The actin cytoskeleton has an important role in the organization and function of the immune synapse during antigen recognition. Dynamic rearrangement of the actin cytoskeleton in response to T cell receptor (TCR) triggering requires the coordinated activation of Rho family GTPases that cycle between active and inactive conformations. This is controlled by GTPase-activating proteins (GAP), which regulate inactivation of Rho GTPases, and guanine exchange factors, which mediate their activation. Whereas much attention has centered on guanine exchange factors for Rho GTPases in T cell activation, the identity and functional roles of the GAP in this process are largely unknown. We previously reported β2-chimaerin as a diacylglycerol-regulated Rac-GAP that is expressed in T cells. We now demonstrate Lck-dependent phosphorylation of β2-chimaerin in response to TCR triggering. We identify Tyr-153 as the Lck-dependent phosphorylation residue and show that its phosphorylation negatively regulates membrane stabilization of β2-chimaerin, decreasing its GAP activity to Rac. This study establishes the existence of TCR-dependent regulation of β2-chimaerin and identifies a novel mechanism for its inactivation.

T cell activation requires presentation of an antigen by antigen-presenting cells (APC) to the T cell receptor (TCR); this event involves the reorganization of several scaffolds and signaling proteins, leading to formation of the immunological synapse (IS) (1). Correct protein redistribution during synapse formation is critical for an efficient T cell response, and it is largely regulated by actin polymerization at the T cell/APC contact site as a result of TCR-regulated Rac-dependent signals (2, 3). Like other Rho GTPases, Rac cycles between a GTP-bound active state and a GDP-bound inactive state. This continuous recycling is regulated by the concerted action of two proteins as follows: GEF, which activates Rac by mediating GDP/GTP exchange (4), and GAP, which induces Rac inactivation by accelerating intrinsic Rac GTPase activity, converting GTP to GDP (5).

Vav-1 is the best studied GEF for Rac, and it has critical roles in T cell-dependent functions (6). In naïve, unstimulated T cells, Vav-1 is in an inactive state through autoinhibition, as the GEF domain is blocked by the N-terminal region (7). This autoinhibition is relieved by TCR-mediated tyrosine phosphorylation (8, 9). Thymocytes from Vav-1-deficient mice have a developmental block, and their mature T cells show severe defects in IS formation, as well as reduced Ca²⁺ influx, IL-2 production, T cell proliferation, and cytotoxic activity (10–13). Although several studies have shown a key role for Vav-1, the mechanisms that govern Rac inactivation downstream of the TCR remain elusive.

The chimaerins are a family of Rho-GAP, with specific activity for Rac. In addition to their catalytic domain, they have an N-terminal SH2 domain and a C1 domain required for interaction with the lipid messenger diacylglycerol (DAG) and with phorbol esters (14). There are two mammalian chimaerin genes (CHN1 and CHN2), which encode the full-length α2- (ARHGAP1) and β2-chimaerins (ARHGAP3), and at least one splice variant each (α1 and β1) that lack the SH2 domain. The α-chimaerins are expressed abundantly in brain and are linked to neuritogenesis and axon guidance (15–20). β2-Chimaerin expression is ubiquitous (21) and is involved in EGF-dependent Rac regulation (22, 23). Experiments in T cells showed that β2-chimaerin participates in chemokine-dependent regulation of T cell migration and adhesion (24). A very recent study implicates chimaerins in the modulation of Rac activity during T cell synapse formation, suggesting that this protein family contributes to DAG-mediated regulation of cytoskeletal remodeling during T cell activation (25).

Determination of the β2-chimaerin crystal structure provided important clues regarding its mechanism of action. In the absence of stimulation, the protein is in an inactive state in which the N-terminal domain maintains a “closed” conformation, blocking Rac binding and concealing the C1 domain (26). These structural data were fully supported by experiments in live T lymphocytes showing that phorbol myristate acetate (PMA)-dependent translocation of β2-chimaerin was less effective than that of its isolated C1 domain (24). These data not only confirmed the lack of accessibility of the β2-chimaerin C1 domain but also...
suggested that there are negative regulatory mechanisms that promote β2-chimaerin release from the membrane.

