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In vivo regulation of human CrkII by cyclophilin A and FK506-binding protein☆

Pulak Ranjan Nath, Guangyu Dong [1], Alex Braiman, Noah Isakov*

The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences and the Cancer Research Center, Ben Gurion University of the Negev, P.O.B. 653, Beer Sheva 84105, Israel

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ABSTRACT

Members of the Crk family of adaptor proteins are key players in signal transduction from a variety of cell surface receptors. CrkI and CrkII are two alternative-spliced forms of a single gene which possess an N-terminal SH2 domain and an SH3 domain that mediate interaction with other proteins. CrkII possesses an additional C-terminal linker region plus an extra SH3 domain, which does not interact with other proteins, but operates as regulatory moiety. Utilizing human Jurkat T cells, we demonstrate that CrkII-SH3N binding of C3G is inhibited by cyclosporin A (CsA) plus FK506 that inhibit the cyclophilin A (CypA) and FK506 binding protein (FKBP) peptidyl-prolyl *cis*-trans isomerases (PPIases; also termed immunophilins), respectively. Jurkat T cells were found to express ~5-fold lower levels of CrkI protein compared to CrkII, but the efficiency of C3G binding by CrkI was ~5-fold higher than that of CrkII, suggesting that the majority of cellular CrkII proteins adopt a conformation that is inaccessible for C3G. Treatment of Jurkat T cells with CsA plus FK506 led to a time-dependent conformational change in overexpressed human $CrkII_{1-236}$ protein-containing FRET-based biosensor, supporting the accumulation of *cis* conformers of human $CrkII_{1-236}$ in the presence of PPIase inhibitors. Our data suggest that the Gly^{219}-Pro-Tyr motif in the human CrkII linker region serves as the recognition and isomerization site of PPIases, and raise the possibility that CsA and FK506 might interfere with selected effector T cell functions via a CrkII-, but not CrkI-dependent mechanisms.

1. Introduction

Adaptor proteins are composed of protein–protein interaction domains and contribute to the specificity of signal transduction events by orchestrating the assembly of multimolecular complexes and linking activated cell-surface receptors to their downstream signaling cascades [1]. The Crk family members are the prototype of adaptor proteins which play critical roles in signal transduction from numerous cell surface receptors [2,3]. In T lymphocytes, Crk proteins were found to associate with the ZAP70 protein tyrosine kinase (PTK) in a cell activation dependent manner [4,5]. In addition, Crk binding to ZAP70 was found to initiate a cascade of events that involve several different effector proteins, lead eventually to actin polymerization and rearrangement of the cytoskeleton [6].

All Crk adaptor proteins possess a single N-terminal Src homology 2 (SH2) domain, which binds phosphotyrosine (pTyr)-containing sequences, followed by one (CrkI) or two (CrkII and CrkL) SH3 domains, which bind left-handed poly-Pro type II helix-containing motifs [7,8]. The two SH3 domains are connected via a linker region containing a single, highly conserved, protein conformation-regulating tyrosine residue, plus several proline residues [9,10]. Phosphorylation of the tyrosine residue (Tyr^{211} and Tyr^{207} in human CrkII and CrkL, respectively) by Abl, or other protein tyrosine kinases (PTKs), promotes intramolecular binding of the linker region to the self-SH2, thereby sequestering the SH2 and SH3N domains [10,11].

Recent *in vitro* studies demonstrated that the conformation of a Gly–Pro motif within the CrkII linker region is under the control of peptidyl-prolyl *cis-trans* isomerases (PPIases) [12–15]. A truncated recombinant protein consisting of the SH3N-linker-SH3C of the

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* Corresponding author.
E-mail address: noah@bgu.ac.il (N. Isakov).
[1] Present address: Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA.

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chicken CrkII (CrkII^{SLS}) was found to undergo *cis-trans* isomerization at Pro^{238} at the linker region Gly^{237}-Pro-Phe motif. Further analysis revealed that CrkII^{SLS} exists in solution in two different geometric conformations, in which the majority of proteins adopts a *cis* conformation at the Pro^{238} and are folded in a closed and inactive conformation (~90%), while a minor fraction (~10%) adopts the *trans* conformation at the Pro^{238} where CrkII^{SLS} adopts an open and active conformation that is available for interaction with binding partners.

More recently, we found that the ability of CrkII to associate with the guanine nucleotide exchange factor for Rap1, C3G, is augmented by the cyclophilin A (CypA) peptidyl-prolyl *cis-trans* isomerase (PPIase), and decreased by the CypA inhibitor, cyclosporin A (CsA) [16]. Furthermore, the ability of the endogenous CrkII, but not CrkI, to interact with C3G in Jurkat T cells was downregulated by a mixture of PPIase inhibitors, CsA and FK506, concomitant with the decreased ability of the T cells to mediate C3G-dependent adhesion and migration [16].

