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ROLE OF CHIMAERINS, A GROUP OF Rac-SPECIFIC GTPase ACTIVATING PROTEINS, IN T-CELL RECEPTOR SIGNALING

María José Caloca^{1,*}, Pilar Delgado², Balbino Alarcón², and Xosé R. Bustelo^{1,*}

¹*Centro de Investigación del Cáncer and Instituto de Biología Molecular y Celular del Cáncer, CSIC-University of Salamanca, Campus Unamuno, E-37007 Salamanca, Spain*

²*Centro de Biología Molecular “Severo Ochoa”, CSIC-Autonomous University of Madrid, Campus Cantoblanco, E-28049, Madrid, Spain*

Abstract

Chimaerins are GTPase-activating proteins that inactivate the GTP-hydrolase Rac1 in a diacylglycerol-dependent manner. To date, the study of chimaerins has been done mostly in neuronal cells. Here, we show that $\alpha 2$ - and $\beta 2$ -chimaerin are expressed at different levels in T-cells and that they participate in T-cell receptor signaling. In agreement with this, we have observed that $\alpha 2$ - and $\beta 2$ -chimaerins translocate to the T-cell/B-cell immune synapse and, using both *gain*- and *loss-of-function* approaches, demonstrated that their catalytic activity is important for the inhibition of the T-cell receptor- and Vav1-dependent stimulation of the transcriptional factor NF-AT. Mutagenesis-based approaches have revealed the molecular determinants that contribute to the biological program of chimaerins during T-cell responses. Unexpectedly, we have found that the translocation of chimaerins to the T-cell/B-cell immune synapse does not rely on the canonical binding of diacylglycerol to the C1 region of these GTPase-activating proteins. Taken together, these results identify chimaerins as candidates for the downmodulation of Rac1 in T-lymphocytes and, in addition, uncover a novel regulatory mechanism that mediates their activation in T-cells.

Keywords

chimaerin; Rac1; T-cell receptor; cell signaling; Vav1; cytoskeleton; diacylglycerol; immune synapse

INTRODUCTION

T-cell receptor (TCR) engagement triggers a complex series of intracellular signaling steps that are essential for proper T-cell development and function [1,2]. This cascade of molecular events is initiated by the antigen-dependent activation of protein tyrosine kinases that, upon phosphorylation of downstream elements such as Vav1, phospholipase C- γ 1 and phosphatidylinositol 3-kinase, promote the activation of multiple biological responses [1,2]. One of these responses is the assembly of the actin cytoskeleton, a process critical for the formation of stable immune synapses between the T-cell and the antigen presenting cell, the polarized secretion

*To whom correspondence should be addressed: Xosé R. Bustelo; E-mail: xbustelo@usal.es or María J. Caloca; E-mail: mjaloca@usal.es, Tel.: +34-923294802, Fax: +34-923294743

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of cytokines, the translocation of a subset of signal transduction molecules to the plasma membrane, and other T-cell responses [3-5].

The essential role of members of the Rac GTPase family (Rac1, Rac2, Rac3 and RhoG) in the regulation of F-actin dynamics is now well established in different cell types [6-10]. In the case of T-cells, Rac1 and Rac2 have been shown to participate in a variety of cell functions, including synapse and lipid raft formation, antigen- and chemokine-triggered polarization, the activation of phospholipase C- γ 1, the proper stimulation of the Ras GDP releasing factor 1 (RasGRP1)/Ras/Erk pathway, and the upregulation of the activity of the nuclear factor of activated T-cells (NF-AT) [7,8,10,11]. The analysis of both *rac2* knockout animals and Rac1-overexpressing transgenic mice in the T-cell compartment has also provided strong genetic evidence supporting the crucial role of these GTPases in T-cell development, T-cell selection, in the assembly of immune responses to antigens by mature T-lymphocytes, and in the differentiation of helper T-cells [7,10,11]. Although much less characterized, there is also information regarding the participation of RhoG in the regulation of the cytoskeleton and NF-AT activities in T-cells [12]. However, *rhoG* deficient mice show no major alterations in T-cell biology [13]. Rac proteins also play roles at the level of antigen presenting cells, since the combined deletion of *rac1* and *rac2* genes impairs the antigen-presenting activity of dendritic cells in mice [10,14].

Like most GTPases of the Ras superfamily, Rac proteins cycle between an inactive, GDP-bound state and an active, GTP-bound state [9]. During cell stimulation, the transition from the inactive to the active state is promoted by GDP/GTP exchange factors (GEFs), a group of enzymes that catalyze the exchange of GDP by GTP on Rac proteins [15-17]. At the end of the signaling cycle, Rac proteins move back to the inactive state by hydrolyzing the bound GTP, a step facilitated by the elevation of their intrinsic GTPase activities by GTPase activating proteins (GAPs) [17,18]. The activity of Rac proteins is also modulated by Rho GDP dissociation inhibitors (RhoGDIs), a third group of molecules implicated in extracting Rac proteins from membranes and keeping them sequestered in the cytosol in the GDP-bound state [19,20]. The importance of this regulatory cycle is demonstrated by observations showing that mutations on Rac proteins that impair any of those regulatory steps promote the formation of either dominant negative or constitutively active proteins that severely disrupt Rac-dependent responses. Likewise, the abnormal upregulation of GEFs or loss of GAP activity has been associated to drastic developmental and functional dysfunctions in different tissues and species [21-25].

While the function of RhoGDIs and Rac GEFs has been extensively studied in T-cells using both signaling and knockout mouse approaches [7,19,26], the analysis of the implication of Rho/Rac GAPs in T-cell-related functions has remained largely unexplored. To start tackling this issue, we decided to investigate the implication of a group of Rac-specific GAPs, the chimaerin family, in TCR signaling. This family is composed of four members in mammals (referred to as α 1-, α 2-, β 1- and β 2-chimaerin) that originate from differential splicing events of two loci, *chn1* (in the case of the α -chimaerins) and *chn2* (in the case of the β -chimaerins) [27-31]. All protein products of these genes are characterized by the presence of a C-terminal GAP catalytic domain and a most N-terminal C1 domain that binds diacylglycerol (DAG) and chemical analogs such as bryostatin or phorbol esters [27-29,32,33]. Previous studies have shown that the C1 domain is important for the subcellular localization of some of these family members [34,35]. The α 2- and β 2-family members have also an N-terminal SH2 domain involved, presumably, in heteromolecular interactions [31,36-38]. α 2-chimaerin has been the focus of recent attention because it has been found that the spontaneous mouse mutation *miffy* maps within the *chn1* locus [39]. This mutation, which is reproduced by the knockout of the *chn1* gene [38-40], induces locomotor problems due to corticospinal axon guidance defects. We hypothesized that the function of chimaerin family members could also extended to the

downmodulation of TCR signaling since these GAPs are regulated by DAG [28,41], an intracellular second messenger that plays pleiotropic roles during T-cell signaling [1]. Furthermore, there was a recent report indicating that one member of this family, $\beta 2$ -chimaerin, plays inhibitory roles in the signaling pathways triggered by the CXCL12 chemokine in T-lymphocytes [42]. Consistent with our hypothesis, we report here that chimaerin family members are expressed in Jurkat T-cells, that they localize in the immune synapse, and that they play inhibitory roles in the context of TCR-mediated signals. In addition, we provide evidence indicating that the upstream regulation of chimaerins in T-cells occurs via DAG-independent mechanisms.