DAG-dependent signaling is critical for the modulation of T cell functions, by virtue of its ability to bind and regulate C1 domain-containing proteins such as protein kinase Cθ, protein kinase D, and RasGRP1 (27). An important issue is to determine how the different DAG-binding proteins discriminate between distinct DAG pools, and how DAG activates certain C1-containing proteins and not others. Some mechanisms that allow discrimination between DAG receptors include the distinct affinity of C1 domains for different DAG pools, association of C1 domain-containing proteins to specific scaffolds, and/or structural determinants in these proteins that limit C1 domain accessibility to membrane DAG (28–30).

To explore the events that contribute to the specific regulation of β2-chimaerin, we studied β2-chimaerin phosphorylation in the context of TCR stimulation. We show that β2-chimaerin is phosphorylated in tyrosine residues after TCR stimulation, and we identify Lck as the Tyr kinase responsible for this phosphorylation. Generation of point mutants identified Tyr-153, at the hinge of the SH2 and C1 domains, as the main tyrosine residue phosphorylated in response to TCR stimulation. Cells expressing a β2-chimaerin mutant defective for Tyr-153 phosphorylation show anomalies in TCR clustering, conjugate formation, NF-AT activation, and IL-2 production that correlate with elevated Rac-GAP activity in this mutant. Subcellular localization analysis of the β2-chimaerin mutants reveals that impairment of β2-chimaerin phosphorylation at Tyr-153 promotes C1-mediated β2-chimaerin stabilization at the plasma membrane, providing a mechanistic explanation for its higher Rac-GAP activity. In summary, our results demonstrate for the first time that tyrosine kinase-mediated negative regulation of β2-chimaerin is elicited by physiological stimulation in T lymphocytes, and suggest that TCR stimulation provides both positive and negative signals for β2-chimaerin activation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Poly-DL-lysine and PMA were from Sigma; Gamma-bind-G-Sepharose beads were from GE Healthcare, and Rac1 activation assay kit was from Upstate Biotechnology, Inc. U73122, PP2, R59949, and Triton X-100 were from Sigma; Gamma-bind-G-Sepharose beads were from GE Healthcare, and Rac1 activation assay kit was from Upstate Biotechnology, Inc.

Rac1 was from BD Transduction Laboratories. Horseradish peroxidase-conjugated goat anti-α, -CD3, and -CD28 from Pharmingen; phosphotyrosine-specific antibodies were from Cell Signaling. Monoclonal antibodies were from Dr. C. Hall (Institute of Neurology, London, UK). The plasmid encoding GFP-fused Vav1 was from Dr. X. R. Bustelo (Centro de Investigación del Cancer, Salamanca, Spain) and that encoding pS6Lck was from Dr. A. C. Carrera (Centro Nacional de Biotecnología).

Cell Culture and Transfection—Human leukemic Jurkat T cells, the Jurkat-derived cell line JCaM-1.6 (defective for Lck expression), and Raji B cells were maintained in RPMI 1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum (Sigma) and 2 mM glutamine (BioWhittaker). Jurkat and JCaM-1.6 cells in logarithmic growth were transiently transfected (1.2 × 10⁵ in 400 μl of complete medium) with 20 μg of plasmid DNA by electroporation using a Gene Pulser (Bio-Rad; 270 V, 975 microfarads). Cells were immediately transferred to 10 ml of complete medium and assayed after 24 h. The human embryonic kidney cell line HEK293 was cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented as above. Cells were transfected using jet-PEI reagent (Polyplus Transfection SAS, Illkirch, France) and assayed 24 h later.

Immunoprecipitation and Western Blot—Transfected cells (4 × 10⁶ cells/ml) were stimulated with PMA (800 ng/ml) or anti-CD3/CD28 antibodies (1 μg/ml each) in complete medium (37 °C, 5 min). For inhibitor treatment, before stimulation cells were incubated with U73122 (1 μM; 37 °C, 30 min) or with PP2 or R59949 (30 μM; 37 °C, 1 h). Stimulated cells were pelleted and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 5 mM EDTA, 2 mM Na₃VO₄, 1 mM Na₂VO₄, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin) for 15 min on ice. After centrifugation (15,000 × g, 4 °C, 15 min), supernatants were assayed for total protein (DC protein assay, Bio-Rad). For immunoprecipitation of CFP or GFP fusion proteins, equal amounts of total cell lysates (300–600 μg) were pre-cleared with Gamma-bind-Sepharose beads (4 °C, 30 min) and incubated with polyclonal anti-GFP antibody that recognizes both fluorescent tags (2 μg, 4 °C, 2 h), and then with Gamma-bind-Sepharose beads (30 μl, 4 °C, 1 h). Beads were washed twice in lysis buffer, once in 0.5 M LiCl, in 150 mM Tris-HCl, and twice in 150 mM Tris-HCl, and immunoprecipitated proteins were eluted in sample buffer. Bound proteins and total lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were blocked, and blots were developed with the indicated antibodies and horseradish peroxidase-conjugated anti-rabbit or mouse antibodies using the ECL detection kit (GE Healthcare).

