negative Jurkat subline J31.13 (14). Cells were grown in RPMI (Life Technologies Italia srl) supplemented with 7.5% fetal calf serum, 2 mM L-glutamine, and 100 IU/ml penicillin. The packaging line, GP+E-86 was grown in Iscove's modified Dulbecco's medium supplemented as above.

Polyclonal rabbit antisera against PLCγ, Fyn, Pyk2, and Lck, and anti-phosphotyrosine mAb, were purchased from Upstate Biotechnology Inc. (Boston, MA). Anti-Fyn mAb was purchased from Transduction Laboratories (Mamhead, United Kingdom). Anti-ZAP-70 and anti-CD3ζ mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mAb suitable for immunoprecipitation of tyrosine-phosphorylated CD3ζ was kindly provided by M. Baniyash. The anti-Vβ8.1 mAb F23.1 (15) was purified from hybridoma culture supernatants using protein G (Amersham Pharmacia Biotech Italia, Milan). Peroxidase-conjugated secondary antibodies were purchased from Amersham Pharmacia Biotech Italia (Milan). Secondary unlabeled antibodies were purchased from Cappel (Durham, NC).

NF-AT/luc contains a trimer of the NF-AT-binding site of the IL-2 promoter upstream of the gene encoding firefly luciferase (16). The expression constructs encoding constitutively active mutants of Fyn, Lck, and Ha-Ras were described previously (17–19). The cDNA encoding F528Fyn was subcloned first into pGEM-3Z (Promega, Madison, WI), then recovered as a *Bam*HI fragment and cloned into the *Bam*HI site of the retroviral vector LXSH (20).

Transfections, Luciferase Assays, and Flow Cytometry—Transfections were carried out as described using a modification of the DEAE-dextran procedure (16). To minimize variability among samples, activation assays were carried out on duplicate aliquots of a single pool of transfected cells. Cells were allowed to recover for 22 h prior to activation and activated either with SEB as described (5) or with a combination of phorbol 12-myristate 13-acetate (100 ng/ml) and A23187 (500 ng/ml). Cells were collected 8–10 h after activation and assayed for luciferase activity using the protocol from Promega and a BioOrbit 1253 luminometer. Luciferase activities in cells tranfected with a control vector were barely above background in the absence of stimulation (0.005–0.020 relative luciferase units). Although transfection with the expression vectors encoding F528Fyn or F505Lck significantly increased the level of basal luciferase activity, this was further enhanced following stimulation with antigen. To normalize the results and compare them among different experiments, basal activities were subtracted from induced activities. The resulting values were expressed as ratio of the SEB-induced activity to maximal phorbol 12-myristate 13-acetate/A23187 induced activity. Cells were analyzed for TCR/CD3 surface expression using a FacsScan flow cytometer (Becton-Dickinson, San Jose, CA) and saturating concentrations of biotinylated B20.1 (anti-Vα2.1 mAb) or F23.1 (anti-Vβ8.1 mAb) and phycoerithrin-labeled streptavidin, or fluoresceinated 145.2c11 (anti-CD3 mAb).

Activations, Immunoblots, Immunoprecipitations, and Kinase Assays—Activations with mAb or SEB were carried out as described (5, 21). The optimal stimulation time using SEB-loaded APCs was determined by a time course analysis of protein tyrosine phosphorylation. The ratio of T cells to APCs was 10:1. Cells ($2–5 \times 10^7$/sample) were lysed in 1% Nonidet P-40 in 20 mM Tris-HCl, pH 8, 150 mM NaCl (in the presence of 0.2 mg/ml sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aproteinin, 1 μg/ml pepstatin, and 10 mM phenylmethylsulfonyl fluoride) and postnuclear extracts were immunoprecipitated using the appropriate antibodies and protein A-Sepharose (Amersham Pharmacia Biotech Italia) or agarose-conjugated anti-mouse antibodies (Sigma Italia srl, Milan). When required, the milder detergents digitonin (1%) or CHAPS (10 mM) were used for cell lysis instead of Nonidet P-40.

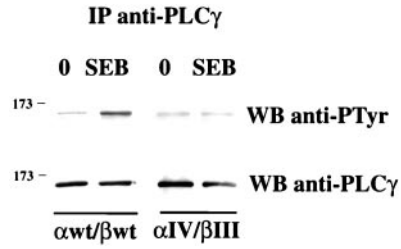

FIG. 1. **Defective phosphorylation of PLCγ in response to SEB in a T cell hybridoma expressing the TCR α-CPM mutant.** Anti-phosphotyrosine immunoblot of PLCγ-specific immunoprecipitates from lysates of T cell hybridomas expressing either a SEB-specific TCR (αwt/βwt) or an α-CPM mutant of the same TCR (αIV/βIII). Cells were either not activated or activated by SEB and antigen presenting cells. After stripping, the filter was reprobed with anti-PLCγ antibodies (*bottom panel*). The migration of molecular mass markers is shown.