MATERIALS AND METHODS

Antibodies and reagents

Mouse monoclonal antibodies to green fluorescent protein and AU5 were obtained from Covance. The mouse monoclonal antibodies to Rac1 and PKC α were obtained from Upstate Biotechnology and BD Transduction Laboratories, respectively. The rabbit polyclonal antiserum to the Vav1 DH domain has been described previously [43]. The polyclonal anti- $\alpha 2$ -chimaerin antibodies were a generous gift from Dr. C. Hall (Institute of Neurology, London, UK). The anti-CD3 mouse monoclonal antibody was from Dako. The anti-TCR CD3 ζ 448 antibody has been described previously [44]. Horseradish peroxidase-conjugated secondary antibodies to rabbit and mouse IgGs were obtained from GE Healthcare. The Cy3-labeled anti-rabbit IgG antibodies, Alexa Fluor 635-labeled phalloidin, and the fluorescent B-cell tracker CMAC (7-amino-4-chloromethylcoumarin) were all from Invitrogen/Molecular Probes. *Staphylococcus* enterotoxin E was obtained from Toxin Technology. Poly-L-lysine was from EMD/Biosciences-Calbiochem. γ -globulin was obtained from Sigma.

Plasmids

pXJ40-FLAG- $\alpha 2$ -chimaerin was kindly provided by Dr. C. Hall. For the generation of the enhanced green fluorescent protein- $\alpha 2$ -chimaerin-encoding vector (pMJC52), the full-length *chn1* cDNA was excised from pXJ40-FLAG- $\alpha 2$ -chimaerin by digestion with Hind III and PstI and ligated into the Hind III/PstI-linearized pEGFP-C3 vector (Clontech Laboratories). For the generation of the enhanced green fluorescent protein- $\alpha 2$ -GAP-encoding vector, a cDNA fragment comprising the $\alpha 2$ -GAP domain (base pairs 761 to 1390) was amplified by PCR using the pXJ40-FLAG- $\alpha 2$ -chimaerin plasmid as template and subcloned into the pEGFP-C3 vector. Expression vectors encoding enhanced green fluorescent protein-tagged $\beta 2$ -chimaerin and mutants were obtained from Dr. M. Kazanietz (University of Pennsylvania Medical School, Philadelphia, USA). Plasmids encoding enhanced green fluorescent protein-CD3 ζ , wild type Vav1 (pJC11), oncogenic Vav1 ($\Delta 1$ -66 mutation, pJC12), Rac1 (pCEFL-AU5-Rac1) and constitutively-active forms (Q61L and F28L mutants) of Rac1 (pCEFL-AU5-Rac1^{Q61L} and pRMP46) have been already described [45-47]. *chn* and *vav1* cDNA sequences were from human and mouse origin, respectively. pNF-AT-*luc* was obtained from Dr. G. Crabtree (Departments of Developmental Biology and Pathology, Stanford University Medical School, Stanford, USA). pRL-SV40 was from Promega. The pGEX vector containing the Rac1 binding domain of PAK1 was provided by Dr. R. Cerione (Cornell University, Ithaca, NY, USA).

Cell culture and DNA transfections

The T-cell Jurkat and lymphoblastoid B-cell Raji cell lines were both cultured at 37 °C in an humidified 5% CO₂ atmosphere in RPMI-1640 supplemented with 10% fetal calf serum plus 100 units/ml of penicillin and streptomycin (Invitrogen-Gibco). For DNA transfections, exponentially growing Jurkat cells were collected, resuspended in 200 μ l of RPMI-1640 to a final concentration of 2×10^7 cells/ml, and electroporated with the indicated plasmids using a Gene Pulser II apparatus (250 V, 950 μ F; BioRad).

Quantitative reverse transcription-polynucleotide chain reactions (RT-PCR)

Total RNA from Jurkat cells was extracted using the RNeasy mini kit (Qiagen). Quantitative RT-PCRs were carried out using the QuantiTect SYBR Green RT-PCR kit (Qiagen) using an iCycler apparatus (Bio-Rad). Primers for the amplification of the $\alpha 2$ -*chn1* cDNA were 5'-GGAGCTACCTCATCCGGGAG-3' (forward) and 5'-TGTGTCTCTTTCAGGACT GGCA-3' (reverse). Primers for the amplification of $\beta 2$ -*chn2* cDNA were 5'-GCAGGCGGATGAG CTTCTT-3' (forward) and 5'-CTCACCCACAAA GTGTTTCCC-3' (reverse). The *gadph* mRNA expression levels in each sample were used as internal control using 5'-GGTCTTACTCCTTGG AGGCCATGTG-3' (forward) and 5'-ACCTAAC TACATCGTTTACATGTT-3' (reverse) primers. Analysis was carried out in six samples, each of them tested in triplicate.

Immunoblotting

Equal amounts of total cellular lysates were diluted 1:1 with SDS-PAGE sample buffer and boiled for 10 min. Lysates were then separated electrophoretically and transferred onto nitrocellulose filters (Schleicher and Schuell). Membranes were incubated with the appropriated antibodies and immunoreactive bands visualized using a standard chemiluminescence detection system (Pierce).

GTP-Rac1 pull-down experiments

Jurkat cells transfected with or without the pMJC52 plasmid for 48 h were disrupted in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 0.5% Triton X-100, 5 mM β -glycerophosphate, 1 mM NaF, 100 μ M Na₂VO₄, a mixture of protease inhibitors (Cømplete, Roche Molecular Biochemicals), and 10 μ g of a glutathione S-transferase (GST) fusion protein containing the Rac1 binding domain of PAK1. Cell lysates were precleared by centrifugation at 14,000 rpm for 10 min at 4 °C and then incubated with glutathione-Sepharose beads (GE Healthcare) for 1 h at 4 °C. After extensive washes, samples were boiled in SDS-PAGE sample buffer, separated by electrophoresis, and the bound Rac1 proteins detected by immunoblotting using anti-Rac antibodies. GTP-Rac levels were quantitated using the Quantity One 1D image analysis software (Bio-Rad).

NF-AT luciferase assays

Exponentially growing Jurkat cells were electroporated with 10 μ g of a firefly luciferase reporter plasmid containing NF-AT sites (pNF-AT-*luc*), 5 ng of a plasmid (pRL-SV40) encoding the *Renilla* luciferase, and the appropriate combination of expression plasmids encoding enhanced green fluorescent fusion molecules when appropriate, 20 μ g of pJC11 was included in the transfections. The total amount of transfected DNA was kept constant in all transfections by supplementing them with pEGFP-C3. 48 h after transfection, cells were either left resting or stimulated with anti-CD3 antibodies (10 μ g/ml) for 8 h. Cells were then harvested and luciferase activities determined using the Dual Luciferase Reporter System (Promega) according to the supplier's instructions. The values of firefly luciferase activity obtained in the different samples were always normalized taking into account the activity of the *Renilla* luciferase obtained in each sample. To confirm comparable expression of proteins in all samples, aliquots of lysates were subjected to SDS-PAGE and immunoblotted with either anti-green fluorescent protein or anti-Vav1 antibodies.