Rac1 Activation Assay—The Rac1 activation assay kit (Upstate Biotechnology, Inc.) was used to precipitate Rac-GTP from cell lysates by binding to the RAC binding domain of PAK-1 fused to glutathione S-transferase (32). Jurkat cells
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transfected with the indicated plasmids were plated (5 × 10^6 cells/ml) onto anti-CD3/CD28 antibody-coated plates (5 μg/ml each) and incubated (37 °C, 15 min). After incubation, medium was removed, and adhered cells were lysed, and equal amounts of cell lysates were processed according to the manufacturer’s protocol. Bound proteins were eluted in loading buffer. The p21-binding domain of PAK-1-PAK-GST-associated Rac was detected with an anti-Rac1 monoclonal antibody; 30 μg of each lysate was probed with the same antibody to detect total Rac.

Time-lapse Confocal Microscopy—For imaging of live Jurkat cells, cells were pelleted at 24 h post-transfection and suspended in HEPES-balanced salt solution (HBSS: 25 mM HEPES-KOH, pH 7.4, 1 mM MgCl2, 1 mM CaCl2, 132 mM NaCl, 0.1% bovine serum albumin) and plated on poly-DL-lysine-coated chamber slides. Slides were mounted on a 37 °C plate on an Olympus Fluoview 1000 confocal microscope, and PMA (200 ng/ml) was added after the first frame; images were captured every 10 s and processed using Adobe Photoshop and ImageJ software.

To monitor Jurkat:APC conjugate formation, Raji cells stably expressing CFP were suspended in HBSS (10 × 10^6 cells/ml) and incubated alone or with Staphylococcus enterotoxin E (SEE, 1 μg/ml; 37 °C, 1 h). At 24 h post-transfection with the indicated GFP- and RFP-fused constructs, Jurkat cells were suspended (0.3 × 10^6 cells/ml) in HBSS containing 2% fetal bovine serum, transferred to poly-DL-lysine-coated chamber slides, and allowed to attach at 37 °C. CFP-expressing Raji cells (2.5 × 10^6 cells/ml) were added to the attached Jurkat cells, and images were recorded every 20 s to monitor conjugate formation. Images were processed as above.

Confocal Microscopy of Fixed Jurkat Cells—For F-actin staining of transfected Jurkat cells conjugated with APC, we incubated Raji cells (10 × 10^6 cells/ml in HBSS) alone or with SEE (1 μg/ml) for 1 h at 37 °C. Cells were washed and suspended in HBSS (10^6 cells/ml). Transfected Jurkat cells were suspended in HBSS (10^6 cells/ml) and incubated with pulsed or unpulsed APC: 1:1 Jurkat:APC; 37 °C, 10 min. Cells were then transferred to poly-DL-lysine-coated coverslips (50 μg/ml) and allowed to attach for 10 min. Cells were fixed with 2% paraformaldehyde (10 min), washed three times with 150 mM Tris-HCl, pH 7.4, permeabilized with PBS, 0.2% Triton X-100 (5 min), washed twice with PBS, blocked in PBS, 1% bovine serum albumin, and incubated with rhodamine-phalloidin (15 min). Coverslips were mounted on an Olympus Fluoview 1000 confocal microscope. Images were processed using Adobe Photoshop software.

Conjugation Assay—Raji cells were incubated alone or with SEE (1 μg/ml, 37 °C, 1–2 h) in HBSS, stained with BODIPY-TR ceramide (5 μM in HBSS, 4 °C, 30 min), washed, and suspended in HBSS (10^6 cells/ml). Jurkat cells transfected with GFP-fused constructs were suspended in HBSS (10^6 cells/ml); 0.25 ml Raji cells were combined with 0.25 ml of Jurkat cells in 6-ml round-bottom tubes and incubated (37 °C, 90 min). Tubes were then vortexed (10 s) to resuspend cells, which were fixed with 0.5 ml of 4% paraformaldehyde. The relative proportion of green/red events in each tube was determined by flow cytometry (Beckman-Coulter). The percentage of conjugation was calculated as the number of green/red events (conjugated transfected Jurkat cells) divided by total green events (total transfected Jurkat cells).