Lysates for *in vitro* autophosphorylation assays were obtained using 3% Nonidet P-40 to disrupt pre-existing intermolecular interactions. Molecular weight markers were purchased from Amersham Pharmacia Biotech Italia. *In vitro* autophosphorylation assays of Fyn, Lck, or ZAP-70 specific immunoprecipitates were carried out in 20 μl of 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 μCi of [γ-^{32}P]ATP, at room temperature for 16 and 4 min for Fyn and Lck, respectively, and at 37 °C for 20 min for ZAP-70. The reaction products were subjected to SDS-PAGE, transferred to nitrocellulose, and exposed to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The filters were subsequently analyzed by immunoblot with anti-Fyn, Lck, or ZAP-70 antibodies to check that similar amounts of specific PTK were recovered in each immunoprecipitate. Immunoblots were carried out using a chemiluminescence detection system (Pierce, Rockford, IL).

Generation of Retroviral Supernatants, Infection of T Cell Hybridomas, and IL-2 Assays—Retroviruses encoding F528Fyn were generated by transfection of the packaging line GP+E-86 and subsequent selection in 500 μg/ml hygromycin (Life Technologies Italia) as described (5). Virus-containing supernatants from drug-resistant pools of transfected GP+E-86 cells were collected from confluent cell layers after 7–10 days. T hybridoma cells (5×10^5) were infected with 1 ml of virus-containing supernatant in the presence of 4 μg/ml Polybrene. After 4 h incubation at 37 °C, the supernatants were replaced with complete medium containing 500 μg/ml hygromycin and kept under selection for 7–10 days. Determination of IL-2 in the culture supernatants of hybridoma T cells activated with increasing concentrations of SEB was carried out as described using the HT-2 indicator cell line (5).

RESULTS

Defective Phosphorylation of PLCγ in Response to SEB in a T Cell Hybridoma Expressing a TCR α-CPM Mutant—We have previouly shown that a T cell hybridoma expressing a chimeric staphylococcal enterotoxin B (SEB)-specific TCR lacking the α chain CPM has a severe impairment in IL-2 production in response to antigenic ligand. This defect could be fully rescued by a calcium ionophore, suggesting that the calcium pathway fails to be activated by the mutant TCR (5). A central molecule in the induction of calcium mobilization is PLCγ, which is activated by phosphorylation on tyrosine residues following TCR triggering and as such induces release of calcium from intracellular stores by generating IP3 from membrane phospholipids. PTKs of the Src, Syk, and Tec families have been implicated, either directly or indirectly, in PLCγ phosphorylation (10, 11, 22–24). We assessed the capacity of the α-CPM TCR mutant to promote PLCγ phosphorylation. PLCγ-specific immunoprecipitates from lysates of T cell hybridomas expressing either wild-type or mutant TCR and activated with SEB were probed by immunoblot with anti-phosphotyrosine antibodies. As shown in Fig. 1, SEB-dependent phosphorylation of PLCγ was completely abolished in the hybridoma expressing the mutant TCR. Hence the defect in the capacity of TCR α-CPM mutant to activate the calcium pathway is due at least in part to the failure of this TCR to activate PLCγ.

Defective Activation of Fyn and Interaction with Tyrosine-

phosphorylated Pyk2 in Response to SEB in a T Cell Hybridoma Expressing the Mutant TCR—Fyn has been implicated both directly and indirectly in the tyrosine phosphorylation of PLCγ (10, 11). A portion of the total cellular Fyn is found in a complex with the TCR, where it interacts through its N-terminal unique region with the γ, ϵ, and ζ chain, independently of the activation state of the TCR (25, 26). Following TCR engagement, Fyn is activated and can in turn phosphorylate a number of substrates, which include, in addition to PLCγ, the adaptors FYB/SLAP-130, SKAP-55, and the PTK Pyk2 (27–29). To determine whether Fyn could be activated by the α-CPM-mutated TCR, Fyn activity was determined using *in vitro* kinase assays of Fyn-specific immunoprecipitates from lysates of the hybridomas expressing either wild-type or mutant receptors and activated with SEB. As shown in Fig. 2*A*, a dramatic reduction in SEB-dependent Fyn activation was observed in cells expressing the mutant TCR, while direct TCR triggering with an anti-TCR mAb (which bypasses antigen presentation) resulted in normal levels of Fyn activation. No significant difference in the basal activity of Fyn was detectable in the hybridomas expressing wild-type and mutant receptor and in the parental hybridoma lacking the TCRαβ (data not shown).