RNA interference

siRNAs to the *chn1* mRNA were purchased from Dharmacon. The siRNA sequences utilized were 5'-CCAAGGCAG CAGAAUACA UdTdT-3' (sense strand) and 5'-A UGU AUUCUGCUGCCUUGGdTdT-3' (antisense strand). As a control, a scrambled siRNA was used in parallel transfections. To introduce the siRNAs in Jurkat cells, exponentially

growing cultures were collected, resuspended in 200 μ l of RPMI-1640 to a final concentration of 2×10^7 cells/ml, and electroporated with 5 nmol of the appropriate siRNA in the Gene Pulser II apparatus as indicated above. NF-AT activation was performed 48 h after the siRNA transfection, as indicated in previous sections.

Confocal microscopy

For subcellular localization studies, Jurkat cells were transfected with enhanced green fluorescent protein-chimaerin encoding vectors. 24 h after transfection, cells were transferred to poly-L-lysine-coated coverslips and allowed to attach for 15 min. Cells were then stimulated with either an anti-CD3 antibody (10 μ g/ml) or phorbol myristate acetate (PMA) (2 μ M, Sigma) for the indicated times, fixed with 3.7% formaldehyde, and analyzed using a laser scanning confocal microscope (LSM 510, Zeiss). Confocal images were collected using the LSM510 software (Zeiss) and finally processed for the preparation of Figures using the Photoshop software (Adobe Systems).

Subcellular fractionation studies

Jurkat cells were treated with 2 μ M PMA for 5 min, harvested, resuspended in a hypotonic lysis buffer (20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 1 mM NaF, 100 μ M Na₂VO₄ and the Cømplete protease inhibitor cocktail) and sonicated. Cell homogenates were subjected to ultracentrifugation to obtain the soluble (cytosolic-enriched) and particulate (membrane-enriched) fractions, as previously described [33]. Equal amounts of protein of each of those fractions were subjected to SDS/PAGE, transferred to nitrocellulose membranes, and immunoblotted.

Conjugate formation and time-lapse fluorescence microscopy

Jurkat cells were transiently transfected by electroporation with the *chn* cDNAs 16 h before being used. Raji cells were loaded at 37 °C for 30 min with CMAC (5 μ M), washed, resuspended in RPMI-1640 supplemented with 5 % fetal calf serum, and preloaded with *Staphylococcus* enterotoxin E (1 μ g/ml) for 30 min at 37 °C. To allow the formation of T-cell/B-cell conjugates, the transfected Jurkat cells (2×10^5 cells/slide) were mixed with an equal number of those Raji cells in a final volume of 50 μ l/slide. Conjugates were obtained by low speed centrifugation, resuspended, and allowed to settle for 15 min at room temperature onto poly-L-lysine-coated coverslips. Attached cells were fixed for 5 min in 2% paraformaldehyde in phosphate-buffered saline solution and then permeabilized for 1 min in the same buffer containing 1% Triton X-100. After blocking Fc receptors with γ -globulin (100 μ g/ml), TCRs were stained serially with anti-TCR CD3 ζ 448 and Cy3-labeled anti-rabbit IgG antibodies. F-actin was also stained using Alexa Fluor 635 phalloidin. Quantitative analysis of conjugates containing enhanced green fluorescent protein proteins at the immune synapse was assessed by fluorescence microscopy. In these experiments, we counted at least 125 different conjugates containing enhanced green fluorescent protein-positive T-cells from three independent experiments. For time-lapse microscopy, glass bottom culture plates (Mat-Tek) were coated with fibronectin (20 μ g/ml) for 20 h at 4 °C and then saturated with Hank's balanced salt solution containing 1% BSA for 30 min at 37 °C. Plates were washed with Hank's balanced salt solution and placed onto the microscope stage. 15×10^4 transiently transfected Jurkat cells resuspended in 1 ml of Hank's balanced salt solution containing 2% fetal calf serum were subsequently allowed to attach for 30 min at 37 °C. *Staphylococcus* enterotoxin E-loaded, red fluorescent protein-expressing Raji cells (1×10^5) were added to the plate containing the Jurkat cells and conjugate formation monitored by collecting pictures of the cultures every 60 sec using an inverted Axiovert 2000 microscope.

Statistical analysis

When three or more than three groups were analyzed, the statistical significance of the results obtained was determined by ANOVA analysis followed by a Bonferroni's multiple comparison test. When data from only two independent experiments were considered, data were analyzed using the Student's *t*-test. *P* values ≤ 0.05 were always considered as statistically significant and indicated as such in the histograms shown in the Figures.

RESULTS

Expression of chimaerin family members in Jurkat T-cells

A previous work reported the expression of the *chn2* mRNA in Jurkat and other lymphoid cell lines [42]. In order to identify whether other family members are expressed in T-cells, we decided to compare the levels of expression of the *chn1* and *chn2* mRNAs in Jurkat cells using quantitative RT-PCR. This analysis confirmed the expression of the *chn2* mRNA in this cell line (Fig. 1A). However, it also revealed that the *chn1* transcript levels were ≈ 60 fold higher than those of its family counterpart (Fig. 1A). Notably, *chn1* mRNA levels were only 2-fold lower than those of the *gadh* housekeeping gene (Fig. 1A), indicating that they were relatively abundant within Jurkat cells. The analysis of the RT-PCR fragments obtained with the *chn1*- and *chn2*-specific primers by agarose gel electrophoresis confirmed that they had the expected sizes (Fig. 1B). Immunoblot analysis confirmed the expression of $\alpha 2$ -chimaerin in these cells (see below, Fig. 3B).

To verify that $\alpha 2$ -chimaerin was active in this cell setting, we performed pull-down experiments to investigate the effects of an enhanced green fluorescent protein (EGFP)-tagged version of this GAP in the levels of active Rac1 present in Jurkat cells. As shown in Fig. 1B, the levels of GTP-Rac1 in TCR-stimulated cells were drastically reduced in cells expressing the EGFP- $\alpha 2$ -chimaerin. These results indicate that $\alpha 2$ -chimaerin works as a bona-fide Rac1 GAP in Jurkat cells.