**RESULTS**

TCR Triggering Induces Tyr Phosphorylation of β2-Chimaerin—β2-chimaerin is expressed in T cells, where it is proposed to regulate Rac activation during T cell synapse formation (25). Moreover, a recent study using COS-1 cells showed that Src-dependent tyrosine phosphorylation negatively regulates β2-chimaerin Rac-GAP functions (31). This led us to determine whether this type of regulation occurs after physiological stimulation of T cells. We transfected Jurkat T cells with a plasmid encoding CFP (cyan fluorescent protein)-β2-chimaerin, stimulated them with anti-CD3/CD28 antibodies, and immunoprecipitated the CFP-fused proteins with an anti-GFP Ab. Phosphorylation levels in the immunoprecipitated proteins were determined with anti-Tyr(P) Ab. We observed robust phosphorylation of β2-chimaerin following activation of the TCR and its co-stimulatory molecule CD28 (Fig. 1A). Addition of PP2, Src family kinase inhibitor (33), prevented this phosphorylation, suggesting that Src family Tyr kinase is responsible for β2-chimaerin phosphorylation.

Lck is Src kinase that is pivotal to TCR function, because it phosphorylates several signaling molecules in the TCR pathway and allows assembly of the scaffolds required for correct T lymphocyte activation (34). To determine the relevance of this Tyr kinase for phosphorylation of β2-chimaerin, we performed immunoprecipitation experiments in JCam 1.6 cells, a Jurkat T cell variant that lacks functional Lck but expresses Fyn and other Src family kinases (35). We observed no phosphorylation of immunoprecipitated β2-chimaerin after CD3/CD28 stimulation of the cells (Fig. 1B). Reintroduction of Lck induced intense phosphorylation of the protein after anti-CD3/CD28 Ab treatment (Fig. 1B). These experiments did not discard β2-chimaerin phosphorylation by an Lck-regulated Tyr kinase.
suggest that Lck is largely responsible for TCR-dependent tyrosine phosphorylation of β2-chimaerin in T cells.

The other chimaerin isoform, α2-chimaerin, has recently received considerable attention, as it is an essential downstream effector of EphA4-dependent axon navigation (17–20). As this isoform is also reported to act as a negative modulator of T cell responses (25), we analyzed whether α2-chimaerin is also subject to tyrosine phosphorylation following T cell activation. Analysis of Jurkat cells expressing green fluorescent protein (GFP)-α2-chimaerin showed TCR/CD28-induced tyrosine phosphorylation, which was not observed in JCaM cells (Fig. 1D). These data suggest that this isoform is also a substrate of Lck-dependent phosphorylation.

Identification of Tyr-153 in β2-Chimaerin as the Lck-phosphorylated Residue—Analysis of the β2-chimaerin amino acid sequence (NCBI NP_004058) with the Motifscanner program reveals Tyr-21, Tyr-31, and Tyr-153 as three potential sites of phosphorylation by Src family kinases. To identify the Tyr residue phosphorylated by Lck in response to TCR triggering, we transfected cells with distinct CFP-β2-chimaerin constructs in which each Tyr residue was mutated independently to Phe. Y21F and Y31F replacements did not impair tyrosine phosphorylation in response to TCR, whereas the Y153F mutant showed TCR/CD28-induced tyrosine phosphorylation, confirming the Rac-GAP function of α2-chimaerin (31), we next compared the Rac-GAP activity of the β2-chimaerin Y153F mutant with that of its wild type (WT) counterpart. We compared Rac-GTP levels induced by CD3/CD28 stimulation in GFP-expressing control Jurkat cells with those of cells transfected with WT β2-chimaerin or the Y153F mutant fused to GFP. In a pulldown assay, WT β2-chimaerin expression reduced TCR-induced Rac-GTP levels, confirming the Rac-GAP function of β2-chimaerin (Fig. 3A). Cells expressing the Y153F mutant showed a marked decrease in Rac-GTP levels, suggesting that Lck-dependent phosphorylation of β2-chimaerin at Tyr-153 negatively regulates its Rac-GAP activity.