To assess whether the defective activation of Fyn was caused by an impairment in the association between Fyn and ζ, ζ-specific immunoprecipitates from lysates of the hybridomas expressing either wild-type or mutant TCR were probed by immunoblot with anti-Fyn antibodies. As shown in Fig. 2*B*, similar levels of Fyn co-precipitated with ζ in both hybridomas, ruling out a defective association between Fyn and ζ. Similar results were obtained from *in vitro* binding assays using a glutathione *S*-transferase fusion protein containing the intracellular domain of the ζ chain (data not shown).

The focal adhesion kinase family PTK Pyk2 is phosphorylated in response to TCR signaling and plays a key role in T cell activation (29, 30). Pyk2 is constitutively associated with Fyn, and as such associates, albeit loosely, with the resting TCR complex. Following TCR engagement, Pyk2 is phosphorylated by Fyn (29). We asked whether Pyk2 was constitutively associated with Fyn in cells expressing the TCR α-CPM mutant, and whether it could be phosphorylated in response to TCR engagement. Fyn-specific immunoprecipitates from lysates of the hybridomas expressing either the wild-type or the mutant TCR and stimulated with SEB were sequentially probed by immunoblot with anti-phosphotyrosine and anti-Pyk2 antibodies. As a control, the same experiment was carried out on cells stimulated with anti-TCR mAb. The results are presented in Fig. 3. Following SEB presentation, a phosphoprotein of about 115 kDa, which was identified as Pyk2, co-precipitated with Fyn in cells expressing the wild-type, but not the mutant TCR (*left panel*). Similar levels of phospho-Pyk2 co-precipitated with Fyn in both hybridomas when the TCR was directly triggered with an anti-TCR mAb (*right panel*). Of note, the association of Pyk2 with Fyn was similar in both hybridomas (*middle panel*). Also a p72-kDa phosphoprotein, which normally co-precipitates with and is phosphorylated by Fyn following TCR engagement (31), is absent from Fyn-specific immunoprecipitates from the hybridoma expressing the mutant TCR following SEB presentation (Fig. 3). Hence the TCR α-CPM mutant is incapable of activating Fyn and promoting phosphorylation of Fyn's downstream substrates.

Tyrosine-phosphorylated PLCγ is found in a complex with ZAP-70, LAT, and Grb2 following TCR engagement (32), suggesting a role for ZAP-70 in PLCγ phosphorylation. In turn ZAP-70 activation requires a Src kinase at two sequential stages, first to generate appropriate docking sites on the CD3/ζ ITAMs, then to phosphorylate a tyrosine residue on ZAP-70

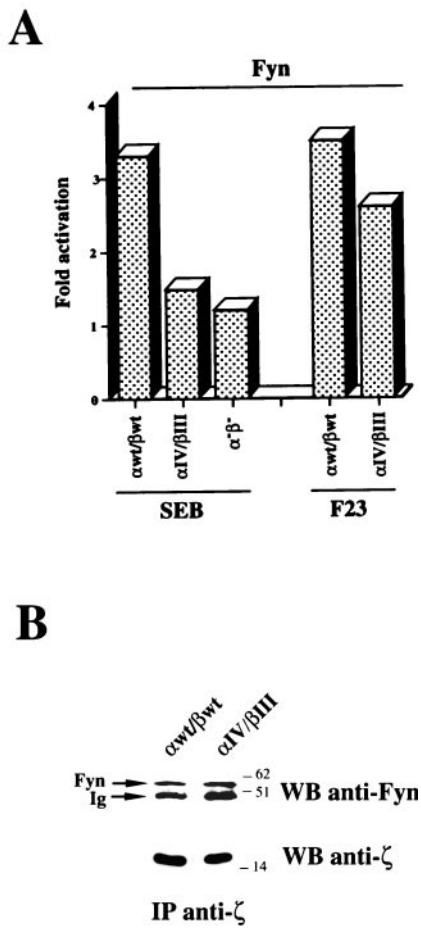

FIG. 2. **Defective activation of Fyn in response to SEB in a T cell hybridoma expressing the TCR α-CPM mutant, but normal association with the TCRζ chain.** *A,* quantitation of Fyn autophosphorylation on *in vitro* kinase assays of Fyn-specific immunoprecipitates from lysates of T cell hybridomas expressing either a SEB-specific TCR (αwt/βwt) or an α-CPM mutant of the same TCR (αIV/βIII), or from the parental hybridoma lacking TCR surface expression ($\alpha^-\beta^-$). Cells were either not activated, or activated by SEB-loaded APCs or by anti-TCR mAb cross-linking (F23). After the kinase reaction, immunoprecipitates were subjected to SDS-PAGE, transferred to a nitrocellulose filter, and exposed and analyzed using a PhosphorImager. The filter was subsequently probed with anti-Fyn mAb. The levels of Fyn immunoprecipitated were quantitated using by laser densitometry. The levels of ^{32}P incorporation by Fyn in each immunoprecipitate were normalized to the respective levels of Fyn. The data show the ratio of Fyn autophosphorylation in activated to resting cells in each cell line. *B,* anti-Fyn immunoblot of CD3ζ-specific immunoprecipitates from lysates of T cell hybridomas expressing either a SEB-specific TCR (αwt/βwt) or an α-CPM mutant of the same TCR (αIV/βIII). After stripping, the filter was reprobed with anti-CD3ζ mAb (*bottom panel*). The migration of molecular mass markers is shown, as well as the migration of Fyn in a total hybridoma T cell lysate separated on the same gel.

