



Cyclophilin A associates with and regulates the activity of ZAP70 in TCR/CD3-stimulated T cells

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Abstract

The ZAP70 protein tyrosine kinase (PTK) couples stimulated T cell antigen receptors (TCRs) to their downstream signal transduction pathways and is *sine qua non* for T cell activation and differentiation. TCR engagement leads to activation-induced post-translational modifications of ZAP70, predominantly by kinases, which modulate its conformation, leading to activation of its catalytic domain. Here, we demonstrate that ZAP70 in TCR/CD3-activated mouse spleen and thymus cells, as well as human Jurkat T cells, is regulated by the peptidyl-prolyl *cis-trans* isomerase (PPIase), cyclophilin A (CypA) and that this regulation is abrogated by cyclosporin A (CsA), a CypA inhibitor. We found that TCR crosslinking promoted a rapid and transient, Lck-dependent association of CypA with the interdomain B region, at the ZAP70 regulatory domain. CsA inhibited CypA binding to ZAP70 and prevented the colocalization of CypA and ZAP70 at the cell membrane. In addition, imaging analyses of antigen-specific T cells stimulated by MHC-restricted antigen-fed antigen-presenting cells revealed the recruitment of ZAP70-bound CypA to the immunological synapse. Enzymatically active CypA downregulated the catalytic activity of ZAP70 in vitro, an effect that was reversed by CsA in TCR/CD3-activated normal T cells but not in CypA-deficient T cells, and further confirmed in vivo by FRET-based studies. We suggest that CypA plays a role in determining the activity of ZAP70 in TCR-engaged T cells and impact on T cell activation by intervening with the activity of multiple downstream effector molecules.

Keywords Cyclophilin A · ZAP70 · Isomerase · T cell activation · Immunophilin · Cyclosporin A

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Abbreviations

C3G	CrkII binding of guanine-nucleotide releasing factor
c-Cbl	Casitas B-lineage lymphoma
cfb3	Cytoplasmic fragment of human erythrocyte band 3
CrkII	CT10 regulator of kinase II
CypA	Cyclophilin A
CsA	Cyclosporin A
FRET	Fluorescence resonance energy transfer
HRP	Horseradish peroxidase
IP ₃	Inositol trisphosphate
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motifs
Itk	Interleukin-2-inducible T cell kinase
JNK	Jun N-terminal kinase
LAT	Linker of activated T cells
Lck	Lymphocyte-specific protein tyrosine kinase
MAPK	Mitogen-activated protein kinase

NFAT	Nuclear factor of activated T cells
Pin1	Peptidyl-prolyl <i>cis</i> – <i>trans</i> isomerase NIMA-interacting 1
PKC α	Protein kinase C α
PLC	Phospholipase C
PPIase	Peptidyl-prolyl <i>cis</i> – <i>trans</i> isomerases
SCID	Severe combined immunodeficiency
Sts1	Suppressor of TCR signaling 1
TCR	T cell antigen receptor
WCL	Whole-cell lysates
ZAP70	ζ -Chain-associated protein of 70 kDa

Introduction

The Syk family protein tyrosine kinase, ζ -chain-associated protein of 70 kDa (ZAP70), plays a critical role in the induction of T cell activation following T cell antigen receptor (TCR) engagement [1]. The crucial role of ZAP70 in the initiation of TCR signaling was observed in ZAP70-deficient humans that suffered from severe combined immunodeficiency (SCID) due to a lack of CD8⁺ T cells and defective activation of their CD4⁺ T cells [2–4]. In addition, knock-out of the mouse ZAP70 resulted in an early developmental arrest of thymic T cells at the CD4⁺/CD8⁺ double-positive stage [5, 6]. In resting T cells, ZAP70 resides within the cytoplasm as a non-phosphorylated and autoinhibited protein. Its activation occurs following TCR stimulation-induced recruitment of ZAP70 to the immunological synapse (IS) where it undergoes a series of post-translational modifications.

Activation of T cells is initiated by TCR engagement with agonistic peptide-bound MHC complex molecules on the surface of antigen-presenting cells (APC). The simultaneous interaction of the T cell coreceptor molecules, CD4 or CD8, with the MHC class II or class I receptors, respectively, positions the CD4/CD8-associated, lymphocyte-specific kinase (Lck) in close proximity with the immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of the CD3 chains [7]. Lck-mediated phosphorylation of the ITAMs' tyrosine residues promotes the interaction of the ZAP70 tandem SH2 domains with doubly phosphorylated ITAMs, leading to a conformational change in ZAP70 that makes it more accessible to phosphorylation by Lck [8]. The phosphorylation of ZAP70 by Lck [9–11] and its autophosphorylation [12–14] on tyrosine residues located at critical positions for secondary structure determination and interaction with binding partners introduces further modifications in its overall conformation in order to unleash its catalytic activity. Activated ZAP70 then phosphorylates TCR downstream effector molecules, including the linker for the activation of T cells (LAT) [15], the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) [16], and

the p38 mitogen-activated protein kinase (MAPK) [17], which promote signal propagation leading to cell activation and proliferation [18].

In general, the correlation between the extent of phosphorylation of ZAP70 and the increase in its catalytic activity suggests that tyrosine phosphorylation serves to positively regulate ZAP70 enzymatic activity. However, depending on the T cell subtype and its mode of activation, ZAP70 may undergo phosphorylation at distinct sites which might impose different effects on the conformation and activity of ZAP70. For example, Lck-mediated phosphorylation of ZAP70-Tyr493, at the activation loop of the catalytic domain, promotes a conformational change that increases ZAP70 activity [13, 19]. In contrast, phosphorylation of ZAP70 on Tyr 292, has a negative regulatory role, as demonstrated by Y292F knock-in studies in primary mouse T cells (where phenylalanine at position 292 serves as a non-phosphorable tyrosine mimetic) which led to enhanced TCR signaling and increased T cell proliferation [14, 20, 21]. Two additional tyrosines that are frequently phosphorylated in TCR-engaged T cells, Tyr315 and Tyr319, are located in the interdomain B region, and appear to play a role in stabilizing the active conformation of ZAP70 [22].

Independently of its enzymatic activity, tyrosine-phosphorylated ZAP70 also serves as an adaptor protein that recruits SH2-containing effector molecules to the IS. These proteins may contribute to the regulation of ZAP70 activity and play significant roles in the propagation of signals downstream of activated TCRs [11, 23–27]. For example, ZAP70-phospho-Tyr319 functions as a binding site for the Lck-SH2 domain, and upon docking, Lck can phosphorylate additional tyrosines on ZAP70 and on neighboring proteins that participate in the TCR-linked signaling cascades [10, 11, 28]. In addition, ZAP70 phospho-Tyr315 serves as a binding site for the SH2 domain of the guanine nucleotide exchange factor, Vav [29], and the CT10 regulator of kinase II (CrkII) adaptor protein [30, 31] in TCR-stimulated T cells. These proteins possess additional protein–protein interaction domains through which they can associate with other effector molecules and recruit them to the IS. Furthermore, the binding of these proteins to ZAP70 may impact on the conformation, subcellular location, and ability of ZAP70 to interact with substrates or other regulatory molecules. Recent studies demonstrated that ZAP70-Tyr126, which undergoes phosphorylation in TCR-stimulated T cells, serves as a binding site for the protein kinase C theta (PKC θ) phosphotyrosine-binding domain and that this interaction is essential for the promotion of proximal TCR signaling events [32]. Phospho-Tyr315 and phospho-Tyr319 of ZAP70 were required for the above interaction, apparently because of their positive role in stabilizing the “open” conformation of ZAP70 which exposes phospho-Tyr126 for the interaction with PKC θ [32]. ZAP70 phospho-Tyr292 also serves as a binding motif for the ubiquitin ligase, c-Cbl (Casitas B-lineage lymphoma),

raising the possibility that c-Cbl mediates the ubiquitination of ZAP70, thereby promoting its degradation and downregulating proximal TCR signals [33, 34].