The present work demonstrates that in spite of the expression of ~5-fold higher levels of CrkII protein, compared to CrkI, the basal C3G binding activity of CrkII in Jurkat T cells is ~5-fold lower than that of CrkI. Furthermore, only the CrkII C3G-binding activity decreases in the presence of PPIase inhibitors, while that of CrkI is unaffected. The data further demonstrate the existence of an evolutionary conserved Gly^{219}-Pro-Tyr motif within the human CrkII linker regions, which serves as the *cis-trans* isomerization site for PPIases.

2. Materials and methods

2.1. Reagents and antibodies

Sandimmune® (cyclosporine, CsA; 50 mg/ml) was from Novartis Pharma AG (Basel, Switzerland) and FK506 (Prograf® or tacrolimus, 5 mg/ml) was from Astellas Pharma Ltd. (UK). Recombinant human CypA, β-mercaptoethanol and Triton X-100 were from Sigma Co. AEBSF, aprotinin and leupeptin were from ICN Biomedicals, Inc. (Aurora, OH). ECL, glutathione-coupled sepharose beads and protein A-sepharose were from Amersham Pharmacia Biotech (Uppsala, Sweden). Mouse anti-Crk (I/II) monoclonal Ab (mAb) was from BD (Transduction Laboratories, Lexington, KY), and mouse anti-phosphotyrosine (pY) mAb (4G10) was from Upstate Biotechnology Inc., (Lake Placid, NY). Mouse anti-human CD3ε mAb (OKT3) was prepared by intraperitoneal injection of the OKT3 hybridoma into athymic nude mice and collection of the ascitic fluid. Rabbit polyclonal anti-C3G Ab was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-β-actin mAb (AC-15) was from Sigma. Horseradish peroxidase (HRP)-conjugated goat Abs directed against mouse or rabbit IgG were from Amersham Pharmacia Biotech Inc.

2.2. Cell lines and culture conditions

Human leukemia Jurkat T cell clone 6.1 and the Jurkat TAg T cell line, which stably expresses the simian virus 40-derived large T antigen, were maintained at a logarithmic growth phase in complete RPMI (RPMI 1640 supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (all from Biological Industries, Beit Haemek, Israel), and 0.5 μM β-mercaptoethanol (Sigma)). Cells were grown in 75 cm^2 growth-area tissue culture flasks (Cellstar, Greiner, Germany) in an atmosphere of 5% CO_2, at 37 °C. TCR stimulation was performed using anti-CD3ε mAbs (OKT3; 1:1000 dilution of ascites, 30 min incubation on ice) plus crosslinking with a secondary Ab (goat anti-mouse IgG, 1:200) for 1 min at 37 °C.

2.3. GST fusion proteins and pull-down assay

pGEX-5X vectors were from Amersham Pharmacia Biotech, and pGEX plasmids encoding different GST-Crk fusion proteins were gifts of Dr. Michiyuki Matsuda (National Institute of Health, Tokyo, Japan). The pGST-C3G was a gift of Dr. Carmen Guerrero (Universidad de Salamanca-CSIC, Salamanca, Spain) [17]. Bacterial expression vectors were transformed into Escherichia *Coli* DH5α™ competent cells and GST-fusion proteins were prepared as described [5]. Pull-down assays were performed by incubation of bead-adsorbed GST or GST fusion proteins (2–10 μg, as indicated) with cell lysates at 4 °C on a rotator for 4 h. The beads were washed (x3) in lysis buffer, resuspended in 2x Laemmli SDS sample buffer and boiled for 5 min. The eluents were subjected to SDS-PAGE under reducing conditions followed by immunoblotting.

2.4. Expression vectors and transient transfection of Jurkat T cells

The phosphorylation indicator of Crk chimeric unit plasmid, Picchu 936x (a gift of Dr. Michiyuki Matsuda, National Institute of Health, Tokyo, Japan) encodes for human CrkII (amino acids 1–236) sandwiched between cyan (CFP)- and yellow (YFP)-emitting variants of green fluorescent protein, in addition to the Ki-Ras-derived CAAX box, which anchors the protein predominantly to the plasma membrane [18]. For DNA transfer into cells, Jurkat TAg cells were maintained at a logarithmic growth phase, washed in serum-free RPMI 1640, resuspended at 7x10^6 cells/ml in transfection buffer (1.8 g/ml sucrose, 2 nM DTT in RPMI-1640) and transferred into 0.4 cm-gap Gene Pulser cuvettes (BioRad) (5x10^6 cells/700 μl/cuvette). Plasmid DNA (10 μg/group, unless otherwise indicated) was added to each cuvette and electroporation was performed using a BioRad Gene Pulser (250 V, 950 μF). The cells were then cultured in 50 ml of complete RPMI 1640 in 145 mm^2 tissue culture plates for 36–48 h.