critical for its function (2, 3, 33). We asked therefore whether Lck and ZAP-70 could be activated in response to SEB by the TCR α-CPM mutant. The activity of both kinases was determined with *in vitro* kinase assays of Lck or ZAP-70 specific immunoprecipitates from lysates of the hybridomas expressing either the wild-type or the mutant TCR and activated with SEB. As shown in Fig. 4, activation of both kinases was impaired in cells expressing the mutant TCR.

Rescue of Signaling from the TCR α-CPM Mutant by Constitutively Active Fyn—Although a reduction in ZAP-70 activity is predictable if Src kinases are impaired, the failure of the mutant TCR to activate Lck suggests that both Fyn and Lck might be implicated in the signaling defect observed with the mutant TCR. To assess this possibility, we used a TCR defective Jurkat

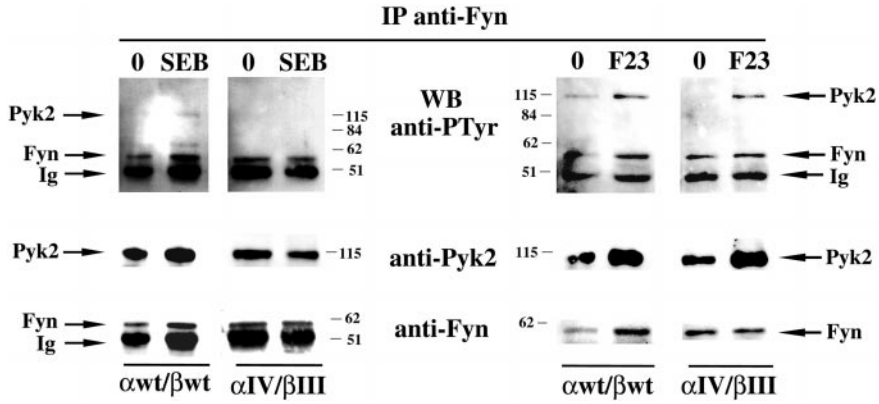

FIG. 3. **Defective phosphorylation of Fyn-associated Pyk2 in response to SEB in a T cell hybridoma expressing the α-CPM-mutated TCR.** *Top*, anti-phosphotyrosine immunoblot of Fyn-specific immunoprecipitates from lysates of T cell hybridomas expressing either a SEB-specific TCR (αwt/βwt) or an α-CPM mutant of the same TCR (αIV/βIII). Cells were either not activated, or activated by antigen presentation (*SEB*; *left*) or by anti-TCR mAb cross-linking (*F23; right*). After stripping, the filter was sequentially probed with anti-Pyk2 (*middle*) and anti-Fyn antibodies (*bottom panel*). The migration of molecular mass markers is shown, as well as the migration of Pyk2 and Fyn in a total hybridoma T cell lysate separated on the same gel.