To confirm that Tyr-153 phosphorylation indeed represents a mechanism that limits β2-chimaerin Rac-GAP function, we
compared the effect of WT and mutant proteins in TCR-triggered Rac-regulated responses. After TCR stimulation, Rac activation is critical for NF-AT transcription, which in turn is necessary for IL-2 production and, finally, cell proliferation (10, 11, 36). Using luciferase reporter assays, we first examined the effect of WT β2-chimaerin and the Y153F mutant on NF-AT activity. As reported previously (25), β2-chimaerin overexpression clearly reduced NF-AT activity after stimulation of Jurkat T cells with anti-CD3 or -CD3/CD28 Ab (Fig. 3A, bottom). We observed similar staining for GFP-β2-chimaerin-expressing Jurkat cells (Fig. 3A, middle), whereas cells expressing GFP-β2-chimaerinY153F showed decreased actin polymerization at the T cell/APC contact zone (Fig. 3A, top). TCR triggering increases the avidity of integrin-mediated adhesion and stabilizes T cell/APC conjugate formation in a process dependent on actin cytoskeleton polymerization (2). We next analyzed whether β2-chimaerin mutant overexpression affected conjugate formation to a greater extent than the WT protein. When Raji cells were SEE-loaded, conjugate formation with Jurkat cells was enhanced, and Vav overexpression increased the percentage of conjugates under basal and stimulation conditions (Fig. 4B). When co-expressed with Vav-1, β2-chimaerin did not markedly decrease conjugate formation, either with SEE-loaded or untreated APC (Fig. 4B). In contrast, overexpression of β2-chimaerinY153F decreased conjugate formation between Vav-expressing Jurkat cells and SEE-loaded APC, without affecting Jurkat/untreated APC conjugate formation. Results were similar when cells were transfected with β2-chimaerinQ32A and -I233A mutants, which have higher Rac-GAP activity than the WT protein because of the lack of intramolecular interactions (26). These data demonstrate that β2-chimaerin phosphorylation at Tyr-153 represents a negative regulatory mechanism for Rac-GAP function of this protein following T cell/APC conjugate formation.

These experiments indicated that impairment of β2-chimaerin phosphorylation at Tyr-153 enhanced Rac-GAP activity, limiting T cell/APC synapse stabilization. We next studied formation of the T cell/APC synapse using real time videomicro-
copy, which allowed simultaneous monitoring of changes in the subcellular localization of the β2-chimaerinY153F construct. We used recruitment of the GFP-fused CD3ζ chain (GFP-CD3ζ) as a marker of IS formation (37). In RFP-overexpressing control Jurkat cells, GFP-CD3ζ relocalized to the contact zone as soon as cells encountered a SEE-loaded APC (Fig. 4, left and top and supplemental movie 1). As soon as they contacted the loaded APC (supplemental movie 1), Jurkat cells extended lamellipodium-like protrusions. In the same conditions, RFP-β2-chimaerinY153F-expressing T cells rapidly formed small filopodium-like protrusions rather than lamellipodia (supplemental movie 2), indicating alterations in cytoskeletal organization. Moreover, in RFP-β2-chimaerinY153F-expressing cells, we detected relocalization neither of this construct (Fig. 4C, right and middle) nor of GFP-CD3ζ to the contact zone (Fig. 4C, right and bottom). Lack of GFP-CD3ζ relocation reflects impaired IS formation, suggesting that β2-chimaerinY153F overexpression has a profound effect on Rac-regulated responses.