2.5. Preparation of cell lysates and immunoprecipitation

Cell lysates were prepared by resuspension of cells in lysis buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml each of leupeptin and aprotinin, 2 mM AEBSF, and 1% Triton X-100), followed by a 20-min incubation on ice. Lysates were centrifuged at 13,000 × *g* for 30 min at 4 °C and the nuclear free supernatants were used for immunoprecipitation.

For immunoprecipitation, primary Abs were pre-adsorbed to protein A-sepharose beads for 1 h at 4 °C. Excess Abs were removed by 3 washes in cold PBS, and Ab-coated beads were incubated with cell lysates for 3 h at 4 °C. Immune complexes were precipitated by centrifugation followed by extensive washing in a lysis buffer. Equal volumes of 2xSDS sample buffer were added to immunoprecipitates or whole cell lysates (WCL), which were vortexed, boiled for 5 min, and fractionated by SDS-PAGE.

2.6. Electrophoresis and immunoblotting

Samples of whole cell lysates, immunoprecipitates, or pulled down proteins were resolved by electrophoresis on 10% polyacrylamide gels using Bio-Rad Mini-PROTEAN II Cells. Proteins from the gel were electorblotted onto a nitrocellulose membrane (Schleicher & Schuell) at 100 V for 1 h in a BioRad Mini Trans-Blot transfer cells. After 1 h blocking with 3% BSA in PBS at 37 °C, the nitrocellulose membranes were incubated with the indicated primary Abs followed by incubation with HRP-conjugated secondary Abs or protein A. Immunoreactive protein bands were visualized using an ECL reagent and autoradiography. Whenever required, nitrocellulose membranes were stripped by incubation in stripping

buffer (100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris/HCl, pH 6.8) for 30 min at 50 °C, followed by 1 h incubation with blocking buffer (3% BSA in PBS).

2.7. Cell treatment with cyclosporine A and FK506

Cyclosporine A (CsA) (Sandimmun®, 50 mg/ml in oil solution) and FK506 (Prograf®; Astellas Pharma US. Inc., 5 mg/ml in ethanol) were diluted in RPMI 1640 culture medium before each experiment. Jurkat Tag cells were cultured in 75 cm^2 growth-area tissue culture flasks at a concentration of $50x10^6$ cells/group in the presence or absence of CsA (5 μg/ml) and/or FK506 (5 ng/ml), and tested on the following day.

2.8. Fluorescence resonance energy transfer (FRET) analysis by FACS

FRET analysis of living cells by flow cytometry was performed according to He et al. [19]. Briefly, cells were transfected with the PICCHUx plasmid constructed to include the human CrkII protein (amino acids 1–236) and yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) on both ends of CrkII, plus an N-terminal CAAX motif [18]. Cells were cultured for 48 h, and then split into two identical groups which were cultured for an additional 24 h in the presence or absence of CsA (5 mM) plus FK506 (5 nM). Cells were then resuspended in FACS-buffer (PBS plus 0.5% BSA and 0.1% NaN_3) for analysis on a FACSCanto II device (BD Biosciences). Excitation of CFP occurred at 405 nm, whereupon emission was detected in the CFP emission window and simultaneously in the YFP emission window. If FRET occurs, CFP emission decreases, while simultaneously YFP-emission increases. This can be visualized by a shift in ratio of YFP/CFP emission intensities. The data was analyzed using Flowjo7.6.3 software.

2.9. Statistical analysis

Statistical analyses were performed by one-way ANOVA using SIGMA STAT 3.11 (Systat Software Inc. 2004, San Jose, CA, USA) or t-test. Values are presented as average ± standard deviation (SD). Data were considered significantly different at $P < 0.05$.