Additional studies demonstrated the existence of phosphorylation-independent mechanisms of regulation of ZAP70, which are mediated by ubiquitinating and deubiquitinating enzymes. For example, the ring finger-type E3 ligase, Nrdp1, can negatively regulate ZAP70 activity by ubiquitinating ZAP70-Lys578, generating a binding site for the suppressor of TCR signaling 1 (Sts1) and Sts2 protein phosphatases which dephosphorylate ZAP70 and downregulate its activity [35]. Abrogation of a distinct ZAP70 ubiquitination site at Lys217 resulted in increased activity of ZAP70 and TCR-downstream events, suggesting a negative regulatory role of this motif in ZAP70-dependent functions [36]. Furthermore, deubiquitinating carboxyl-terminal hydrolases, such as Usp9X [37] and Otud7b [38], can catalyze the deubiquitination of ZAP70 and facilitate T cell activation, while oxidation of ZAP70-Cys575 has an impact on ZAP70 stability and activity [39]. The above studies and additional information substantiate the assumption that the conformation of ZAP70, which is critical for its function, is subjected to regulation by multiple post-translational mechanisms that may potentially act in synergism or antagonism.

In the present work, we provide data supporting the existence of an additional mechanism of regulation of ZAP70 in TCR-activated T cells, which is mediated by the peptidyl-prolyl *cis-trans* isomerase, CypA, the predominant cyclophilin in T cells. We found that CypA interacts with the ZAP70 interdomain B region in a TCR activation-dependent manner. CypA-ZAP70 interaction requires an active Lck and is inhibited by the CypA inhibitor, CsA. TCR crosslinking promotes the recruitment of the ZAP70-associated CypA to the TCR/CD3 receptor complex within the IS. Functional studies revealed that CypA, but not the Pin1 PPIase, downregulates the catalytic activity of ZAP70, an event that is inhibited by CsA in normal T cells but not in CypA-deficient T cells. We suggest that CypA regulates ZAP70 activity by isomerization, physical interaction or intervention with the ability of ZAP70 to bind ATP or its substrate proteins, that CypA-mediated regulation of ZAP70 can be reversed by CsA, and that the CsA-regulated CypA-mediated effect on ZAP70 may synergize with the effect of CypA/CsA on the calcineurin/NF-AT signaling cascade which promotes T cell immunosuppression.

Results

TCR crosslinking promotes the association of CypA with ZAP70

To identify CypA-binding partners in TCR-engaged T cells, which might play a role in T cell activation and are targets

for regulation by CypA we immunoprecipitated CypA from lysates of resting and OKT3-stimulated Jurkat T cells and immunoblotted the samples with phospho-Tyr-specific mAbs. A prominent protein band of 70 kDa was observed in lysates of activated but not resting Jurkat T cells. Reblotting of the membrane with anti-ZAP70 mAbs indicated that this protein band corresponds to ZAP70 (Fig. 1A). To ascertain that the CypA-associated 70 kDa protein band represents ZAP70, we repeated the experiment using a ZAP70-deficient Jurkat T cell subline, P116. Anti-CypA mAbs were found to coimmunoprecipitate a ZAP70 immunoreactive protein from OKT3-stimulated Jurkat, but not P116 T cell lysates (Fig. S1A). In addition, heterologous expression of GST-CypA and Myc-ZAP70 in Jurkat T cells followed by anti-GST immunoprecipitation and anti-Myc immunoblotting reconfirmed the association between CypA and ZAP70 in OKT3-treated, but not resting T cells (Fig. 1B). An immunoprecipitation study in Jurkat T cells involving early and late time points of T cell activation demonstrated that binding of CypA to ZAP70 is transient and peaks at ~60 s post-TCR crosslinking (Fig. 1C). Coimmunoprecipitation of ZAP70 with CypA was also observed in lysates of TCR-triggered C57BL/6 J mouse spleen and thymus lymphocytes (Fig. 1D, E), suggesting that CypA binding to ZAP70 is a physiological phenomenon. Finally, by utilizing an appropriate isotype control, we validated the specificity of ZAP70 coimmunoprecipitation by CypA, and a reverse coimmunoprecipitation study revealed the ability of ZAP70 to coimmunoprecipitate CypA from OKT3-stimulated Jurkat cells (Fig. S1B).

To define the subcellular location of CypA-bound ZAP70 in TCR-engaged T cells, we stained the mouse spleen and thymus cells, as well as Jurkat T cells, with CypA- and ZAP70-specific Abs followed by a confocal microscope analysis. Cell staining with p-Tyr-specific mAbs verified the efficiency of the TCR stimulation by showing tyrosine-phosphorylated proteins at the membrane of the stimulated, but not resting Jurkat T cells (Fig. S1C). We found that in resting T cells, CypA and ZAP70 reside predominantly in the cytosol. In contrast, TCR crosslinking led to translocation and colocalization of the two proteins at the plasma membrane of the mouse spleen and thymus and Jurkat T cells (Figs. 1F–I, S1D–E).

TCR stimulation promotes CypA binding to the ZAP70 interdomain B region in an Lck-dependent manner

TCR ligation triggers rapid phosphorylation of multiple effector proteins on tyrosine residues, hence we suspected that CypA-ZAP70 interaction might be regulated by one or more protein tyrosine kinases (PTKs). One of the major candidates for this phosphorylation is the Lck PTK which associates with the cytoplasmic tails of the CD4 and CD8