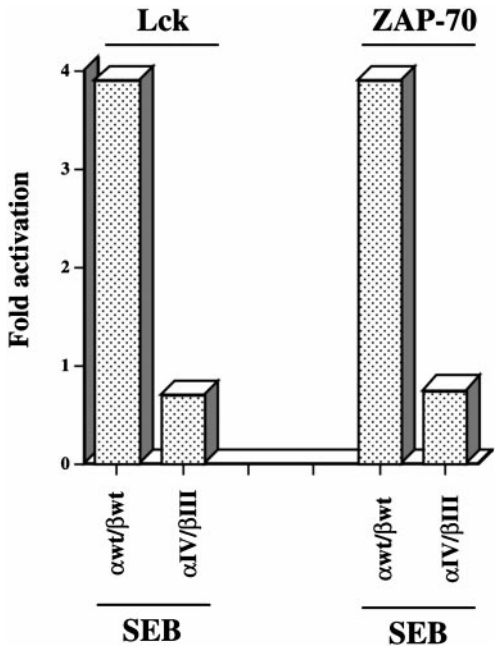

FIG. 4. **Defective activation of Lck and ZAP-70 in response to SEB in a T cell hybridoma expressing the TCR α-CPM mutant.** Quantitation of Lck and ZAP-70 autophosphorylation on *in vitro* kinase assays of Lck- or ZAP-70-specific immunoprecipitates from lysates of T cell hybridomas expressing either a SEB-specific TCR (αwt/βwt) or an α-CPM mutant of the same TCR (αIV/βIII). Cells were either not activated, or activated by SEB-loaded APCs. After the kinase reaction, immunoprecipitates were subjected to SDS-PAGE, transferred to a nitrocellulose filter, and exposed and analyzed using a PhosphorImager. The filter was subsequently probed with anti-Lck or anti-ZAP-70 mAb. The levels of Lck or ZAP-70 immunoprecipitated were quantitated by laser densitometry. The levels of ^{32}P incorporation by Lck or ZAP-70 in each immunoprecipitate were normalized to the respective levels of immunoprecipitated protein. The data show the ratio of Lck or ZAP-70 autophosphorylation in activated to resting cells in each cell line.

T cell model. Expression of the mutant TCR in these cells shows a defect in SEB-dependent activation of the transcription factor NF-AT, which could be rescued by a calcium ionophore (5). We used this model to ask whether bypassing antigen-specific TCR triggering by forced expression of constitutively active forms of Fyn or Lck could restore NF-AT activation by the TCR α-CPM mutant. Cells were transiently co-transfected with expression vectors encoding wild-type or chimeric TCR αβ, a NF-AT/luciferase reporter, and either empty vector or an expression vector encoding the constitutively active F528Fyn mutant lacking the negative regulatory tyrosine C-terminal residue. After recovery, cells were activated with SEB. Similar experiments were carried out using a vector encoding the constitutively active F505Lck mutant. The results are shown in Fig. 5. Expression of F528Fyn restored the levels of NF-AT activation induced by SEB in cells expressing the mutant TCR to levels comparable to those induced by the wild-type TCR. Expression of F528Fyn also resulted in an enhancement of SEB-dependent NF-AT activation by the wild-type TCR (Fig. 5). No significant increase in the NF-AT response to SEB was observed either in the presence of F505Lck (Fig. 5), or by a constitutively active Ras mutant (data not shown). Hence the introduction of constitutively active Fyn effectively complements the signaling defect in the α-CPM mutant receptor.

To confirm this finding and analyze biochemically the effects of F528Fyn expression we cloned the cDNA encoding F528Fyn in a retroviral vector. Retroviral supernatants containing this construct were used to infect the T cell hybridomas expressing either the wild-type TCR or the TCR α-CPM mutant. Stable transfectants were selected in the presence of hygromycin. Flow cytometric analysis revealed no differences in the levels of surface TCR in these cells as compared with the respective parental lines (data not shown). As shown in Fig. 6*A*, the levels of Fyn in total cells lysates were significantly increased in the F528Fyn hybridomas, which is expected due to the presence of a long terminal repeat-driven F528Fyn expression construct. Probing the same filter with anti-phosphotyrosine antibodies showed a considerably more complex phosphoprotein pattern in the F528Fyn-transduced hybridomas (Fig. 6*A*). Furthermore, *in vitro* kinase assays of Fyn-specific immunoprecipitates from hydridomas expressing the wild-type or mutant TCR, and from their respective F528Fyn transfectants, showed a large increase in Fyn activity in the F528Fyn-transfected cells (Fig. 6*B*). Immunoblot analysis of similar Fyn-specific immunoprecipitates with anti-phosphotyrosine antibodies, followed by anti-Pyk2 antibodies (not shown), revealed higher levels of Fyn-associated, phospho-Pyk2 in the F528Fyn hybridomas (Fig. 6*C*). Furthermore, anti-phosphotyrosine immunoblots of ζ-specific immunoprecipitates showed constitutive ζ chain phosphorylation in F528Fyn-expressing cells (data not shown).

To understand whether constitutively active Fyn could restore the IL-2 response to SEB in cells expressing the α-CPM-mutated TCR, the hybridomas expressing the wild-type or mu-