3. Results and discussion

3.1. The combined effect of cyclosporin A and FK506 inhibits C3G binding to CrkII in Jurkat T cells

Previous studies demonstrated that CrkII can interact with both cyclophilin A and FKBP PPIases, and that PPIase inhibitors, such as

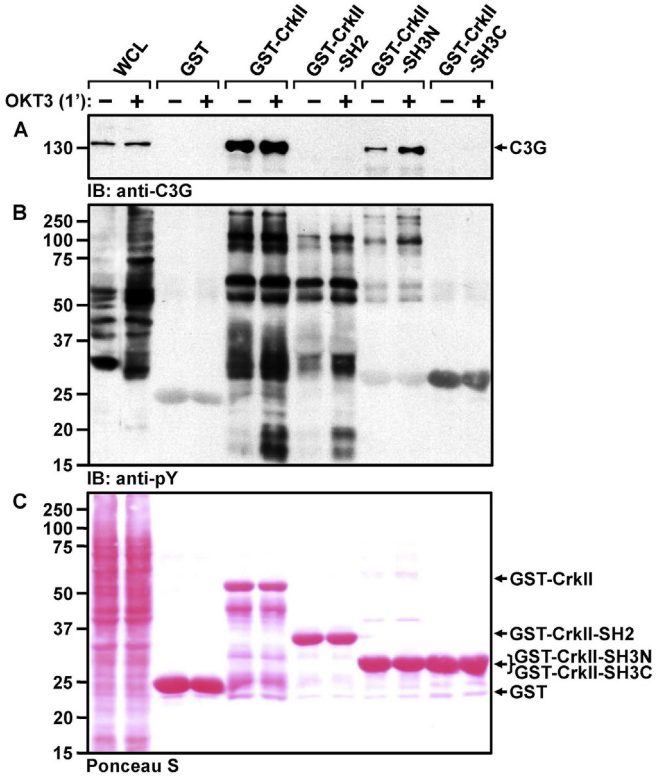

Fig. 1. Crk binding to C3G is mediated by the Crk-SH3N domain. Resting and anti-CD3ε Ab (OKT3)-stimulated (1 min) Jurkat T cells (10^7/group) were lysed in ice-cold lysis buffer on ice, and cell lysates were then incubated with bead-immobilized GST fusion proteins (10 μg/group) on a rotator at 4 °C for 3 h. After centrifugation and thorough washings, the beads were resuspended in SDS sample buffer and bound proteins were eluted from the beads by 5 min boiling in SDS sample buffer. Samples were subjected to SDS-PAGE under reducing conditions, followed by electrotransfer to a nitrocellulose membrane. The membrane was sequentially immunoreacted with mouse anti-C3G (A) and anti-phospho-tyrosine (pY) (B) mAbs. Reversible Ponceau S staining was performed in order to validate equal loading and transfer of protein samples during the Western blot experiment (panel C). Samples of whole cell lysates (WCL, equivalent to $2.5x10^5$ cells/group) were boiled and electrophoresed in parallel on the same gel. Molecular weight markers (in kDa) are indicated on the left and arrows mark the position of the indicated protein bands. Results are representative of five independent experiments.