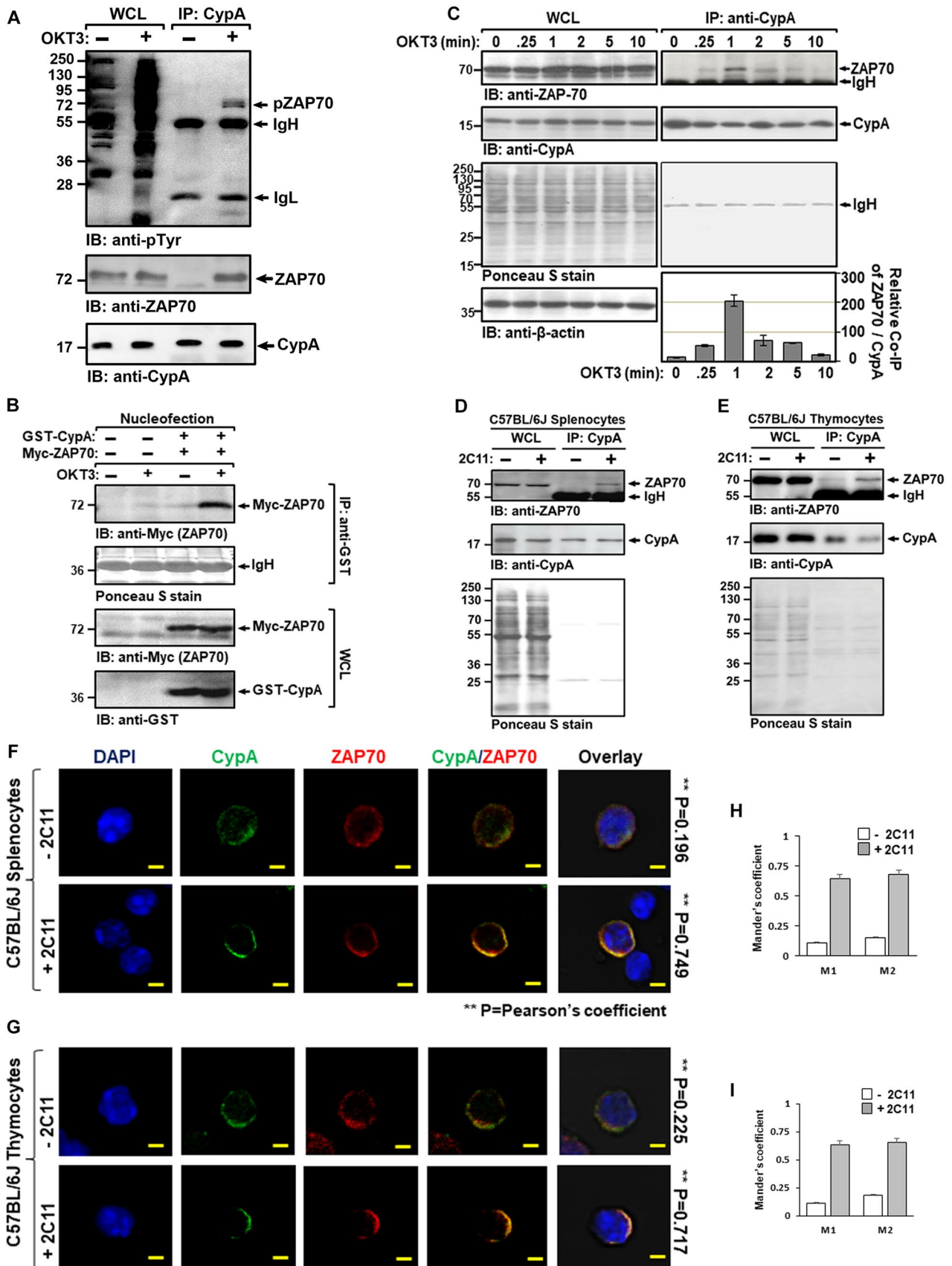


Fig. 1 TCR/CD3 crosslinking in T cells promotes the association of cyclophilin A and ZAP70. **A** Jurkat T cells were stimulated by TCR/CD3 crosslinking (using OKT3 mAbs) for 1 min, followed by cell lysis and immunoprecipitation, as described in Materials and Methods. Whole-cell lysates and CypA immunoprecipitates were then subjected to SDS-PAGE under reducing conditions and immunoblotting with anti-pTyr mAbs. Presence of ZAP70 and CypA on the same membrane was determined by membrane stripping and reblotting with the indicated Abs. **B** Jurkat T cells co-transfected with GST-CypA- and Myc-ZAP70-encoding eukaryotic expression vectors were stimulated with OKT3 mAbs for 1 min and cell lysates were subjected to immunoprecipitation using anti-GST mAbs. Samples were immunoblotted sequentially with anti-Myc mAbs and anti-GST mAbs. Membrane staining with Ponceau S monitored the equal loading of proteins in all lanes. **C** Jurkat T cells were stimulated with OKT3 mAbs for the indicated time intervals and lysates were then subjected to CypA immunoprecipitation. Samples were immunoblotted sequentially with ZAP70-, CypA-, and β -actin-specific mAbs. Membrane staining with Ponceau S determined the equal loading of proteins in all lanes, and densitometry analysis determined the relative amount of ZAP70 that coimmunoprecipitated with CypA. **D, E** C57BL/6 J mouse spleen (**D**) and thymus (**E**) cells were stimulated with 2C11 (anti-CD3 ϵ) mAbs for 1 min and cell lysates were then subjected to immunoprecipitation using anti-CypA mAbs. Samples were immunoblotted sequentially with anti-ZAP70 and anti-CypA mAbs. Membrane staining with Ponceau S monitored equal loading of proteins in all lanes. **F–I** C57BL/6 J mouse spleen and thymus cells were stimulated with 2C11 (anti-CD3 ϵ) mAbs as before, fixed, permeabilized and incubated with rabbit anti-CypA and mouse anti-ZAP70 mAbs. After washing, the cells were immunostained with Alexa Fluor 488-conjugated anti-rabbit- and Alexa Fluor 546-conjugated anti-mouse-IgG and counterstained with DAPI. The cells were analyzed using a confocal laser microscope (scale bar equals 2 μ m) (**F, G**). CypA-ZAP70 colocalization was quantified using the ImageJ plugin, and Pearson's coefficient values (**P) for resting and stimulated cells are indicated on the right side of each panel. **H, I** A graph representing Mander's coefficient values, M1 (red overlap with green) and M2 (green overlap with red). Results are representative of three independent experiments. Representative graphs show mean \pm SD (n=9). Molecular weight markers (in kDa) are indicated on the left of each panel of immunoblot. Arrows mark the position of the indicated protein bands. *IB*, immunoblot; *IgH*, Ig heavy chain; *IgL*, Ig light chain; *IP*, immunoprecipitates; *WCL*, whole-cell lysates

coreceptors. Lck recruits to the TCR/CD3, upon coreceptor binding to MHC, and phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on the TCR/CD3 chains, as well as ZAP70 [40]. To test the involvement of Lck in CypA-ZAP70 interaction, we compared the ability of CypA to coimmunoprecipitate ZAP70 from wild-type Jurkat vs. the Lck-deficient Jurkat subline (JCaM.1). We observed that CypA coimmunoprecipitated ZAP70 from lysates of OKT3-treated Jurkat, but not JCaM.1 T cells (Fig. S2A). The results suggest that the in vivo interaction between CypA and ZAP70 is dependent on Lck, which is known to phosphorylate ZAP70 in TCR-triggered T cells [40]. Furthermore, we immunoprecipitated CypA from anti-CD3 ϵ (2C11)-stimulated mouse spleen cells, and incubated the bead-immune-complexes in the presence or absence of calf-intestinal alkaline phosphatase (CIP) for 1 h. As observed above, CypA coimmunoprecipitated ZAP70 from lysates of

2C11-treated mouse spleen cells. However, CIP treatment of the immune complexes resulted in ZAP70 dissociation from the immune complexes (Fig. S2B) suggesting that CypA-ZAP70 interaction is dependent on the phosphorylation of ZAP70, CypA, or both proteins.

Previous studies demonstrated that CypA mediates its association with IL-2-inducible T-cell kinase (Itk), a predominant tyrosine kinase in T cells, by interacting with the N-SH2 domain in the Itk regulatory domain [41]. Hence, we speculated that CypA association with ZAP70 tyrosine kinase could also be through the ZAP70 regulatory region. A pull-down assay using various GST-ZAP70-deletion mutants demonstrated that CypA can be pulled down only by the full-length ZAP70 and the interdomain B region within the regulatory domain ZAP70 (Fig. 2A).