Negative modulation of CD3- and Vav1-dependent NF-AT activity by chimaerins in Jurkat cells

To further characterize the implication of chimaerins in the modulation of TCR signals, we decided to use both *gain*- and *loss-of-function* approaches to test the roles of these GAPs in the modulation of NF-AT, a signaling route activated by the TCR that is crucial for both T-cell activation and differentiation [48]. To this end, we first used luciferase reporter assays to test the effect of overexpressing $\alpha 2$ - and $\beta 2$ -chimaerin in the levels of NF-AT activity of Jurkat cells. As shown in Fig. 2A, the overexpression of any of these two chimaerin family members resulted in a significant reduction of the NF-AT activity induced by the stimulation of Jurkat cells with soluble anti-CD3 antibodies. Since the activation of NF-AT in Jurkat cells requires the activity of the Rho/Rac exchange factor Vav1 [49], we also studied the effect of the overexpression of chimaerins in the activation of NF-AT mediated by the ectopic expression of this GEF. As initially reported [49], Vav1 overexpression promoted the stimulation of the transcriptional activity of NF-AT, both in non-stimulated and anti-CD3-stimulated cells (Fig. 2B). Such activity was severely repressed upon the co-expression of either $\alpha 2$ - or $\beta 2$ -chimaerin with Vav1 in Jurkat cells (Fig. 2B). These repressions were not due to improper expression of the ectopic proteins, because immunoblot analysis indicated that all proteins used in the experiments had similar levels of expression among different transfections (Fig. 2A,B; insets). These results indicate that, at least under overexpression conditions, chimaerin family members have negative regulatory roles in the regulation of the TCR- and Vav1-mediated pathways that lead to NF-AT activation.

To corroborate these results under more physiological conditions, we decided to perform *loss-of-function* studies to evaluate the specific role of $\alpha 2$ -chimaerin in this signaling route. To this end, we evaluated the levels of NF-AT activity in Jurkat cells in which the expression of both the *chn1* mRNA and $\alpha 2$ -chimaerin protein, but not of the *chn2* transcripts, were lowered $\approx 60\%$ using siRNA-mediated techniques (Fig. 3A,B). As negative control, we measured the NF-AT activity in Jurkat cells electroporated with a scrambled siRNA. Luciferase-based NF-AT reporter assays indicated that the *chn1* knockdown induced a 3-fold increase in the basal levels of NF-AT activity of non-stimulated Jurkat cells (Fig. 3C). Likewise, the reduction of $\alpha 2$ -chimaerin expression induced a 2-fold increase in the levels of NF-AT activity of anti-CD3-stimulated Jurkat cells (Fig. 3C). Taken together, these results indicated that $\alpha 2$ -chimaerin does modulate negatively specific output signals from the TCR.

The GAP activity of chimaerins is required for the inhibition of NF-AT signaling

We next used a collection of $\beta 2$ -chimerin mutants already available in our laboratory to investigate the role of its three known structural domains in the regulation of NF-AT activity. This collection included either missense or small intradomain deletions mutants with inactive SH2, C1 or GAP domains (Fig. 4A, proteins labeled as 2, 3 and 4, respectively) and truncated mutants lacking the SH2-C1 region, the entire SH2 domain plus downstream sequences, or the GAP domain (Fig. 4A, proteins labeled as 5, 6 and 7, respectively). In addition, we included an $\alpha 2$ -chimerin mutant containing exclusively the catalytic GAP domain. To facilitate the read-out of these experiments, we measured NF-AT activities always in the presence of overexpressed Vav1 because, under these conditions, the levels of NF-AT activity were more robust than in non-transfected, TCR-stimulated cells (see **Figs. 2 and 4B**). These experiments indicated that the $\beta 2$ -chimaerin SH2 region was totally dispensable for the inhibitory activity of this protein (Fig. 4B, left panel, compare proteins 2, 5 and 6 with protein 1). In contrast, the GAP domain was essential for the downmodulation of NF-AT signals, since $\beta 2$ -chimaerin proteins lacking this domain were totally inactive in this cellular response (Fig. 4B, left panel, compare proteins 4 and 7 with 1). In agreement to these results, the activity of a $\beta 2$ -chimaerin mutant protein containing only the GAP domain was indistinguishable from that of its wild type counterpart (Fig. 4B, left panel, compare proteins 5 and 1). Similar results were obtained when an analogous $\alpha 2$ -chimerin mutant was included in the experiments (Fig. 4C, left panel). Interestingly, the deletion of the entire $\beta 2$ -chimaerin GAP domain seemed to generate dominant negative mutants, since the levels of NF-AT activity in Jurkat cells were significantly increased when Vav1 was co-expressed with a C-terminally truncated $\beta 2$ -chimaerin containing exclusively the SH2-C1 domains (Fig. 4B, left panel, compare bars of protein 7 with the bars of Vav1 alone). However, this effect was not observed with $\beta 2$ -chimerin mutants containing small deletions inside the GAP region (Fig. 4B, left panel, protein 4), a discrepancy probably due to the lower levels of expression of the latter mutant when compared to the $\beta 2$ -chimerin lacking the entire GAP region (Fig. 4B, right panel, compare lanes 4 and 7).

Whereas the analysis of the SH2 and GAP domains gave a clear-cut picture in all these experiments, we observed that the role of the C1 domain was more complex. Thus, we found that the mutation of this domain decreased, although it did not abolish, the biological activity of the full-length $\beta 2$ -chimaerin protein (Fig. 4B, left panel, compare proteins 3 and 1). However, we observed that the activity of the isolated GAP domain was similar with or without an attached C1 region (Fig. 4B, left panel, compare proteins 5 and 6), indicating that the C1 region was totally dispensable for the activity of the N-terminally truncated $\beta 2$ -chimaerin proteins. Immunoblot analysis confirmed that all these proteins were properly expressed in all these experiments (Fig. 4B,C, right panels).

To confirm that the downmodulation of the NF-AT pathway by chimaerins is mediated by their GAP activities and, consequently, relies on the reduction in the levels of active, GTP-loaded

Rac1 in Jurkat cells, we analyzed the effect of the overexpression of chimerins in the NF-AT activity triggered by constitutively active versions of Rac1. To this end, we co-transfected chimaerins with Rac1^{Q61L}, a commonly used GTPase-deficient mutant or, alternatively, with Rac1^{F28L}, a protein that shows an accelerated rate of nucleotide exchange and, thereby, is preferentially loaded with GTP. However, unlike Rac1^{Q61L}, this mutant maintains its GTPase activity intact [50,51]. As shown in Supplementary Fig. S1, $\alpha 2$ and $\beta 2$ -chimerins were incapable of downmodulating the NF-AT activity elicited by the GTPase-deficient Rac1 mutant. Instead, they showed a significant, although not complete inhibition of the NF-AT activity induced by the rapid cycling Rac1 mutant. Taken together, these results indicate that the downmodulation of the NF-AT pathway by chimaerins is mediated by its GAP domain and, consequently, relies on the catalysis of GTP hydrolysis on active Rac1.