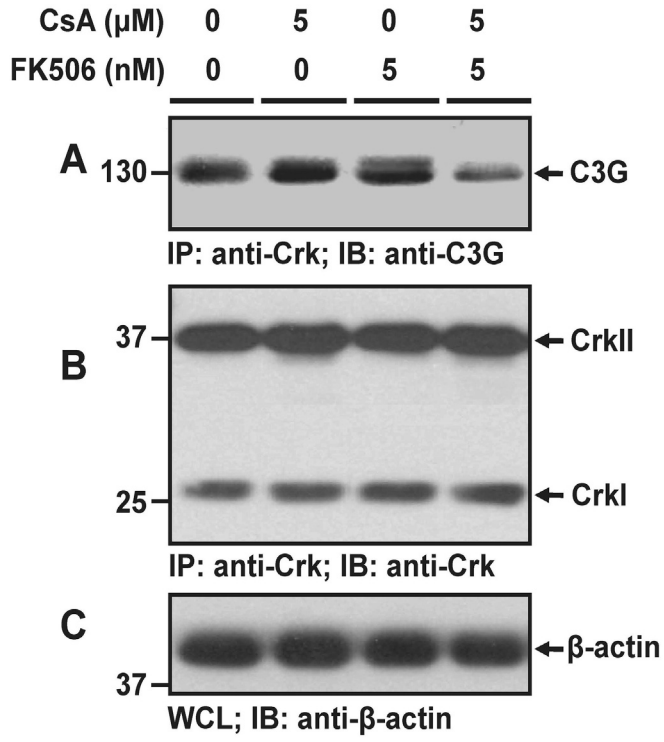

Fig. 2. The combined effects of CsA and FK506 results in inhibition of C3G binding to CrkII. Jurkat T cells (10^7/group) were cultured without or with CsA (5 μg/ml), FK506 (5 ng/ml), or a mixture of the two drugs for 24 h. The cells were then incubated for 30 min in lysis buffer on ice, and the soluble fraction was further incubated with protein A-sepharose beads pre-coated with anti-Crk mAbs on a rotator at 4 °C. After 3 h, the beads were extensively washed in cold lysis buffer, bound proteins were eluted by boiling for 5 min in SDS sample buffer and then subjected to SDS-PAGE under reducing conditions. Proteins were then electroblotted onto a nitrocellulose membrane that was immunoreacted with mouse anti-C3G (A), mouse anti-Crk (B) and mouse anti-β-actin (C) mAbs. Molecular weight markers (in kDa) are indicated on the left and arrows mark the position of the indicated protein bands. Results are representative of three independent experiments. Whole cell lysates (WCL, equivalent to 5×10^5 cells/group) were boiled and electrophoresed in parallel on the same gel and immunoblotted for β-actin, as a control for usage of equal amounts of proteins in the immunoprecipitation.

CsA and FK506, can downregulate C3G binding to CrkII [16]. Thus, in contrast to the process of calcineurin-mediated regulation of NF-AT, which is inhibited by complexes of either CypA-CsA or FKBP-FK506 [20], inhibition of C3G binding to CrkII, which is regulated by both CypA and FKBP, requires the concomitant inhibition of both types of PPIases. To test this assumption, we first verified whether C3G interacts with the CrkII-SH3N domain, which is highly sensitive to a regulation by PPIases [12,13]. Utilizing a battery of GST fusion proteins, we found that the CrkII-SH3N, but not the CrkII-SH2 or -SH3C domain can pull down C3G from a lysate of Jurkat T cells (Fig. 1). While CrkII-SH2 does not seem to be involved in binding of C3G, TCR/CD3 stimulation of the cells slightly increased CrkII binding to C3G, suggesting that T cell activation-dependent posttranslational modification of CrkII and/or C3G alters the protein(s) conformation in a manner that increases their attraction forces.

We then treated Jurkat T cells with CsA (5 μM), FK506 (5 nM), or a mixture of both drugs for 24 h, lysed the cells, and tested the ability of CrkII to coimmunoprecipitate C3G. We found (Fig. 2) that FK506 had no effect on CrkII binding to C3G and CsA had only a minor inhibitory effect. In contrast, a mixture of both drugs induced a significant decrease in the ability of CrkII to coimmunoprecipitate C3G. The fact that CsA and FK506 did not induce complete inhibition of CrkII binding to C3G may suggest the usage of sub-optimal concentration of drugs or the involvement of additional CrkII conformation-regulating mechanisms that are insensitive to CsA and FK506.

3.2. CsA plus FK506 inhibit C3G binding to CrkII by modulating the CrkII, but not C3G, binding properties

The indirect inhibitory effect of CsA plus FK506 on C3G binding to CrkII might reflect changes in PPIase-mediated alterations in the conformation of CrkII and/or C3G. To distinguish between these two possibilities, we used bead-immobilized GST-C3G and GST-CrkII fusion proteins to pull down CrkII and C3G, respectively, from a lysate of non-treated and CsA plus FK506-treated Jurkat T cells. Since the cells were incubated with the drugs for 24 h, the conformation of the endogenous proteins could potentially be affected by the drugs, but not the conformation of the GST fusion proteins that originated from bacteria. We found that CsA plus FK506 inhibited CrkII binding to GST-C3G, but not C3G binding to GST-CrkII. The results suggest that the inhibitory effect of CsA plus FK506 on C3G binding to CrkII is due to the drug effect on PPIase-mediated conformational regulation of CrkII, but not C3G (Fig. 3).