PMA Induces Lck-mediated Tyrosine Phosphorylation of β2-Chimaerin at Tyr-153—DAG binding to the β2-chimaerin C1 domain is a critical mechanism for enzyme activation (24, 38). The impairment in IS formation in β2-chimaerinY153F-expressing cells impedes assessment of the effect of Tyr-153 phosphorylation on C1-mediated functions. For this reason, we used PMA, a pharmacological DAG analog that binds to the β2-chimaerin C1 domain, to analyze the effect of Tyr-153 phosphorylation on C1-dependent membrane binding of β2-chimaerin. We first assessed the effect of PMA treatment on β2-chimaerin tyrosine phosphorylation. As for TCR/CD28 stimulation, PMA treatment of Jurkat T cells induced Tyr phosphorylation of immunoprecipitated CFP-β2-chimaerin; this phosphorylation was sensitive to PP2 treatment (Fig. 5A). In contrast, we detected no PMA-induced phosphorylation in JCaM 1.6 cells (Fig. 5B). Re-introduction of Lck in these cells induced robust phosphorylation of β2-chimaerin after PMA treatment (Fig. 5B). Neither Y21F nor Y31F replacement decreased β2-chimaerin tyrosine phosphorylation in response to PMA, whereas Y153F mutation resulted in severe impairment of PMA-dependent tyrosine phosphorylation (Fig. 5C), as observed after TCR triggering. Finally, β2-chimaerinY153F was not phosphorylated in Lck-deficient JCaM cells reconstituted with Lck, a difference from WT β2-chimaerin (Fig. 5D). These results confirm that Tyr-153 is the main β2-chi-
This suggests that impairment of Tyr-153 phosphorylation facilitates PMA-induced translocation of β2-chimaerin, either through enhanced exposure of the C1 domain or by stabilizing the “open” protein conformation at the membrane.

We then compared the translocation kinetics of β2-chimaerin Y153F with those of a construct bearing an I130A mutation that disrupts intramolecular interactions, allowing exposure of the C1 region (26). We co-transfected Jurkat cells with the two mutants, fused to GFP or RFP, and used videomicroscopy to assess membrane localization in live cells after PMA treatment. Translocation kinetics were similar for GFP-β2-chimaerin Y153F and the RFP-β2-chimaerin I130A mutant, with relocalization to the membrane by 1-min post-PMA addition (Fig. 6B). These data confirm that, as for the I130A mutant, impairment of Tyr-153 phosphorylation facilitates C1-mediated membrane localization.

C1-mediated DAG Binding Is Inversely Related to β2-Chimaerin Tyr Phosphorylation—The previous experiments suggested a model in which Lck-dependent phosphorylation of β2-chimaerin acts as a negative regulatory mechanism for this protein, leading to its dissociation from the membrane and reducing its GAP activity. As the C1 domain promotes β2-chimaerin Rac-GAP activation by virtue of its ability to bind membrane DAG, we reasoned that regulation of β2-chimaerin activity by the C1 domain could be inversely related to its regulation by tyrosine phosphorylation.

To test this correlation in the context of physiological stimulation, we examined the effect of triggering TCR-dependent Tyr phosphorylation of β2-chimaerin in conditions that alter DAG generation. Treatment of Jurkat cells with the PLC inhibitor U73122, which reduces DAG levels after TCR stimulation (39), induced greater Tyr phosphorylation of immunoprecipitated GFP-β2-chimaerin than in untreated cells (Fig. 7A). DAG generated after TCR triggering is rapidly metabolized into phosphatidic acid by diacylglycerol kinases (40). Treatment of cells with the DAG kinase inhibitor R59949, which increases DAG levels following TCR triggering, resulted in decreased Tyr phosphorylation of β2-chimaerin (Fig. 7A). These data show that DAG generation negatively regulates Tyr phosphorylation of β2-chimaerin, suggesting that phosphorylation takes place when there is

**FIGURE 5. Lck Limits β2-Chimaerin GAP Function.**

A, Jurkat T cells were transiently transfected with control (CFP) or CFP-β2-chimaerin plasmids. Cells were treated with PMA (800 ng/ml, 5 min) and, where indicated, with PP2 (30 μM, 1 h). Cells were lysed, and CFP-β2-chimaerin was purified by immunoprecipitation (IP) with anti-GFP Ab. Tyr phosphorylation of the immunoprecipitated protein was determined by immunoblotting with anti-Tyr(P) Ab (top). Blots were reprobed with anti-GFP Ab as a control (bottom). B, Jurkat and JCaM cells were transfected with CFP-β2-chimaerin and, in the case of JCaM cells, co-transfected with p56Lck where indicated. Cells were treated with vehicle or PMA, and Tyr phosphorylation of immunoprecipitated CFP-β2-chimaerin was determined as in A. Blots of immunoprecipitated protein and total lysates (TL) were developed with the indicated Ab as controls. C, Jurkat cells were transfected with the indicated plasmids, and Tyr phosphorylation was determined as described in A. D, JCaM cells were transfected with plasmids encoding p56Lck and either CFP-β2-chimaerin or a CFP-β2-chimaerin Y153F mutant. Cells were treated and analyzed as in A. All experiments shown are representative of three independent assays with similar results.