Chimaerin proteins do not translocate to the plasma membrane upon TCR engagement

We next investigated the mechanism of regulation of chimaerins during TCR signaling. Previous works have shown that the upregulation of the activity of chimaerins during signal transduction occurs concurrently with their shuttling from the cytosol to either the Golgi apparatus or the plasma membrane. This translocation occurs through the binding of the chimaerin C1 domain to DAG and, in the case of the Golgi apparatus, on the additional binding of $\beta 2$ -chimaerin to the Golgi-localized Timp21-I protein [34,35,37]. To study whether a similar regulation occurred in T-cells, we monitored the changes in the subcellular localization of EGFP-tagged $\alpha 2$ - and $\beta 2$ -chimaerin upon the treatment of Jurkat cells with anti-CD3 antibodies for 5 min. As positive control, we used an EGFP fused to RasGRP1, a DAG-dependent GEF for Ras that has been shown before to translocate to the plasma membrane of lymphocytes upon similar stimulation conditions [52,53]. Unexpectedly, we found that the stimulation of the TCR did not induce any translocation of chimaerin family members to membrane structures (Fig 5A, see first and second rows of panels from top). Similar results were obtained when Jurkat cells were stimulated during shorter and longer incubation times with anti-CD3 antibodies (data not shown). In contrast, the EGFP-RasGRP1 fusion protein readily moved to the plasma membrane of stimulated Jurkat cells under identical experimental conditions (Fig. 5A, lower panels).

Since high doses of the DAG analog PMA can also trigger changes in the intracellular distribution of $\beta 2$ -chimaerin [33,34], we decided to monitor the effect of this compound on the subcellular localization of $\alpha 2$ -chimaerin, $\beta 2$ -chimaerin and RasGRP1. As expected [33,34, 42,54], EGFP- $\beta 2$ -chimaerin and, to a larger extent, EGFP-RasGRP1 redistributed from the cytoplasm to the plasma membrane upon the administration of PMA to Jurkat cells (Fig 5B, middle and bottom panels, respectively). By contrast, EGFP- $\alpha 2$ -chimaerin kept its cytosolic localization under the same experimental conditions (Fig. 5B, upper panels). To eliminate the possibility that the defective translocation of $\alpha 2$ -chimaerin was due to either the fusion of the N-terminal EGFP or to the overexpression conditions, we analyzed the behavior of the endogenous protein using subcellular fractionation and immunoblotting techniques. As positive control, we used in these experiments protein kinase C (PKC) α , a protein previously shown to undergo shifts from the cytoplasm to the plasma membrane upon treatment of T-cells with phorbol esters [55]. Under conditions in which the endogenous PKC α effectively translocated to particulate fractions (Fig. 5C, compare middle and bottom immunoblots in the right column), the subcellular distribution of $\alpha 2$ -chimaerin remained unchanged between non-stimulated and PMA-stimulated cells (Fig 5C, compare middle and bottom immunoblots in the left column). Taken together, these observations indicate that chimaerins, unlike other C1-containing proteins such as RasGRP1 and PKCs, do not translocate to the plasma membrane upon TCR stimulation. In addition, they indicate that the role of the C1 domain in the regulation of the biological activity of full-length chimaerin proteins shown previously in Fig. 4B does not relies in its ability to tether these proteins to specific subcellular compartments.

Recruitment of chimaerins to the T-cell/B-cell immune synapse

Based on these above observations, we decided to monitor whether the subcellular localization of chimaerins could be influenced by the formation of the immune synapse between Jurkat cells and superantigen-loaded Raji cells. Using time-lapse fluorescence microscopy, we observed that both the EGFP- $\alpha 2$ - and EGFP- $\beta 2$ -chimaerin proteins were evenly distributed in the cytosol of non-stimulated T-cells (Fig. 6A, left panels). However, unlike the case of the stimulation with soluble anti-CD3 antibodies (see above), these proteins rapidly moved to the immune synapse upon the initial Jurkat cell/B-cell contact (Fig. 6A, third panels from left). The localization of the EGFP-chimaerin proteins at the immune synapse was maintained for at least 10-15 min after the first contact (Fig. 6A). This translocation was specific, since the non-chimeric EGFP did not undergo such changes under identical experimental conditions (data not shown, but see below, Fig. 6B,C).

To characterize the translocation of EGFP-tagged chimaerins to the immune synapse in more detail, we resorted to confocal microscopy experiments using formaldehyde-fixed T-cell/B-cell conjugates (see Materials and Methods). As negative and positive controls, we used Jurkat cells expressing EGFP alone and a CD3 ζ -EGFP fusion protein, respectively. Since the latter protein is associated to the TCR, it is a good marker to follow up the clusterization of this antigen receptor upon the recognition of the antigen presenting cell. After transfection of the appropriate EGFPs and conjugate formation with superantigen-loaded Raji cells, cells were fixed and stained with anti-CD3 ζ antibodies and Alexa Fluor 635-labeled phalloidin to track the localization of the CD3 ζ subunit and F-actin in the immune synapse, respectively. As a complementary approach, we also measured the distribution of the fluorescence intensity of each protein along a defined axis of the T-cell in order to obtain a more quantitative view of the translocation of both the EGFPs and CD3 ζ . These experiments indicated that $\alpha 2$ - and $\beta 2$ -chimaerins were detected in the cytosol and the immune synapse of unconjugated and conjugated T-cells, respectively (Fig. 6B,C; first and second rows of panels from top, respectively). As expected, the endogenous CD3 ζ (data not shown) and the ectopically expressed CD3 ζ -EGFP (Fig. 6B,C, third row of panels) were homogenously distributed in the plasma membrane of non-engaged T-cells but, upon conjugation, both of them redistributed preferentially towards the immune synapse (Fig. 6B,C, third row of panels). F-actin was not detected in non-engaged T-cells but, as in the previous cases, accumulated at the T-cell/B-cell contact upon conjugate formation (Fig. 6B, see panels in the four column from left). In contrast, the non-chimeric EGFP molecule remained in the cytosol and the nucleus both in non-conjugated and conjugated T-cells (Fig. 6B,C; bottom panels).

To measure these translocation events in more detail, we counted the percentage of EGFP-chimaerin molecules present in the immune synapses of paraformaldehyde fixed T-cell/B-cell conjugates. As a positive control, we also estimated the percentage of translocation of the TCR CD3 ζ subunit to the same cellular structures in the EGFP-positive cells. These experiments indicated that 80% of the conjugated T-cell/B-cell complexes showed the EGFP- $\alpha 2$ -chimaerin at the immune synapse, a percentage similar to that shown by CD3 ζ molecules (Fig. 6D). Similar results were obtained with EGFP- $\beta 2$ -chimaerin, although the translocation of this chimaerin family member was slightly less efficient (59% of all conjugates; Fig. 6D). However, under these conditions, we also observed that the localization of TCR CD3 ζ at the immune synapse was also reduced to $\approx 67\%$ of conjugation events (Fig. 6D). In agreement with the previous results, the non-chimeric EGFP protein showed no migration to the immune synapse (Fig. 6D). These results indicate that the subcellular localization of chimerins is modulated by the formation of the immune synapse with antigen presenting cells.