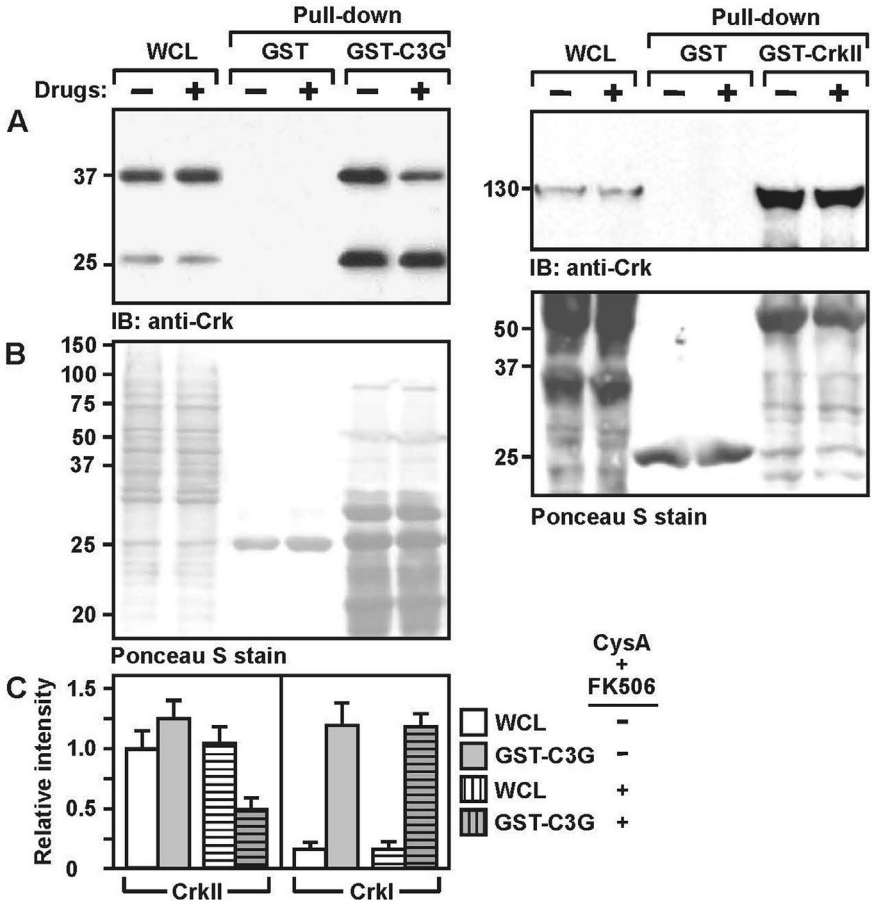

Fig. 3. CrkII exhibits weaker C3G binding activity, as compared to CrkI, which is further decreased by immunophilin inhibitors. Jurkat T cells (10^7/group) were cultured with or without CsA (5 μg/ml) plus FK506 (5 ng/ml) for 24 h. After 30 min of incubation in lysis buffer on ice, equal volumes of cell lysates were incubated with bead-immobilized GST or GST-C3G fusion proteins (10 μg/group). After 4 h of incubation on a rotator at 4 °C, the beads were extensively washed with cold lysis buffer and bound proteins were eluted by boiling for 5 min in SDS sample buffer and subjected to SDS-PAGE under reducing conditions. Proteins were then electroblotted onto a nitrocellulose membrane that was immunoreacted with mouse anti-Crk mAbs (A). Whole cell lysates (WCL, equivalent to 5×10^5 cells/group) were boiled and electrophoresed in parallel on the same gel. Reversible Ponceau S staining was performed in order to validate equal loading and transfer of protein samples during the Western blot experiment (B). Molecular weight markers (in kDa) are indicated on the left and arrows mark the position of the indicated protein bands. Results are representative of three independent experiments. The relative intensity of the protein band signals was determined by ImageJ (v1.45) and the average values obtained in three independent experiments are presented as a bar graph (C).

3.3. Jurkat T cell CrkI is expressed at a lower level, compared to CrkII, but exhibits a higher C3G binding affinity which is insensitive to PPIase inhibitors

Previous work [21,22], including our own studies (Fig. 2) demonstrated that the Crk-SH3N domain mediates C3G binding. CrkI and CrkII possess an identical SH3N domain, but the ligand binding properties of SH3N appeared to be dependent on the protein's conformation in CrkII, but not in CrkI. Thus, all cellular CrkI, as well as the *trans* conformer of CrkII, adopt an extended 'open' conformation and are available for interaction with their ligands, while the *cis* conformer of CrkII acquires a 'closed' conformation that is not accessible for interaction with C3G. Immunoblot analysis of Jurkat T cell lysates revealed that although the relative expression levels of CrkII is about ~5-fold higher than that of CrkI, GST-C3G pulled down similar quantities of both proteins, suggesting that CrkI binding affinity to C3G is ~5-fold higher than that of the endogenous CrkII (Fig. 3). Furthermore, Jurkat T cell treatment with CsA plus FK506 further decreased the amount of CrkII that interacted with C3G, but had no effect on CrkI binding to C3G (Fig. 3).