Association of CypA with activated ZAP70 promotes CypA recruitment to the TCR within the IS

The ZAP70 PTK resides in the cytoplasm of resting T cells. Following TCR stimulation it translocates to the cell membrane and undergoes phosphorylation which peaks at ~60 s [1]. Since CypA association with ZAP70 occurs in a similar time kinetic post-TCR stimulation we tested whether the membrane-translocating ZAP70 pulls with it the CypA protein. Jurkat T cells were stimulated with OKT3 for 60 s and their lysates were subjected to immunoprecipitation using phospho-CD3 ζ (pCD3 ζ)-specific Abs. As previously reported, ZAP70 and Lck coimmunoprecipitated with the pCD3 ζ from TCR-activated T cells (Fig. 2B) [1, 42]. In addition, CypA was found to coimmunoprecipitate with pCD3 ζ (Fig. 2B). CypA recruitment to the cell membrane was dependent on the presence of ZAP70 and did not occur in ZAP70-deficient P116 T cells (Fig. 2B). Immunofluorescence studies utilizing similar cells and activation conditions confirmed the colocalization of CypA and pCD3 ζ at the plasma membrane of activated Jurkat T cells (Fig. 2C–F). To test whether the localization of CypA at the membrane of TCR-activated T cells occurs via its association with ZAP70 or perhaps by its direct interaction with pCD3 ζ , the experiment was repeated using ZAP70-deficient P116 T cells. We found that the lack of ZAP70 disrupted the ability of CypA to coimmunoprecipitate with pCD3 ζ (Fig. 2B) or colocalize with it at the plasma membrane (Fig. 2D), suggesting the requirement of ZAP70 for the association of CypA with the activated TCR.

We further speculated a tripartite complexing of CD3 receptor-ZAP70-CypA upon TCR crosslinking. In order to detect CD3 in both resting and activated cells, we utilized an antibody against total CD3 for the immunofluorescence studies. Since ZAP70 interacts with CD3 ζ as well as CD3 ϵ ITAM motifs [43] we used a combination of anti-CD3 ϵ , -ZAP70, and -CypA Abs to immunostain OKT3-stimulated

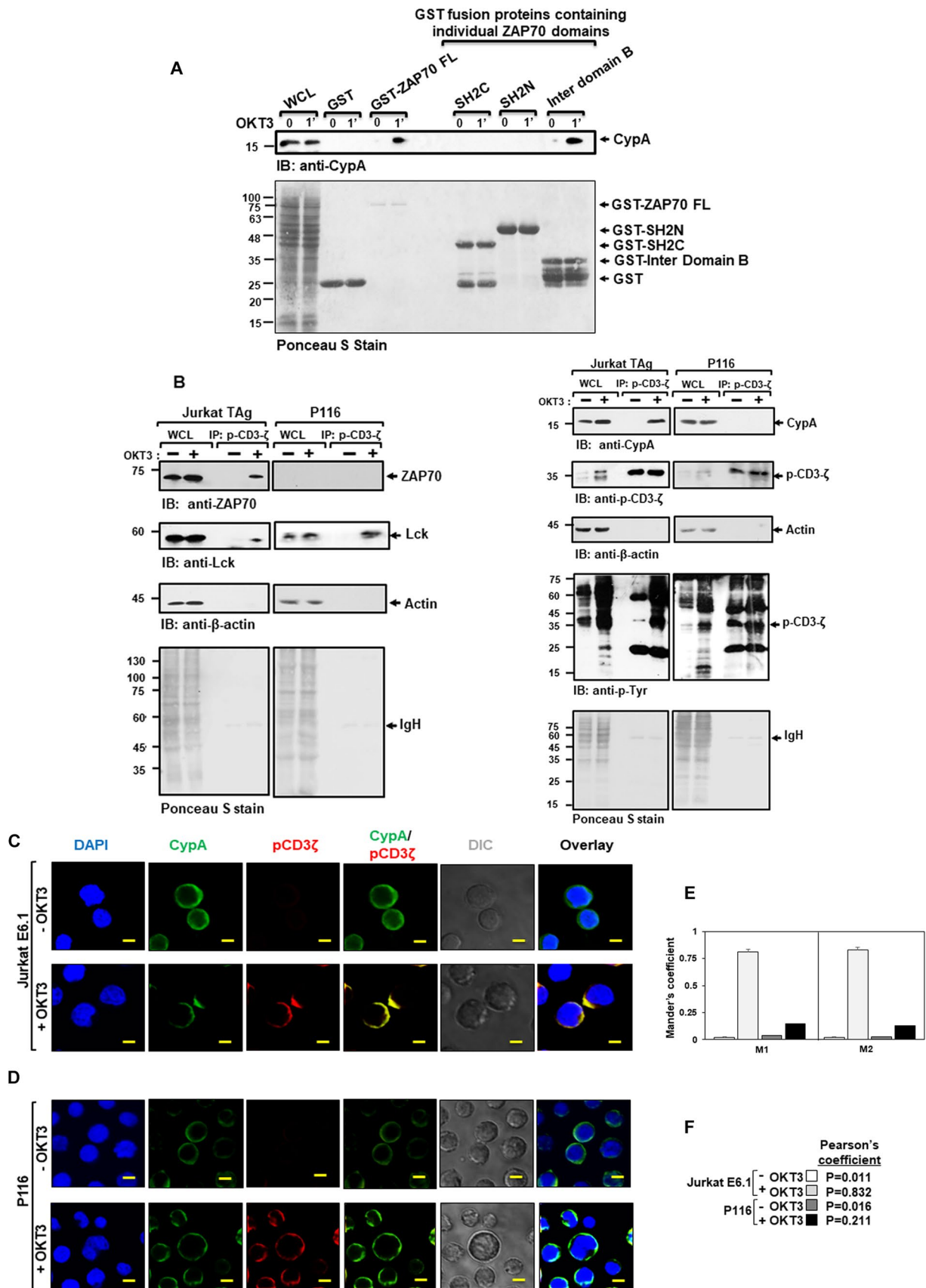


Fig. 2 TCR/CD3 stimulation promotes CypA binding to the ZAP70 interdomain B region and recruitment of CypA to the phospho-CD3 ζ -associated ZAP70. **A** Resting and OKT3-stimulated Jurkat T cell lysates were subjected to pulldown assay using bead-immobilized GST proteins that are fused to full-length or truncated products of ZAP70 (10 μ g/group), as described in material and methods. Samples were subjected to SDS-PAGE under reducing conditions, followed by protein electrotransfer to a membrane. The membrane was immunoreacted with anti-CypA mAbs and stained with Ponceau S in order to visualize the GST-immobilized fusion proteins. **B** Jurkat T cells and ZAP70-deficient P116 T cells were stimulated with OKT3 mAbs for 1 min followed by cell lysis and immunoprecipitation using anti-pCD3 ζ mAbs. Whole-cell lysates and pCD3 ζ immunoprecipitates were then divided into two groups and subsequently subjected to SDS-PAGE under reducing conditions on an 8% and 12% polyacrylamide gels. The 8% gel-transferred nitrocellulose membrane was sequentially immunoblotted with ZAP70-, Lck-, and β -actin-specific mAbs, and the 12% gel-transferred nitrocellulose membrane was sequentially immunoblotted with CypA-, pCD3 ζ -, and β -actin-, and pTyr-specific mAbs. Ponceau S staining of the membranes determined equal loading of proteins in all lanes. **C–F** Jurkat and P116 T cells were stimulated with OKT3 mAbs for 1 min. After fixation and permeabilization, the cells were incubated with rabbit anti-CypA and mouse anti-pCD3 ζ mAbs, followed by immunostaining with Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 546-conjugated anti-mouse Ig Abs, and counterstained with DAPI. Cells were analyzed using a confocal laser microscope (**C**, **D** scale bar equals 2 μ m) and comparative colocalization of CypA and pCD3 ζ in Jurkat vs. P116 cells was quantified using the ImageJ plugin, *JACoP*. Mander's coefficient values for M1 (red overlap with green) and M2 (green overlap with red) (**E**) and Pearson's coefficient values (**F**) are indicated. Results are representative of three independent experiments. Representative graphs show mean \pm SD ($n=9$). Molecular weight markers (in kDa) are indicated on the left of each panel of immunoblot. Arrows mark the position of the indicated protein bands. *IB*, immunoblot; *IgH*, Ig heavy chain; *IgL*, Ig light chain; *IP*, immunoprecipitates; *WCL*, whole-cell lysates