Structural determinants of the translocation of chimaerins to the T-cell/B-cell immune synapse

To characterize the molecular determinants responsible for the translocation of chimaerins to this structure, we investigated the ability of the previously used chimaerin mutant proteins to translocate to the areas of T-cell/B-cell contact. These experiments indicated that the GAP region was not required for this translocation event, because β 2-chimaerin molecules containing an inactive GAP domain translocated to the immune synapse at rates similar to those shown by the wild type protein (Fig. 7A,B, compare first and second panels from top; Fig. 7C). In concordance with these observations, we observed that the β 2- or the α 2-chimaerin GAP domains could not translocate to the immune synapse in the absence of the N-terminal regions (Fig. 7A,B, see third and fourth panels from top, respectively; Fig. 7C). Instead, a truncated β 2-chimaerin protein containing only the N-terminal SH2 and C1 domains did show optimal anchoring to the immune synapse (Fig. 7A,B, fifth panel from top; Fig. 7C), suggesting that the SH2, the intervening sequence (ITS) and/or the C1 were involved in this translocation event. The subsequent analysis of the β 2-chimaerin C1 point mutant eliminated the possible participation of the C1 region in this response, because this protein migrated as efficiently as its wild type counterpart to the immune synapse (Fig. 7A,B, sixth panel from top; Fig. 7C). Intriguingly, we also observed that β 2-chimaerin proteins carrying a mutation in the SH2 phospho-tyrosine binding pocket could also translocate to the immune synapse (Fig. 7A,B, seventh panel from top), suggesting that the ITS alone or in combination with some of the other N-terminal domains contribute to this translocation step (see Discussion). In the absence of T-cell/B-cell conjugation, the EGFP-chimaerin proteins were visualized exclusively in the cytosol or, alternatively, both in the cytosol and the nucleus (in the case of the isolated GAP regions, Fig. 7A, left column). As controls for these experiments, we observed that the endogenous CD3 ζ subunit and F-actin concentrated in the immune synapse in the majority of the T-cell/B-cell conjugates screened by confocal microscopy (Fig. 7A-C). In contrast, the non-chimeric EGFP remained in the cytosol and the nucleus both in the non-conjugated and conjugated cells (Fig. 7A,B, bottom panels). Taken together, these results indicate that the translocation of chimaerins to the immune synapse is not mediated by the interaction of these proteins with activated Rac1 (via the GAP region), DAG (via the C1 domain) or other intracellular phospho-proteins (via the SH2 domain) that could be present at high local concentrations in the immune synapse.

The differential behavior of chimaerin in terms of membrane translocation between anti-CD3-stimulated and B-cell-conjugated Jurkat cells indicated that these GAPs may require specific signals to become physically close to plasma membrane. Given the high content of F-actin in the immune synapse, we hypothesized that cytoskeletal-dependent signals may contribute to this translocation event. To explore this possibility, we investigated whether chimaerins could move to the plasma membrane in non-conjugated cells under conditions of high levels of F-actin polymerization induced by the expression of the oncogenic, constitutively active version of Vav1. α 2- and β 2-chimaerin did translocate to the membrane under these conditions. However, such translocation was circumscribed to areas of the plasma membrane enriched in both Vav1 and F-actin molecules (Fig. S2). Taken together, these results suggest that the effective translocation of chimaerins to the plasma membrane of T-cells requires conditions that trigger high, or spatially concentrated levels of F-actin polymerization.

DISCUSSION

We have shown in this work that chimaerins participate in the modulation of the signaling output of the TCR, the receptor of T-cells in charge of T-cell development, T-cell selection, and antigen recognition [1]. In agreement to this function, we have observed that α 2- and β 2-chimaerin migrate to the immune synapse in the Jurkat:Raji-superantigen system. In addition,

we have shown using both *gain*- and *loss-of-function* approaches that chimaerin family members participate in the downmodulation of TCR- and Vav1-dependent pathways leading to NF-AT stimulation. The use of chimaerin mutant proteins indicate that their three structural domains contribute differently to the TCR-related functions of chimaerins and, in addition, that their importance may vary depending on the type of biological process analyzed. In the case of NF-AT responses, we observed that the SH2 domain is irrelevant and the GAP region essential for the effective action of $\alpha 2$ - and $\beta 2$ -chimaerin on this route. The function of the C1 domain is more complex, being important for the activity of the full-length protein but totally dispensable for the N-terminally truncated mutants. While the requirement of the catalytic domain is fully consistent with the role of chimaerins as Rac1-specific GAPs, the results obtained with SH2 and C1 mutants can be rationalized taking into consideration the $\beta 2$ -chimaerin crystal structure [56]. This model indicates that the inactive form of $\beta 2$ -chimaerin adopts an auto-inhibitory, “closed” conformation in which the C1 and GAP domains are buried inside the molecule by overlapping sequences of the N-terminus. Due to this, the phospholipid binding surface of the C1 is not exposed to the exterior of the protein and, in addition, the GAP region is inactive due to both a catalytically inefficient conformation and the occlusion of the GTPase binding site [56]. According to this model, the binding of the C1 to DAG, PMA or other possible ligands would unleash that inhibitory structure, leading to the opening of the molecule and the exposure of the GAP domain in a catalytically compatible structure [56]. Based on this model, the lack of requirement of the C1 domain for the activity of the mutants containing the isolated GAP domain observed in our experiments could be explained because, due to the removal of the inhibitory N-terminal sequences, these mutants are already in a fully active conformation. Likewise, the regulatory role of the C1 in the case of full-length GAP proteins would reflect its importance for eliminating the auto-inhibitory structure of these GAPs during signal transduction. In the same regulatory context, the full activity of the SH2 mutants in NF-AT downmodulation is an indirect confirmation of the passive role of this domain in the process of chimaerin activation inferred from the crystal structure of these proteins.

Interestingly, we have observed that chimerins translocate to the immune synapse during TCR signaling. Using a collection of chimaerin mutants, we have also observed that this tethering step is C1 independent, suggesting that DAG signaling is not directly involved in this signaling step. This is not a property that can be generalized to all C1-containing proteins, because PKD1 does require DAG for effective translocation to the immune synapse [57]. However, it should be noted that the translocation of PKC θ to the immune synapse appears to be cytoskeletal-dependent rather than DAG-mediated [58,59]. A role of the F-actin cytoskeleton in the translocation of RasGRP1 to the plasma membrane has been also demonstrated [54]. It is likely that a similar mechanism may occur in the case of chimaerins, since we have observed that these GAPs can effectively move to membrane areas under artificial conditions of F-actin polymerization, both in T-cells (this paper) and non-hematopoietic cells (i.e., COS1 cells, unpublished data). The results presented in this work also indicate that the structural motif(s) involved in the translocation of chimerins to the immune synapse are localized in their N-terminus. Thus, the isolated GAP domains of either $\alpha 2$ - or $\beta 2$ -chimerin do not translocate to this structure upon T-cell/B-cell conjugation. By contrast, a truncated version of $\beta 2$ -chimerin encompassing the SH2-ITS-C1 region translocates as efficiently as the wild type protein to the immune synapse. Several observations indicate that the ITS region is probably involved in this regulatory step. Thus, we have observed that a $\beta 2$ -chimerin protein containing a mutation in the SH2 that eliminates its phosphotyrosine binding activity (although not the overall folding of the domain) still translocates to the immune synapse, suggesting that this tethering step does not involve a canonical phosphotyrosine-dependent protein-protein interaction event. Furthermore, we have observed that $\beta 1$ -chimerin, an isoform that lacks the SH2 domain but contains most of the ITS, also translocates to the immune synapse. However, this translocation is lost upon the deletion of $\beta 1$ -chimerin ITS (data not shown). Despite this evidence, all our

efforts aimed at demonstrating that the isolated ITS intervening region translocates to the immune synapse have yielded negative results. At this moment, we do not know whether this lack of translocation is due to a technical problem derived from the generation of the EGFP-ITS (i.e., lack of proper folding) or, alternatively, that is due to the need for cooperating actions by other domains present in the chimaerin structure. Further studies will be needed to address this important regulatory issue.