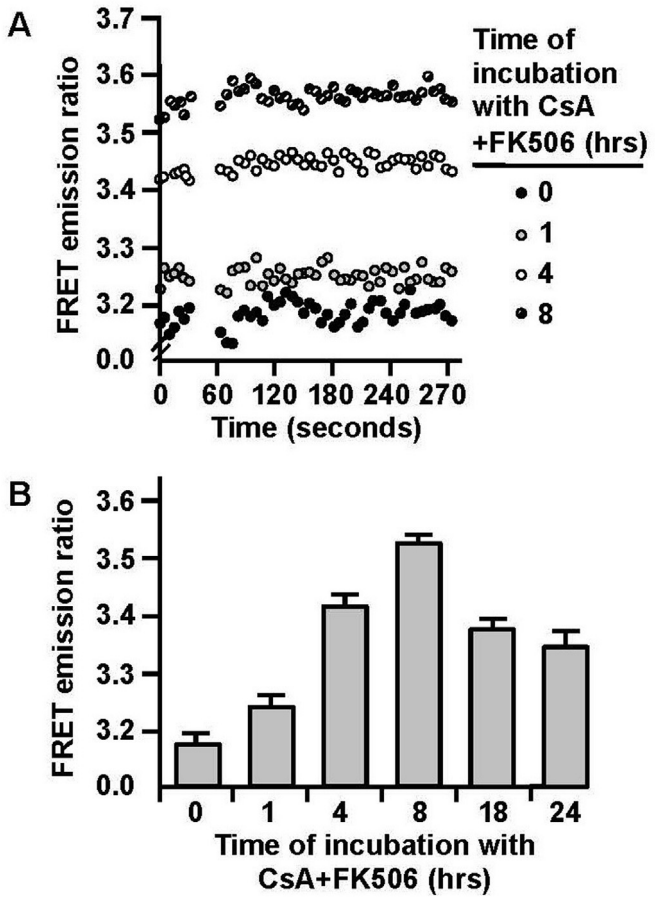

Fig. 4. Time kinetic effects of CsA plus FK506 on the Jurkat T cell-expressed $CrkII_{1-236}$ FRET-based biosensor. Jurkat T cells (2×10^7/group) were transiently transfected with the Picchu 936x plasmid encoding the human $CrkII_{1-236}$ sandwiched between CFP and YFP (10 μg/2×10^7 cells). After 48 h in culture, the cells were washed and resuspended in complete RPMI. Cell suspension was split into six identical aliquots and recultured for additional 24 h in the absence (untreated control) or presence of CsA (5 mM) plus FK506 (5 nM) added during the last 1, 4, 8, 18, or 24 h of culture. FACS-based FRET analysis of the Picchu 936x-expressing Jurkat T cells was performed using a BD FACSCanto™ II Flow Cytometer. Live cells were gated according to forward and sideward scatter (FSC/SSC), adjusted photomultiplier tube (PMT) voltages and compensated for CFP and YFP to specifically assess FRET in double positive cells. Emission values were analyzed by FlowJo Ver. 7.6.3 software. Measurements were taken at 5-sec intervals and each point in the graph indicates the average emission ratio of ~10,000 cells (A). The average FRET values observed in the control and drug-treated cells are presented as a bar graph (B). Results are representative of four independent experiments.

3.4. Jurkat T cell treatment with CsA plus FK506 leads to a time-dependent conformational change in an overexpressed FRET-based biosensor containing the human $CrkII_{1-236}$ protein

We have previously shown that the $CrkII_{1-236}$-containg FRET-based biosensor is sensitive to the effect of CsA plus FK506 [16]. To substantiate these data and further analyze the time-dependent effect of CsA plus FK506 on the conformation of $CrkII_{1-236}$, we used Jurkat T cells transfected with the Picchu 936X-encoded FRET-based biosensor and a BD FACSCanto™ II Flow Cytometer to measure CsA plus FK506-induced time dependent changes in fluorescence emission. The results demonstrate that 1 h treatment of Jurkat T cells with CsA plus FK506 already resulted in increased FRET (Fig. 4). The maximal increase in FRET occurred at 8 h post-drug addition, and levels of FRET remained higher than those in untreated cells for at least 24 h.

3.5. The PPIase substrate-recognition site in the human CrkII is localized at the Gly^{219}-Pro-Tyr motif within the linker region

In vitro studies using recombinant proteins indicated that the chicken $CrkII^{SLS}$ is prone to PPIases-mediated *cis-trans* isomerization at the Gly^{237}-Pro^{238} peptide bond [12,13]. The Pro^{238}-containing motif in the chicken CrkII (Gly^{237}-Pro-Phe) is only partially conserved in the human CrkII (Gly^{236}-Pro-Ile) and the nature of the residue that immediately follows the Pro (Phe *vs.* Ile, in chicken and