Jurkat T cells. While the three proteins were found to be distributed in the same subcellular compartments, stain overlapping analysis revealed a striking colocalization of CypA-ZAP70, ZAP70-CD3 ϵ , and CypA-CD3 ϵ in OKT3-stimulated cells, in comparison to the staining results in resting Jurkat T cells (Fig. 3A–D). To test whether CypA recruits to the IS of T cells triggered by peptide antigen-loaded APC, by virtue of its interaction with ZAP70, we utilized CH7C17 Jurkat T cells, which express the influenza hemagglutinin (HA) peptide-specific TCR, and co-cultured them with antigen-fed LG2 cells, as APC. LG2 cells fed with the HA 307–319 peptide formed conjugates with the CH7C17 T cells in which CypA and ZAP70 colocalized at the T cell-APC contact area (Fig. 3E). The response was antigen-specific since colocalization of CypA-ZAP70 did not occur in LG2 cells fed with a mutated peptide, HA(K316E). CypA-ZAP70 also colocalized with the F-actin binding protein, phalloidin, which serves as an IS-specific marker. These results suggest a potential regulatory role for CypA in the vicinity of the ZAP70-TCR complex within the IS, where CypA might regulate IS-residing effector molecules that impact on TCR-downstream signaling cascades.

CypA inhibits ZAP70 catalytic activity in vitro

To test the direct effect of CypA on ZAP70 activity we performed an in vitro radioactive kinase assay on ZAP70 immunoprecipitates from 1 min OKT3-treated Jurkat T cells and 2C11-treated C57BL/6 J mouse spleen and thymus T cells. Preincubation of ZAP70 from all three sources with enzymatically active recombinant CypA (rCypA) inhibited ZAP70 autophosphorylation activity in a concentration- (Figs. 4A, C, S3A, C) and time- (Figs. 4B, D, S3B, D) dependent manner. A linear CypA-mediated inhibition of the ZAP70 catalytic activity was observed in the three different sources of ZAP70 (Fig. 4E, F). Furthermore, an augmented ZAP70 kinase activity was noted in OKT3-stimulated CypA-deficient Jurkat T cells when compared to that of wild-type cells (Fig. 4G, H). These data suggest that CypA functions as a negative regulator of ZAP70 enzymatic activity.

To test whether ZAP70 is sensitive to isomerases in general, or whether its regulation is selective to CypA, we repeated the assay comparing the effects of CypA vs. Pin1 PPIase on the catalytic activity of ZAP70. We found that CypA, but not Pin1, downregulated the autophosphorylation activity of ZAP70 (compare Fig. S4A vs. S4B). A control experiment validated that rPin1 is catalytically active by showing its ability to modulate the activity of PKC α , a known Pin1 substrate [44] (Fig. S4C). Inclusion of a ZAP70 substrate, the cytoplasmic fragment of human erythrocyte band 3 (cfb3) [45] in the in vitro kinase assay further demonstrated that rCypA inhibited the ability of ZAP70 to phosphorylate an exogenous substrate in a concentration- (Fig. S3C) and time- (Fig. S3D) dependent manner. We also noticed that the inclusion of recombinant CypA in the ZAP70 kinase assay system did not result in phosphorylation of CypA, negating the possibility of reciprocal regulation of CypA by ZAP70 (Figs. 4, S3). While the above studies suggest that CypA inhibits the phosphorylation activity of ZAP70, they do not rule out the possibility that the reduction in ZAP70 phosphorylation is due to rCypA-mediated dephosphorylation of ZAP70. This hypothesis was tested by co-incubation of enzymatically active rCypA with an inactive, radiolabeled, phospholabeled ZAP70. We found (Fig. S4D) that rCypA was unable to dephosphorylate ZAP70, supporting the assumption that CypA is a conformational regulator of ZAP70.

CsA reverses the regulatory effect of CypA on ZAP70

CsA is a potent immunosuppressive drug that mediates high-affinity binding to CypA and inhibits its enzymatic activity [46, 47]. To test whether CypA binding to ZAP70 is affected by CsA, we treated C57BL/6 J-derived spleen and thymus cells and Jurkat T cells with CsA and