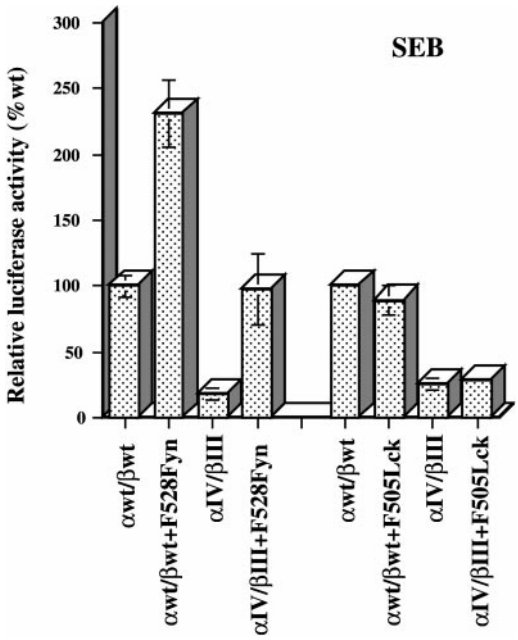

FIG. 5. **Rescue of NF-AT activation in response to SEB in a T cell hybridoma expressing the TCR α-CPM mutant by constitutively active Fyn.** Relative luciferase activity in lysates of J31.13 cells transiently co-transfected with a NF-AT/luciferase reporter, expression plasmids encoding the α and β chains of a SEB-specific TCR, or the corresponding α-CPM mutated TCR, and either empty vector or an expression construct encoding a constitutively active Fyn (or Lck) mutant. After recovery, cells were either exposed to APC alone, or to APC loaded with SEB. Luciferase activity induced by treatment with SEB was normalized to maximal activity induced by a combination of phorbol 12-myristate 13-acetate and A23187. Relative SEB dependent luciferase activities in J31.13 cells coexpressing wild-type TCR and constitutively active Fyn (αwt/βwt+F528Fyn), or expressing the TCR α-CPM mutant, alone (αIV/βIII) or in combination with constitutively active Fyn (αIV/βIII + F528Fyn), are expressed as % of the value obtained in cells expressing the wild-type TCR alone (αwt/βwt). The same experiment was carried out with a constitutively active Lck mutant (*right*). The results obtained on duplicate samples of two representative experiments for each constitutively active mutant are shown.

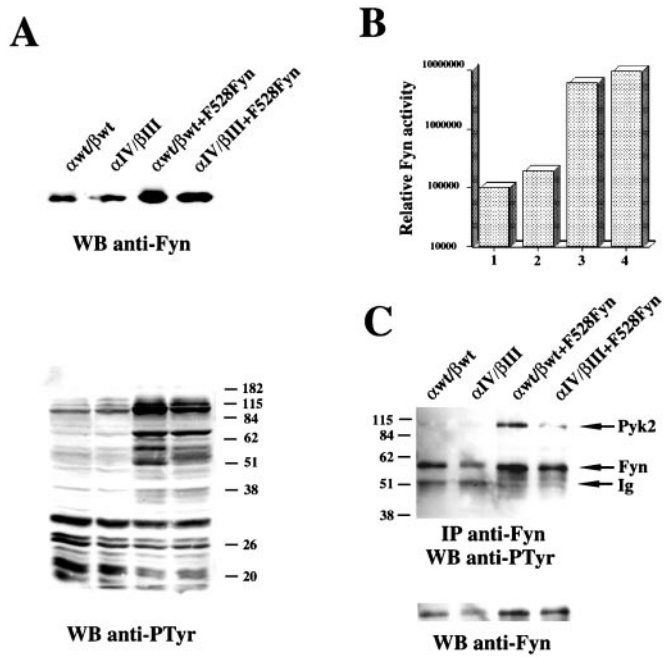

FIG. 6. **Generation and characterization of T cell hybridomas expressing a constitutively active Fyn mutant.** *A,* anti-Fyn (*top*) or anti-phosphotyrosine (*bottom*) immunoblot of total lysates from T cell hybridomas expressing either a SEB-specific TCR (αwt/βwt) or an α-CPM mutant of the same TCR (αIV/βIII), or stable transductants infected with a retroviral construct encoding a constitutively active Fyn mutant (F528Fyn). Equal amounts of proteins were loaded in each lane. *B,* quantitation of Fyn autophosphorylation on *in vitro* kinase assays of Fyn-specific immunoprecipitates from lysates of resting T cell hybridomas expressing either a SEB-specific TCR (*column* 1) or an α-CPM mutant of the same TCR (*column* 2), or stable transductants infected with a retroviral construct encoding a constitutively active Fyn mutant (F528Fyn; *columns* 3 and 4). After the kinase reaction, immunoprecipitates were subjected to SDS-PAGE, transferred to a nitrocellulose filter, and exposed and analyzed using a PhosphorImager. The filter was subsequently probed with anti-Fyn mAb. The levels of Fyn immunoprecipitated were quantitated by laser densitometry. The levels of ^{32}P incorporation by Fyn in each immunoprecipite were normalized to the respective levels of Fyn. *C,* anti-phosphotyrosine immunoblot of Fyn-specific immunoprecipitates from lysates of resting T cell hybridomas expressing either a SEB-specific TCR (αwt/βwt) or an α-CPM mutant of the same TCR (αIV/βIII), or the respective transductants expressing F528Fyn. After stripping, the filter was sequentially probed with anti-Pyk2 (*bottom*) and anti-Fyn antibodies (not shown). The migration of molecular mass markers is shown, as well as the migration of Pyk2 and Fyn in a total hybridoma T cell lysate separated on the same gel.

tant TCR, and their respective counterparts expressing F528Fyn, were assayed for the capacity to produce IL-2 in response to SEB stimulation. As shown in Fig. 7, the amount of IL-2 detected in the culture supernatant of the hybridoma expressing the mutant receptor and stimulated with SEB was close to background. On the other hand, the IL-2 response to SEB induced by the mutant TCR was fully restored in the presence of F528Fyn. Similar to the results obtained in the Jurkat T cell model, an enhancement of the response elicited by wild-type TCR in the presence of F528Fyn was also observed (Fig. 7). Collectively, the results strongly suggest that the signaling dysfunction in the α-CPM mutant TCR is caused at least in part by a defective activation of Fyn, resulting in abortive downstream signaling, and leading to an impairment of the signals involved in calcium mobilization.