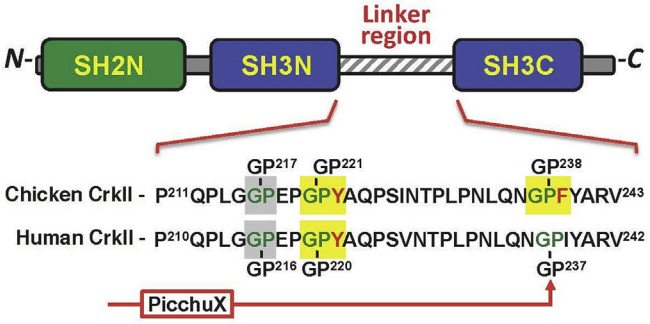

Fig. 5. The $G^{219}PY$ sequence in the CrkII-linker region is a substrate recognition motif for PPIases. A schematic representation of the domain architecture of the chicken and human CrkII, which includes an N-terminal Src homology 2 (SH2) domain and two SH3 domains (SH3N and SH3C) interspersed by a 40-50-residue PPIase-sensitive linker region. Amino acid sequence of the chicken and human CrkII linker region is shown, emphasizing Gly–Pro sequences that represent potential recognition sites for cyclophilin A (CypA) [24]. *In vitro* studies demonstrated that CypA induces *cis-trans* isomerization of a truncated chicken CrkII carboxy-terminus ($CrkII^{SLS}$) at the Gly^{237}-Pro-Phe motif [12]. The homologous sequence in the human CrkII, Gly^{236}-Pro-Ile, was found to be insensitive to PPIase-mediated isomerization [11,28]. The differential sensitivity of the chicken *vs.* human $CrkII^{SLS}$ to PPIases was attributed to the nature of the amino acid that immediately follows the Pro residue; Phe and Ile, in the chicken and human CrkII, respectively. However, *in vivo* studies demonstrated that a full-length human CrkII, as well as a truncated protein (residues 1–236) are subjected to a regulation by PPIases [16], while human CrkI, a natural splice variant of CrkII that includes residues 1–204, is insensitive to PPIases [16]. The CrkII Gly^{219}-Pro-Tyr motif is an evolutionary conserved sequence found in all known vertebrate CrkII proteins. Furthermore, Phe^{239} (in the chicken CrkII Gly^{237}-Pro-Phe motif) and Tyr^{221} (in the human CrkII Gly^{219}-Pro-Tyr motif) are structurally related aromatic residues can be interchangeable in many proteins without affecting their biological activities. It is suggested therefore that the Gly^{219}-Pro-Tyr motif is a favorable PPIase binding and isomerization site that is shared by CrkII proteins in a majority of vertebrate species.

human CrkII, respectively) may possibly account for the difference in the folding kinetics of the two peptides [23]. Furthermore, substitution of Phe^{239}-to-Ile stabilized the chicken protein and decreased its C3G binding ability, while substitution of Ile^{239}-to-Phe in the human protein had an opposite effect; it destabilized the protein and increased its C3G binding ability [23]. Based on these studies, it has been concluded that *cis-trans* isomerization of Pro^{238} determines the extent of ligand binding by the chicken, but not human CrkII [23].

The present studies support the model in which conformational changes in human CrkII are also subjected to a regulation by PPIases. However, usage of a human $CrkII_{1-236}$ protein in the FRET assay indicates that its isomerization involves a Pro residue that is distinct from Pro^{237} (which corresponds to Pro^{238} in the chicken CrkII) (see Fig. 5). Furthermore, previous studies [16] and the present findings (Fig. 3) suggest that CrkI, which is identical to the N-terminal 204 residues of CrkII [8], is insensitive to the effect of PPIases or their inhibitors. It appears therefore that the site of isomerization of the human CrkII is located between amino acids 205 and 236, which includes two Gly–Pro motifs, representing putative favorable recognition sites for CypA [24,25]. However, the second motif in the human CrkII is highly similar to the chicken CrkII motif, since replacement of Phe-by-Tyr represents a conservative substitution between two similar aromatic residues that were shown to be interchangeable in a variety of proteins without affecting their biological functions [26,27]. In contrast, the third residue in the first motif, Glu, is a non-aromatic negatively charged acidic residue representing a non-conserved replacement of Phe. Preliminary data indicated that substitution of Tyr^{221} by Ala abolished the ability of $CrkII_{1-236}$ to undergo CsA plus FK506-induced conformational changes in resting Jurkat T cells, further supporting our assumption that the Gly^{219}-Pro-Tyr motif in the human CrkII linker region serves as the recognition site for CypA and the site of *cis-trans* protein isomerization. It should be emphasized that the Gly^{215}-Pro-Phe motif is found only in the chicken CrkII protein. In contrast, the Gly^{219}-Pro-Tyr motif observed in the human CrkII appears to be an evolutionary conserved sequence found in CrkII proteins from all vertebrate species tested. The results suggest therefore that CrkII regulation by *cis-trans* isomerization is not restricted to chickens, but is a general regulatory mechanism of CrkII in the majority of vertebrate species.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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