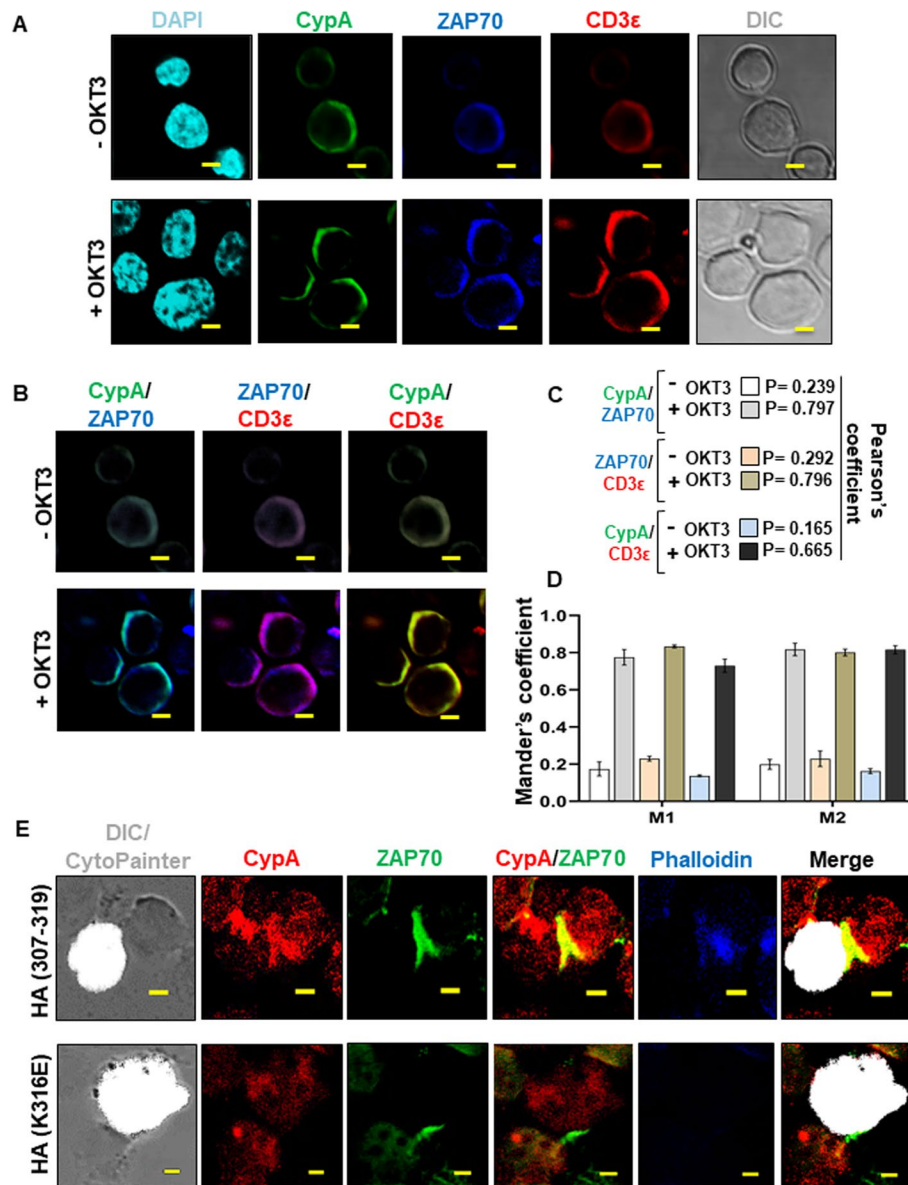
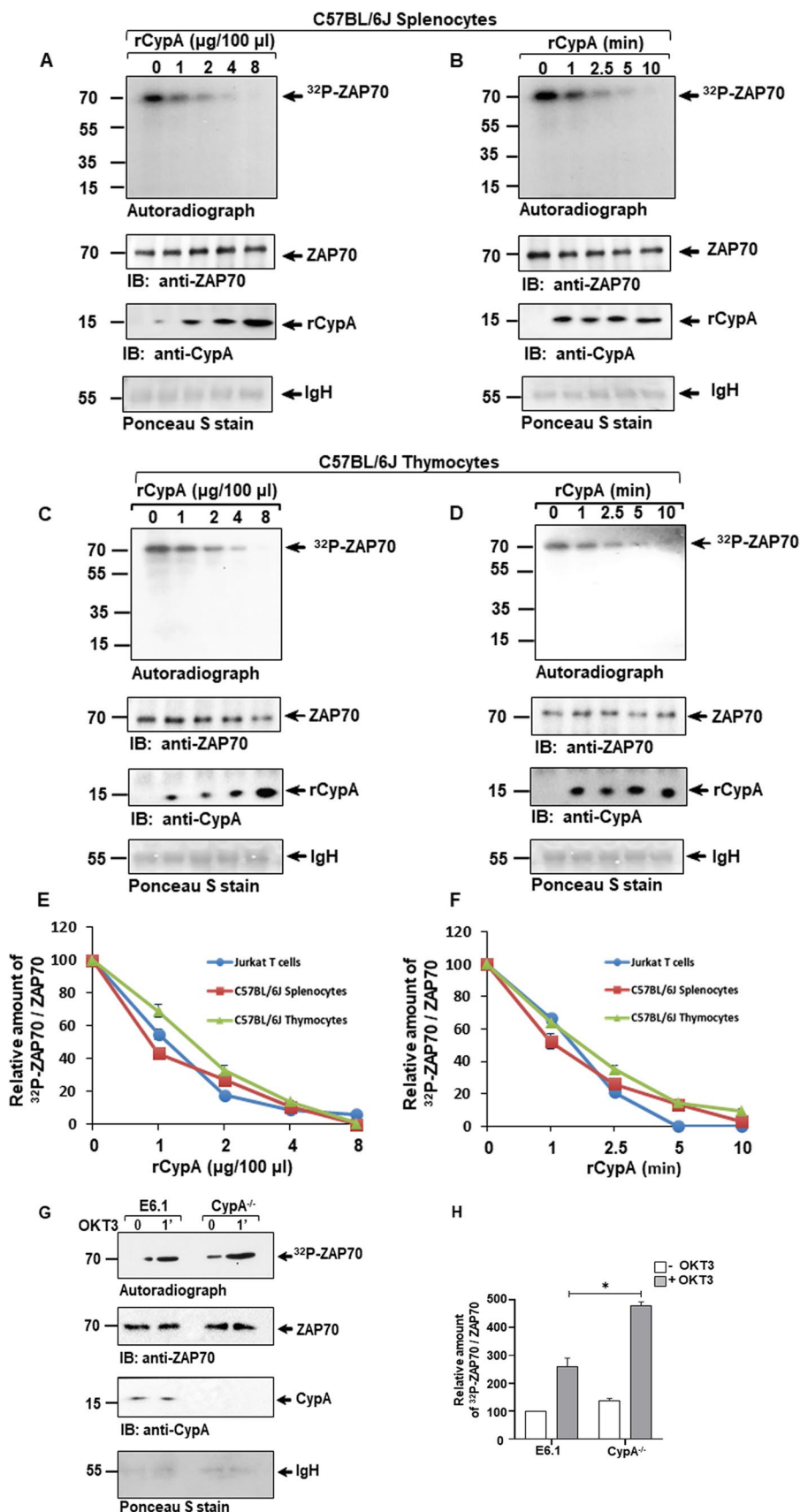


Fig. 3 ZAP70-bound CypA recruits to the TCR and stimulation by peptide-loaded MHC antigens on the surface of APC induces CypA-ZAP70 colocalization at the IS. **A–D** Jurkat T cells were stimulated with OKT3 mAbs for 1 min or left untreated. Fixed and permeabilized cells were then incubated with rabbit CypA-, mouse ZAP70-, and goat CD3ε-specific Abs, followed by immunostaining with Alexa Fluor 488-conjugated anti-rabbit-, Alexa Fluor 633-conjugated anti-mouse-, and Alexa Fluor 546-conjugated anti-goat-Ig Abs and counterstained with DAPI. The cells were analyzed using a confocal laser microscope (**A, B**; scale bar equals 2 μm). The extent of colocalization of CypA and either ZAP70 or CD3ε was determined using the ImageJ plugin, *JACoP*. Pearson's coefficient values (**C**) and Mander's coefficient values (±SD) M1 and M2 for the respective protein colocalization (**D**) are indicated ($n=9$). **E** Conjugate formation between Jurkat T cells, clone CH7C17, and APCs (B cell line, LG2) was performed as described in Materials and Methods. Following antigen (HA peptide 307–319, or an inactive HA peptide, HA K316E) pre-pulsing and cocubation for 5 min, T cells and APCs were fixed, permeabilized, and analyzed by confocal microscopy (scale bar equals 2 μm). Immunofluorescence staining was performed using primary Abs; mouse anti-ZAP70, and rabbit anti-CypA, followed by Alexa Fluor 633-conjugated phalloidin and secondary Abs, Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 546-conjugated anti-rabbit IgG. CytoPainter stain was detected at 405 nm wavelength. Results are representative of three independent experiments

subsequently stimulated the cells with anti-CD3 (2C11/OKT3) mAbs, followed by immunoprecipitation of CypA. We found that cell treatment with CsA inhibited the ability of CypA to associate with ZAP70 (Fig. 5A, S5A, S6A).

The effect of CsA on the ability of CypA to associate with ZAP70 was also tested by cell staining and immunofluorescent imaging using confocal microscopy. The results demonstrated strong CsA-mediated inhibition of CypA

Fig. 4 CypA downregulates the catalytic activity of ZAP70. **A–F** C57BL/6 J mice-derived spleen and thymus cells were stimulated with 2C11 mAbs for 1 min. Subsequently, cell lysates were subjected to ZAP70 immunoprecipitation and ZAP70-containing beads were incubated in the presence of the indicated concentrations of enzymatically active recombinant human CypA (rCypA) for 10 min at 37 °C (**A, C**), or with 4 μg/ml of rCypA for indicated time intervals (**B, D**). Samples were then subjected to a ZAP70 kinase assay in the presence of [γ - 32 P]-ATP, as described in Materials and Methods. Samples were resolved by SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and developed by autoradiography and immunoblotting using anti-ZAP70 and anti-CypA mAbs. Ponceau S staining of the membrane determined equal loading of proteins in all lanes. pZAP70 and ZAP70 protein band signals were quantified using the ImageJ software, and the relative amounts of pZAP70/ZAP70 in Jurkat cells, and mouse spleen and thymus cells (\pm SD) were presented in a graph (**E, F**). **G** E6.1 Jurkat T cells and CypA-deficient Jurkat T cells were stimulated with OKT3 mAbs for 1 min. Cell lysates were then subjected to a ZAP70 kinase assay in the presence of [γ - 32 P]-ATP, as previously described. Ponceau S staining monitored equal loading of proteins in all lanes. pZAP70 and ZAP70 protein band signals were quantified using the ImageJ software, and the relative amounts of pZAP70/ZAP70 in E6.1 vs CypA-deficient Jurkat cells were presented in a bar graph (**H**). Graph values show mean \pm SEM, * p < 0.05, using Student's unpaired t test. Molecular weight markers (in kDa) are indicated on the left and arrows mark positions of the indicated protein bands. Results are representative of three independent experiments. *IB*, immunoblot; *IgH*, Ig heavy chain