DISCUSSION

The α-CPM within the αβ TCR has a specialized role during T cell development in that TCRs with a mutated α-CPM are unable to drive thymocytes to undergo positive selection (6, 34). Studies of α-CPM mutant receptors have shown that positive selection requires a prolonged activation of the Erk pathway and recruitment of proximal signaling components into glycolipid rafts within the plasma membrane (34). In addition, structural characterization of the α-CPM-mutated TCR used in this study has revealed that, although expressed at normal levels at the cell surface, the mutant TCR associates only loosely with both CD3δ and -ζ (5, 6). Genetic ablation of CD3δ or -ζ results in defective thymocyte development (35–37). However, in the case of CD3ζ, this effect appears related to its key role in TCR·CD3 complex assembly and expression at the cell surface, as deletion of its cytoplasmic tail or of individual ITAMs does not affect TCR signaling (38, 39). Similar results have been obtained in T cell models expressing TCR·CD3 complexes with mutations or deletions in the cytoplasmic domain of CD3ζ (40). Furthermore, although TCR signaling is impaired in CD3δ-deficient thymocytes (41), the cytoplasmic domain appears dispensable for antigen-dependent effector functions in a CTL clone (42), suggesting a partial redundancy among the ITAMs. Nevertheless, preferential coupling of individual TCR/CD3 components with specific signaling pathways is likely, especially in conditions where they are all expressed so that no "emergency" compensation is required. The data presented above show that the signaling defect in the T cell hybridoma expressing the α-CPM-mutated TCR is at least in part dependent on the failure of Fyn to become activated. A significant part of the total intracellular content of Fyn is constitutively associated with the ζ chain, as well as with the ϵ and γ chains of the

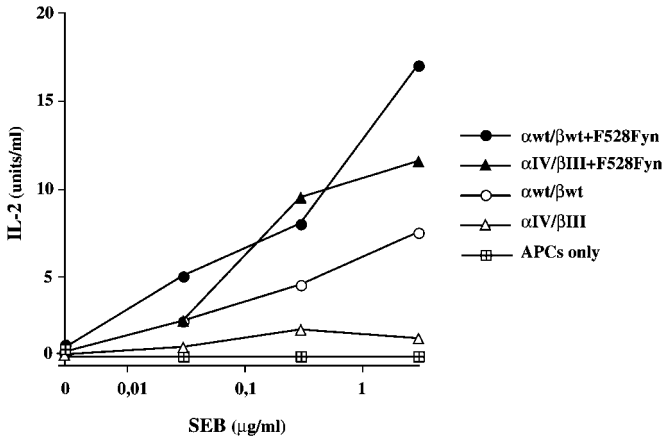

FIG. 7. **Rescue of IL-2 production in response to SEB in a T cell hybridoma expressing the α-CPM-mutated TCR by constitutively active Fyn.** Levels of IL-2 in supernatants of T cell hybridomas expressing either a SEB-specific TCR (αwt/βwt) or an α-CPM mutant of the same TCR (αIV/βIII), or their respective transductants expressing F528Fyn. Cells were either exposed to APC alone, or to APC loaded with increasing concentrations of SEB.

CD3 complex (25, 26). Interestingly, no association with CD3δ has been detected.[2] Since Fyn is stably associated with ζ in the T cell hybridomas expressing either the wild type or the α-CPM mutant TCR (Fig. 2), another mechanism is required to explain the defective activation of Fyn by the mutant receptor. The fact that the ζ chain associates poorly with the α-CPM-mutated TCR may explain the failure of this receptor to activate Fyn. In fact, coupling the TCR·CD3 complex to Fyn activation might require stable association of ζ with the TCR·CD3 complex.