In addition to the DAG-independent translocation of chimerins to the immune synapse, a totally unexpected result of our work is the observation that $\alpha 2$ -chimaerin and $\beta 2$ -chimaerin do not translocate to the plasma membrane or the Golgi apparatus upon the stimulation of Jurkat cells with soluble anti-CD3 antibodies. Furthermore, we could not see any translocation of $\alpha 2$ -chimaerin upon treatment of cells with PMA although, in this case, $\beta 2$ -chimaerin did show an enrichment in the cellular membranes of Jurkat cells. This behavior is in contrast to previous results in this GAP family in other cell types [34,35,37]. Several observations indicate that the lack of regulation of chimaerin by DAG in Jurkat cells is not an experimental artifact. Thus, we have demonstrated that the DAG-regulated RasGRP1 does shift from the cytosol to the plasma membrane under identical experimental conditions. Using subcellular fractionation experiments, we have also demonstrated that the endogenous PKC α can move to membrane-enriched fractions of PMA-stimulated Jurkat cells in the same conditions in which the endogenous $\alpha 2$ -chimaerin fails to do so. These two observations rule out the possibility that the lack of translocation of these GAPs during anti-CD3-mediated TCR signaling could be due to the lack of DAG generation or, alternatively, to artifacts derived from the use of overexpressed proteins. Given that the isolated C1 domains of $\alpha 2$ - and $\beta 2$ -chimaerin show affinities to DAG analogs similar or even higher than C1 domains of PKCs and RasGRP1 [32,33,60], we believe that the differential behavior of these GAPs is probably a reflection of the high-energy cost required for releasing the auto-inhibitory structure of chimaerins [56]. This interpretation is consistent with the observation that $\beta 2$ -chimaerin requires in vivo ≈ 100 -fold higher doses of PMA than PKC α for its effective translocation to cellular membranes [34].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A **B** **FIGURE 1.**

Expression and GAP activity of *chn* family genes in Jurkat cells. (A) *Left panel*, determination of the expression levels of *chn1* and *chn2* mRNAs by quantitative RT-PCR in Jurkat T-cells, as indicated in Materials and Methods. The values represent the percentage of each transcript relative to the expression levels of the *gadph* mRNA used as control. Bars and error bars represent, respectively, the mean $\pm$ s.e.m. of all determinations performed ($n = 6$). *Right panel*, analysis by agarose gel electrophoresis of the size of the *chn1*, *chn2* and *gadph* cDNA fragments generated in the RT-PCR experiments. ϕ X174, a sample of HaeIII-digested ϕ X174 DNA used as molecular size marker. The size of each cDNA fragment is shown in the right. bp, basepairs. (B) *Left panel*, effect of α 2-chimaerin on the levels of activated, GTP-bound

Rac1 present in T-cells. Control Jurkat cells or cells expressing EGFP- α 2-chimaerin were left untreated (-) or stimulated (+) with anti-CD3-antibodies and subjected to pull-down experiments to detect the levels of GTP-bound Rac1 proteins (top panel). As control, aliquots of the same total cellular lysates were subjected to immunoblot analysis with anti-Rac1 and anti-EGFP antibodies to visualize the levels of expression of Rac1 (middle panel) and EGFP- α 2-chimaerin (lower panel) in each sample. Chn, chimaerin; PD, pull-down; TCE, total cellular extract. *Right panel*, quantification of GTP-Rac1 levels in control and α 2-chimaerin-expressing Jurkat cells. Values are expressed as fold induction vs. control unstimulated cell and represent the mean $\pm$ standard error ($n = 3$; **, $P \leq 0.01$).

FIGURE 2.

Chimaerin overexpression leads to the downmodulation of the transcriptional activity of NF-AT stimulated by both the TCR and Vav1. **(A)** Effect of the overexpression of chimaerins in anti-CD3-stimulated NF-AT levels. Jurkat cells expressing the indicated EGFP molecules were either left non-stimulated or stimulated with anti-CD3 antibodies and NF-AT activities determined as indicated in Materials and Methods. Results are expressed as fold change respect to the NF-AT activity present in non-stimulated cells expressing a non-chimeric EGFP ($n = 4$; ***, $P \leq 0.001$). The expression of the EGFP molecules used in one of these experiments was determined by immunoblot using anti-EGFP antibodies (inset). Similar expression levels were obtained in the other experiments (data not shown). Arrows indicate the migration of EGFP-tagged chimaerins (upper panel) and the non-chimeric EGFP (lower panel). **(B)** Effect of the

overexpression of chimaerins in Vav1-stimulated NF-AT levels. Jurkat cells expressing the indicated EGFP molecules were either left non-stimulated or stimulated with anti-CD3 antibodies and NF-AT activities determined as indicated in Materials and Methods. Results are expressed as fold change respect to the NF-AT activity present in non-stimulated cells expressing the non-chimeric EGFP ($n = 4$; ***, $P \leq 0.001$). The expression of the ectopic molecules used in one of these experiments was determined by immunoblot with appropriate antibodies (inset). Similar expression levels were obtained in the other experiments (data not shown). Arrows indicate the migration of EGFP-tagged chimaerins (upper panel), non-chimeric EGFP (middle panel), and untagged Vav1 (lower panel).

FIGURE 3.

The endogenous $\alpha 2$ -chimaerin contributes to the regulation of the NF-AT route in Jurkat cells. (A) Effective knockdown of *chn1* transcripts using a siRNA approach. Jurkat cells were electroporated with the indicated siRNAs and, 48 h later, *chn1* and *chn2* mRNA levels determined by quantitative RT-PCR. Values represent the variation in transcript levels of each *chn* transcript when compared to those observed in Jurkat cells transfected with a scrambled siRNA. (B) $\alpha 2$ -chimaerin protein levels in siRNA-transfected Jurkat cells. Jurkat cells were transfected as above and, 48 h later, treated (+) or untreated (-) with anti-CD3 antibodies for 8 h. After this period, cells were lysed and total cellular extracts analyzed by immunoblot with antibodies specific to $\alpha 2$ -chimaerin. WB, western blot. (C) Effect of the *chn1* knockdown on

the NF-AT route. Jurkat cells were electroporated with the luciferase reporter plasmids and the indicated siRNA molecules and subsequently stimulated with anti-CD3 antibodies. NF-AT activities were then determined in total cell extracts derived from the transfected cells using the firefly luciferase assay (see Materials and Methods). Values are expressed as fold change variations of NF-AT activity in the indicated experimental samples when compared to that displayed by non-stimulated cells that had been transfected with the scrambled siRNA ($n = 3$; *, $P \leq 0.05$).