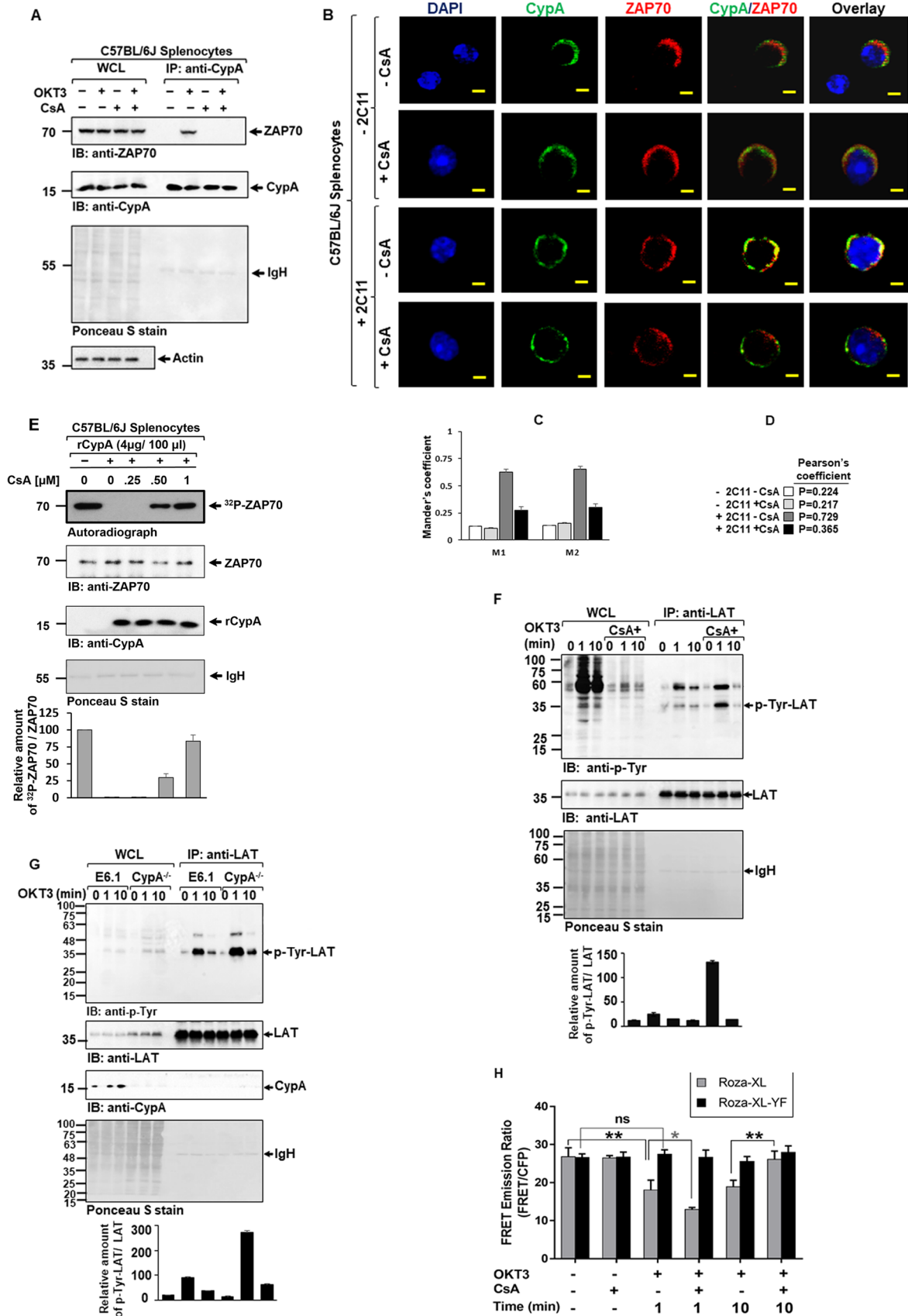


Fig. 5 CypA ablation or its inhibition by cyclosporin A annuls the effect of CypA on ZAP70 activity. **A** C57BL/6 J-derived spleen cells were treated with CsA and stimulated with 2C11 mAbs for 1 min. Whole cell lysates and CypA immunoprecipitates were resolved by SDS-PAGE under reducing conditions followed by sequential immunoblotting with ZAP70-, CypA-, and β -actin-specific mAbs. Membrane staining with Ponceau S determined equal loading of proteins in all lanes. **B** CsA-treated or untreated C57BL/6 J spleen cells were left unstimulated or stimulated using 2C11 mAbs for 1 min. Fixed and permeabilized cells were then incubated with rabbit anti-CypA and mouse anti-ZAP70 mAbs, followed by immunostaining with Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 546-conjugated anti-mouse Ig Abs and counterstained with DAPI. The cells were analyzed using a confocal laser microscope. Scale bar equals 2 μ m. **C–D** Quantification of the extent of colocalization of CypA and ZAP70 was performed using the ImageJ plugin, *JACoP*. Mander's coefficient values for M1 (red overlap with green) and M2 (green overlap with red) (\pm SEM) (**C**) and Pearson's coefficient values (**D**) are indicated ($n=9$). **E** C57BL/6 J-derived spleen cells were stimulated as before and cell lysates were subjected to ZAP70 immunoprecipitation. The ZAP70-containing beads were incubated in the presence or absence of enzymatically active recombinant human CypA (rCypA; 4 μ g/100 μ l) plus the indicated concentrations of CsA, for 10 min at 37 $^{\circ}$ C. Samples were then subjected to a radioactive ZAP70 kinase assay in the presence of [γ - 32 P]-ATP followed by SDS-PAGE under reducing conditions and protein electroblotting onto nitrocellulose membranes. The membranes were developed by autoradiography and immunoblotting using anti-ZAP70 and anti-CypA mAbs. Ponceau S staining of the nitrocellulose membrane monitored the equal usage of ZAP70 mAbs. **F–G** Jurkat T cells that were left untreated or treated with CsA (**F**), and CypA-deficient Jurkat T cells (**G**) were stimulated with OKT3 mAbs for indicated time intervals. Cell lysates were then subjected to LAT immunoprecipitation and samples of whole-cell lysates and immunoprecipitates were subjected to SDS-PAGE on 10% acrylamide gels under reducing conditions followed by sequential immunoblotting using anti-p-Tyr (**F**, **G**), anti-LAT (**F**, **G**), and anti-CypA (**G**) mAbs. Membrane staining with Ponceau S determined the equal loading of proteins in all lanes. pTyr-LAT and LAT protein band signals were quantified using the ImageJ software and the relative amounts of pTyr-LAT/LAT (\pm SD) were presented in a bar graph. **H** Jurkat T cells were nucleofected with ROZA-XL or ROZA-XL-YF plasmids. 48 h post-transfection, the cells were left untreated or treated with CsA, and subsequently stimulated with OKT3 mAbs for indicated time intervals or left unstimulated. Cells were then analyzed by FACS at CFP excitation wavelength (405 nm) and simultaneous emission was detected in the CFP and YFP emission spectra. FRET data analysis was performed using FlowJo v10.7 software and data (\pm SEM) were presented in a bar graph. Data are representative of three independent experiments. * $p < 0.05$ and ** $p < 0.01$, using Student's unpaired t test. Molecular weight markers (in kDa) are indicated on the left of each panel of immunoblot. Arrows mark the position of the indicated protein bands. *IB*, immunoblot; *IgH*, Ig heavy chain; *IP*, immunoprecipitates; *WCL*, whole-cell lysates

colocalization with ZAP70 in C57BL/6 J mouse spleen and thymus cells and Jurkat T cells (Figs. 5B–D, S5B–D, S6B–D). A major mechanism by which CsA-CypA complexes contribute to immunosuppression is by binding to and inhibition of calcineurin and its downstream signaling pathway that controls IL-2 gene transcription. However, the above findings also suggest the involvement of CypA in the regulation of ZAP70. We tested the effect of