Fyn has been shown to be an important component of the TCR signaling machinery. Expression of constitutively active Fyn enhances T cell responsiveness to TCR-mediated stimulation and contributes to NFAT activation, while inhibitory effects are elicited by overexpression of a kinase-dead Fyn mutant (7, 8, 17, 43). Furthermore, genetic ablation of Fyn results in impaired TCR signaling in thymocytes and splenic T cells (44, 45). Interestingly, Fyn appears specifically implicated in both IP3-dependent and IP3-independent calcium responses. IP3-dependent targets of Fyn are PLCγ and the IP3 receptor, which are both implicated in calcium release from intracellular stores (10–12). A role for Fyn in calcium mobilization independent of phosphatidylinositol bisphosphate hydrolysis has also been demonstrated (13). Furthermore, expression of constitutively active Fyn obviates the requirement for a calcium ionophore in a pharmacological model of NF-AT activation (8). The property of promoting TCR-dependent calcium flux is specific to the T cell isoform of Fyn (FynT) and has been mapped to its catalytic domain (46). Furthermore, coexpression of Fyn and ζ in a heterologous cell model is sufficient for reconstitution of calcium mobilization (9). Of note, these effects are specifically elicited by Fyn, and not by the related kinase Lck. Hence, although Lck activation is also impaired in the T cell hybridoma expressing the TCR α-CPM mutant, restoration of defective TCR function by a calcium ionophore (5), as well as the specific complementation of the defect by constitutively active Fyn, suggests that the impairment of Lck activation might not be causal to the mutant TCR dysfunction at least in the hybridoma. This might be due to the unique features of superantigen binding to the TCR in this system, which neither requires nor involves CD4 (47). In support of this possibility, triggering of TCR signaling with staphylococcal enterotoxins has been shown to be unaffected in Lck defective T cells (48). Furthermore, T cells from $Fyn^{-/-}$ mice showed reduced responses to SEB (44). It should be noted that, despite the reduction in PTK activation in response to SEB, the PKC/Ras/MAP kinase pathway is still operational, as shown by the restoration of NF-AT activation by calcium ionophore alone in cells expressing the α-CPM-mutated TCR (5), suggesting that the requirements for Ras activation may be less stringent, possibly because of the redundancy in the TCR signaling components involved in recruitement of Grb2·Sos complexes (49). In this regard, we should point out that $CD4^+/CD8^+$ thymocytes expressing an α-CPM-mutated receptor are capable of activating Erk in response to a negatively selecting ligand, but show a specific defect in Erk activation in response to a positive selection ligand (34). For this TCR, SEB is a negatively selecting ligand and it is therefore expected that the mutant TCR can activate the Erk pathway. Furthermore, there are likey other differences between a T cell hybridoma and a $CD4^+/CD8^+$ thymocyte. In support of this possibility, a significant basal level of Erk2 phosphorylation was detected in the T cell hybridoma expressing the TCR α-CPM mutant which was not observed in $CD4^+/CD8^+$ thymocytes.[2]

Mice expressing the α-CPM-mutated TCR harbor a severe defect in positive selection (6). Although a defective activation of mitogen-activated kinases, but not of stress-activated kinases, was observed in thymocytes expressing the α-CPM mutant when exposed to a positively selecting ligand, the primary defect could be traced to the very first steps of TCR signaling, and specifically to recruitment and phosphorylation of signaling proteins in lipid rafts (34). These specialized subdomains of the plasma membrane enriched in cholesterol and glycosphingolipids have been recently shown to be critically involved in PTK-dependent signaling pathways (50). Lck and Fyn have been shown to be interchangeable in terms of ITAM phosphorylation in a heterologous cell model (51). Furthermore, the TCRζ chain is phosphorylated, albeit weakly, in Lck-defective peripheral T cells (52), suggesting a functional redundancy among Src family PTKs. A severe block in thymocyte development was, however, observed only in mice lacking Lck or expressing a dominant negative Lck mutant (53, 54), while both thymocyte development and positive selection are unaffected in mice lacking Fyn (44, 45). On the other hand, a role for Fyn in thymocyte development is supported by the markedly more severe phenotype of developing $Lck^{-/-}Fyn^{-/-}$thymocytes as compared with thymocytes lacking only Lck, with a very early block at the double negative stage (55, 56). Furthermore, a constitutively active form of Fyn could partially substitute for Lck in thymocyte development and positive selection, suggesting a cooperation between the two PTKs (56). Hence, although the defective functional coupling of Fyn with the TCR α-CPM domain might not be causal in the defect in positive selection in thymocytes expressing this TCR, this possibility cannot be completely ruled out. Alternatively, the same function might be subserved by different Src kinases in thymocytes and T lymphocytes. In this context, the failure of the mutant TCR to activate Lck might be relevant to the defective phosphorylation of signaling proteins in lipid rafts in positively selected thymocytes expressing the TCR α-CPM mutant.

Acknowledgments—We thank Sonia Grassini and Barbara Hausmann for excellent technical assistance, Giancarlo Benocci for secretarial assistance, Antonio Lanzavecchia for the gift of APC, and John L. Telford for productive discussions and critical reading the manuscript.

[2] C. Ulivieri and C. T. Baldari, unpublished data.

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