**Impairment of Tyr-153 Phosphorylation Promotes C1-mediated Membrane Stabilization of β2-Chimaerin—**Crystallographic analysis showed that the β2-chimaerin C1 region is buried in the inactive conformation as the result of intramolecular interactions (26). Inaccessibility of the C1 domain in the inactive protein prevents PMA-dependent β2-chimaerin translocation to the cell membrane at the PMA concentrations that induce relocalization of the isolated β2-chimaerin C1 domain (24). Our experiments here indicated that PMA addition induced rapid phosphorylation of β2-chimaerin at Tyr-153; we therefore tested whether lack of phosphorylation affected PMA-dependent translocation kinetics. Confocal analysis of live Jurkat T cells co-expressing WT RFP-β2-chimaerin and GFP-β2-chimaerin Y153F confirmed lack of β2-chimaerin response to PMA treatment, whereas β2-chimaerin Y153F showed rapid, sustained PMA-dependent relocalization to the membrane (Fig. 6A, left and middle). This suggests that impairment of Tyr-153 phosphorylation

facilitates PMA-induced translocation of β2-chimaerin, either through enhanced exposure of the C1 domain or by stabilizing the “open” protein conformation at the membrane.

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The expression of β2-chimaerin in T cells (24) suggested a link between Rac-dependent actin dynamics and DAG generation in this activation process. Here we identify a mechanism that limits the negative function of β2-chimaerin in Rac-mediated responses after TCR triggering. Lck-dependent phosphorylation of β2-chimaerin on Tyr-153 in response to TCR stimulation negatively regulates C1-mediated β2-chimaerin stabilization at the membrane, and thus its Rac-GAP activity. Our data demonstrate dynamic regulation of β2-chimaerin during T cell activation, which would ensure the correct Rac activation required for this process. These results define additional complexity in DAG-dependent functions by showing a role for DAG in the control of TCR-induced cytoskeletal remodeling.

The negative effect of tyrosine phosphorylation on β2-chimaerin GAP activity confirms a report using EGF-stimulated COS cells (31). Nevertheless, there are notable differences between that study and the results presented here, suggesting that the mechanisms by which tyrosine phosphorylation regulates β2-chimaerin differ depending on cell context and/or stimulus. In contrast to the clear phos-
phorylation we observed in Jurkat T cells after TCR triggering, EGF-dependent tyrosine phosphorylation of β2-chimaerin is detected in COS cells only after treatment with tyrosine phosphatase inhibitors, suggesting that it is a very transient event (31). As to the identity of the phosphorylated residue, Tyr-21 appears to be the main residue phosphorylated in response to EGF; in our system, mutation of Tyr-21 to Phe did not diminish PMA- or TCR-dependent tyrosine phosphorylation of β2-chimaerin (Fig. 2 and Fig. 5C). Moreover, whereas PMA treatment induced clear β2-chimaerin phosphorylation in Jurkat T cells (Fig. 5A), Kai et al. (31) reported that similar treatment does not phosphorylate the protein when overexpressed in COS cells. This concurs with our experiments in HEK293 cells, in which β2-chimaerin was phosphorylated only when co-expressed with the T cell-specific Src kinase Lck (Fig. 1C). These observations suggest that, in addition to the Lck-induced phosphorylation of β2-chimaerin at Tyr-153, additional residues can be phosphorylated in a more transient manner and/or by other Src kinases.

Kai et al. (31) also reported phosphorylation of endogenous protein in rat cerebellum cells, supporting β2-chimaerin regulation by Src-dependent tyrosine phosphorylation as a potential general mechanism. A reduction in β2-chimaerin Rac-GAP activity because of Src-dependent Tyr phosphorylation would thus cooperate with increased activation of the Rac-GEF to promote the enhanced Rac activity required for Src-induced transformation (44).