FIGURE 4.

Structural determinants of the inhibitory activity of chimaerins on the NF-AT pathway. **(A)** Schematic representation of the β 2-chimaerin mutant proteins used in these experiments. A α 2-chimaerin mutant analogous to protein 5 is not shown. ITS, intervening sequence. **(B) Left panel**, inhibitory activity of β 2-chimaerin mutants on the NF-AT route. Non-stimulated and anti-CD3-stimulated Jurkat cells overexpressing the indicated combinations of chimaerin and Vav1 proteins were subjected to NF-AT luciferase determinations. Values are expressed as fold change variations of NF-AT activity in the indicated experimental samples when compared to that displayed by non-stimulated cells that had been transfected with a vector encoding the non-chimeric EGFP ($n = 3$; ***, $P \leq 0.001$; **, $P \leq 0.01$). **Right panel**, expression levels of the ectopically expressed wild type and β 2-chimaerin mutants (upper panel), EGFP (middle panel) and Vav1 (lower panel) obtained in one of these experiments using the indicated antibodies (right). The migration of the proteins is indicated by arrows on the left. Similar expression levels were obtained in the other experiments (data not shown). **(C) Left panel**, inhibitory activity of the isolated α 2-chimaerin GAP domain on the NF-AT route. Non-stimulated and anti-CD3-stimulated Jurkat cells overexpressing the indicated combinations of Vav1, wild type chimaerins or isolated chimaerin GAP domains were subjected to NF-AT luciferase determinations. Values are expressed as fold change variations of NF-AT activity in the

indicated experimental samples when compared to that displayed by non-stimulated cells that had been transfected with a vector encoding the non-chimeric EGFP ($n = 3$; **, $P \leq 0.01$). *Right panel*, expression levels of the ectopically expressed wild type chimaerin and GAP domains (first and second panels from top, respectively), EGFP (third panel) and Vav1 (lower panel) obtained in one of these experiments using the indicated antibodies (right). The migration of proteins is indicated by arrows on the left. Similar expression levels were obtained in the rest of experiments (data not shown).

FIGURE 5.

Subcellular localization of chimaerin family members in Jurkat cells. (**A,B**) Jurkat cells electroporated with vectors encoding the indicated EGFP-tagged proteins (left) were cultured for 24 h, attached to coverslips, stimulated with either anti-CD3 (**A**) or PMA (**B**) for 5 min, fixed, and analyzed by confocal microscopy. Microscopic images of untreated and stimulated cells are shown in the left and middle panels, respectively. The white lines shown in the middle panels highlight the areas of the cells used to measure the distribution of fluorescence intensities of the indicated EGFP molecules (right panels). *Right panels*, profiles of the distribution of the fluorescence intensities for EGFP- $\alpha 2$ -chimaerin (**A**) and EGFP-RasGRP1 (**B**) along the axis highlighted in white in the middle panels. NS, non-stimulated. (**C**) Subcellular distribution of endogenous $\alpha 2$ -chimaerin (left panels) and PKC α (right panels) in normal and PMA-treated Jurkat cells. Untransfected Jurkat cells were incubated with (+) or without (-) with PMA for 5 min, lysed in a hypotonic buffer, and soluble and particulate fractions obtained as described in Materials and Methods. Equal amounts of these fractions were then separated electrophoretically and subjected to immunoblot analysis with the indicated antibodies.

FIGURE 6.

Recruitment of chimaerins to the immune synapse upon TCR clustering. **(A)** Changes in the subcellular distribution of EGFP-tagged $\alpha 2$ - (upper panels) and $\beta 2$ -chimaerin (lower panels) fusion proteins followed in real time during the formation of T-cell/B-cell synapses. Jurkat cells expressing the indicated proteins were subjected to conjugate formation with red fluorescent protein-expressing Raji B-cells and analyzed by time-lapse fluorescence microscopy, as described in Materials and Methods. The 0 time point was set just before the first contact between the T-cell and the antigen presenting B cell. Representative images taken from the indicated times after synapse formation (top) are shown. Asterisks indicate the B-cells involved in the immune synapse with the T-cell. **(B)** Co-localization of chimaerin proteins

with TCR components in the immune synapse. Jurkat cells transfected with the indicated EGFP molecules (left) were incubated with CMAC-labeled Raji cells previously loaded with superantigen. Conjugates formed were plated onto coverslips, fixed, stained with anti-CD3 ζ antibodies and Alexa Fluor 635-labeled phalloidin, and subjected to confocal fluorescence microscopy. Images show in green the localization of the EGFP molecules, in red the distribution of the CD3 ζ subunit and in purple the localization of polymerized actin. A merge of the above images is shown on the right column, where the areas of overlap between EGFP molecules and CD3 ζ are seen as yellow areas. The white arrows shown in the panels of the left and right columns indicate the cell areas used to measure the distribution of fluorescence intensities of the indicated EGFP molecules and/or CD3 ζ shown in **Fig. 6C**. **(C)** Distribution of intensities corresponding to EGFP (green) and CD3 ζ (red) in non-conjugated (left panels) and conjugated (right panels) Jurkat cells along the areas indicated in **Fig. 6B**. The bar shown in the right panels indicates the area of the immune synapse, as assessed by the distribution of the CD3 ζ marker. **(D)** Quantification of the percentage of localization of the indicated EGFP molecules in immune synapses, as indicated in Materials and Methods ($n = 3$, each including at least 125 independent T-cell/B-cell conjugates expressing each of the indicated chimaerin constructs). IS, immune synapse.

FIGURE 7.

Translocation of chimaerins to the immune synapse requires an intact N-terminal domain. (A) Jurkat cells transfected with either EGFP-wild type β 2-chimaerin or the indicated EGFP-tagged β 2- and α 2-chimaerin mutants (left). 16 h later, cells were incubated with CMAC-labeled, superantigen-loaded Raji cells to allow the formation of T-cell/B-cell conjugates. Conjugates were then plated onto coverslips, fixed, stained with anti-CD3 ζ antibodies and Alexa Fluor 635-labeled phalloidin, and analyzed by confocal fluorescence microscopy. Images show in green the localization of EGFP-tagged molecules, in red the distribution of the CD3 ζ subunit and in purple the localization of polymerized actin. A merge of the above images is shown on the right panels, where the areas of overlap between EGFP molecules and CD3 ζ

are seen in yellow color. The white arrows shown in the panels of the right column indicate the cell areas used to measure the distribution of fluorescence intensities of the indicated EGFP molecules and/or CD3 ζ shown in **Fig. 7B**. **(B)** Distribution of the fluorescence intensities corresponding to EGFP (green) and CD3 ζ (red) in conjugated Jurkat cells along the areas indicated in **Fig. 7A**. The bar indicates the area of the immune synapse, as assessed by the distribution of the CD3 ζ marker. **(C)** Quantification of the percentage of localization of the indicated EGFP molecules in immune synapses, as indicated in Materials and Methods ($n = 3$, each including at least 125 independent T-cell/B-cell conjugates expressing each of the indicated chimaerin constructs).