Tyr-153 mutation to Phe not only renders β2-chimaerin more active, but it also facilitates PMA-dependent membrane translocation, implying that phosphorylation at this residue negatively regulates either C1 domain exposure and/or membrane interaction. Dephosphorylation of Tyr residues responsible for intramolecular interactions is the classical activation mechanism for Src family kinases (45). The presence of an SH2 domain that folds over the C1 domain in the inactive β2-chimaerin conformation suggests that a similar mechanism could be responsible for the transition from a closed/inactive to an open/active conformation. Our experiments nonetheless demonstrate that β2-chimaerin phosphorylation increases after cell stimulation. In addition, Tyr-153, which is located at the hinge between the SH2 and C1 domains, is poorly accessible in the inactive β2-chimaerin conformation, indicating that this Tyr can only be phosphorylated when the protein is in an open/active conformation. Our results suggest a model in which Tyr-153 phosphorylation takes place when β2-chimaerin is in an active state, promoting its dissociation from the plasma membrane. The constitutive Tyr phosphorylation of β2-chimaerinF215G, a DAG binding-defective mutant, as well as the increased phosphorylation of the WT protein in cells treated with a PLC inhibitor, further suggest a scenario in which C1-mediated membrane stabilization and Lck-dependent Tyr phosphorylation of β2-chimaerin are inversely related processes.

The chimaerin family includes two isoforms (α2- and β2-chimaerin), each of which has at least one splice variant (α1 and β1). Our experiments showing TCR-induced Tyr phosphorylation also for α2-chimaerin suggest similar negative regulation for both α2- and β2-chimaerin isoforms. Indeed, the Tyr-153 residue is conserved in α2-chimaerin (Tyr-143). Type 1 chimaerins (α1 and β1), which lack the SH2 domain and most of the inter-SH2-C1 region including Tyr-153 (or Tyr-143 for α1-chimaerin), are localized constitutively in the membrane and/or cytoskeletal fraction of various cell types, in contrast with the cytosolic localization of type 2 isoforms (15, 46, 47). This indicates that the splice variants lack the mechanisms responsible for negative regulation of membrane localization, which could include Tyr phosphorylation.

Our data demonstrate a mechanism for protein inactivation but do not explain how the protein achieves an open/active conformation. Recent studies suggest a role for the SH2 domain of α2-chimaerin in its activation through protein-protein interactions. Generation of α2-chimaerin-deficient mice showed that this isoform is a downstream effector of EphA4, with an essential function in EphA4-dependent axon navigation during neuron network formation (17–19). EphA4-mediated α2-chimaerin activation thus appears to be achieved by SH2 domain-dependent binding to EphA4. This interaction might be necessary to make the Rac-GAP domain available for Rac binding and subsequent inactivation.

Additional studies identified the Nck adaptor protein as an additional α2-chimaerin SH2 domain partner (18, 48, 49). Through pulldown experiments, we confirmed that both α2- and β2-chimaerin interact with Nck1 in Jurkat T cells, and we identified the Nck SH3 region as responsible for this interaction. Nck binds to the LAT-SLP76 complex (50) and the CD3ε chain (51), recruiting several proteins that contribute to cytoskeletal remodeling such as PAK1 (52, 53), WASP/N-WASP (54–56), and WIP (57). Nck-mediated recruitment of β2-chimaerin would provide a mechanism to localize this Rac-GAP near the T cell/APC contact zone, where actin polymerization is very active. β2-Chimaerin would thus form part of the multiprotein complex that is engaged in proximity to the TCR and is responsible for dynamic regulation of the cytoskeleton. This interaction would also facilitate recruitment of β2-chimaerin near Lck and PLCγ, which we show are two key modulators of β2-chimaerin stabilization at the membrane. Additional experiments are needed for detailed characterization of the nature and functional consequences of Nck-β2-chimaerin interaction in the context of T cell activation.

Our experiments showing negative regulation of β2-chimaerin by Lck-dependent phosphorylation in response to TCR triggering suggest a role for Lck in the regulation of both positive and negative signals in Rac activation. Vav1, the Rac-GEF with a key function in Rac activation after TCR triggering, localizes near the receptor by binding to SLP76, where Lck contributes to Vav1 activation (7, 8, 58). Lck would thus contribute to Rac activation by promoting Vav1 GEF function, as well as by inactivating β2-chimaerin GAP activity.

In summary, our studies support a model in which β2-chimaerin-mediated Rac inactivation is dynamically regulated by signals initiated by TCR activation, such as DAG generation and Lck-mediated phosphorylation. The continuous activation/inactivation of β2-chimaerin would help to maintain the
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Rac on/off cycle, necessary to guarantee correct F-actin polymerization/dem polymerization dynamics at the immune synapse, critical for efficient and accurate T cell responses.


Lck Limits β2-Chimaerin GAP Function