CsA on the ability of rCypA to attenuate ZAP70 catalytic activity. The inclusion of CsA at the rCypA-ZAP70 pre-incubation step reversed the inhibitory effect of CypA on ZAP70 autophosphorylation in a concentration-dependent manner in C57BL/6 J mouse spleen and thymus cells and in Jurkat T cells (Figs. 5E, S5E, S6E). The results suggest that CsA-mediated regulation of CypA in activated T cells may affect the catalytic activity of ZAP70 and potentially modulate signal transduction pathways downstream of the TCR.

Previous studies demonstrated that CypA is a negative regulator of the Itk in T cells and that CsA can augment the tyrosine phosphorylation of PLC γ 1, the primary substrate of Itk [41]. In analogous to this system, we tested the effect of CsA on ZAP70-mediated phosphorylation of LAT, the immediate physiological substrate of ZAP70 in TCR-stimulated T cells [15]. Immunoprecipitation of LAT from OKT3-treated Jurkat T cells revealed its rapid phosphorylation at 60-s post-TCR/CD3 stimulation. Pretreatment of the cells with CsA augmented the tyrosine phosphorylation of LAT (Fig. 5F). CsA had no effect on the phosphorylation level of LAT at a longer temporal post-TCR stimulation (10 min) when the amount of ZAP70-associated CypA was negligible. Higher tyrosine phosphorylation of LAT was also observed in TCR/CD3-stimulated CypA-deficient Jurkat T cells, relative to the phosphorylation level observed in wild-type cells (Fig. 5G). These results suggest that ZAP70-mediated phosphorylation of LAT in vivo, which occurs at an early time-point post-TCR stimulation, is subjected to regulation by CypA.

To substantiate the latter findings showing in vivo effects of CypA/CsA on ZAP70-mediated LAT phosphorylation, we transfected Jurkat T cells with the ROZA-XL plasmid which functions as a biosensor of ZAP70 activity (see Fig. S7). FACS analysis of ROZA-XL-expressing cells revealed that OKT3 stimulation led to a decrease in their FRET values which reflects increased ZAP70 activity (Fig. 5H). No change in FRET was observed in OKT3-stimulated cells that express the ROZA-XL-YF plasmid which encodes a ZAP70 activation-insensitive protein [48]. To ensure the accuracy of FRET readings, the transfection efficiency of ROZA-XL/YF was validated by FACS analysis, and equal numbers of ROZA-XL/YF positive cells were used in each experimental group. Interestingly, pretreatment of the ROZA-XL-expressing cells with CsA further reduced the OKT3 stimulation-induced FRET values, suggesting that inhibition of CypA at an early time-point post-TCR stimulation increases the activity of ZAP70 (Fig. 5H). The results suggest the involvement of CypA in the regulation of an early T cell activation response which leads to the downregulation of ZAP70 catalytic activity, an effect that can be abrogated by the CypA inhibitor, CsA.

Discussion

CypA is a member of the immunophilins that catalyzes the reversible *cis*–*trans* conversion of peptide bonds containing the amino acid proline. The two major groups of mammalian immunophilins, the cyclophilins and FK506-binding proteins (FKBPs), function as chaperons and assist newly synthesized proteins to undergo proper folding and acquire a conformation that is essential for their stability, localization, and biological function. The T lymphocyte immunophilins, CypA and FKBPs, are of particular interest because of their ability to bind the CsA and FK506 (tacrolimus) compounds, respectively, and promote immunosuppression by inhibition of T cell activation [47, 49]. Triggering of the TCR stimulates a rapid phospholipase C (PLC)-mediated breakdown of inositol phospholipids, resulting in the production of second messengers, including inositol trisphosphate (IP₃), which promotes the rise in intracellular free Ca²⁺ concentration and the activation of calcineurin [50]. Once activated, the calcineurin associates with and dephosphorylates the nuclear factor of activated T cells (NFAT) [51], which then translocates to the nucleus, binds to selected DNA promoter regions, and initiates the transcription of interleukin-2 (IL-2) [52] and other proinflammatory cytokines [53].

Inhibition of calcineurin by immunophilin-drug complexes [54, 55] hinders NFAT translocation to the nucleus and inhibits the formation of IL-2 and other cytokines which are crucial for the maintenance, survival, differentiation, and activation of distinct T cell subtypes [56]. While inhibition of calcineurin activity is a major mechanism by which immunophilin-drug complexes induce immunosuppression, immunophilins were found to impair T cell functions by intervening with the activity of several additional effector molecules. Functional studies demonstrated that immunophilin-drug complexes block the activation of Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs) in TCR-stimulated T cells, via a calcineurin-independent mechanism [57]. p38 is a direct substrate for activated ZAP70 in TCR-engaged T cells, and phosphorylation of p38 initiates a negative feedback loop that promotes the dissociation of ZAP70 from CD3 ζ and negatively regulates TCR proximal signals [58]. Based on the above data, the effect of immunophilin-drug complexes on p38 may be indirect, reflecting the effect of the immunophilin-drug complexes on ZAP70, which is an upstream regulator of p38.

Studies by Brazin et al., [41] revealed that CypA can form a stable complex with Itk, an essential kinase for signal transduction downstream of activated TCR, which plays a key role in T cell activation, proliferation, and differentiation [59–61]. CypA binding to Itk induces a

proline-dependent conformational switch within the Itk SH2 domain leading to the inhibition of Itk enzymatic activity and modulation of the Itk ligand recognition, a mechanism that can be disrupted by CsA [41]. Further studies revealed that irrespective of CsA, CypA can down-regulate TCR signal strength in CD4+ T cells [62]. CT10 regulator of kinase II (CrkII) adaptor protein is an additional TCR-coupled signaling protein that is regulated by CypA. In vitro studies of a recombinant protein consisting of the SH3N-linker-SH3C of the chicken CrkII showed that this peptide can undergo CypA-mediated *cis*–*trans* isomerization at the linker region Pro238 residue [63, 64]. The *cis* conformer of CrkII is autoinhibited due to the intramolecular interaction between its two SH3 domains. In contrast, the *trans* conformer of CrkII acquires an extended conformation in which its SH2 and SH3 domains are available for interaction with binding partners. TCR stimulation promotes direct physical interaction between CrkII and ZAP70, which is mediated by the Crk-SH2 domain and phospho-Tyr315 in the ZAP70, an interaction that is dependent on the presence of an active Lck [27, 30]. Both CypA and FKBP were found to associate with CrkII in resting Jurkat T cells and regulate its conformation [65]. In addition, CypA increased the ability of CrkII to interact with the guanine-nucleotide releasing factor, C3G, which promotes integrin-mediated cell adhesion and migration [65]. As a result, CsA/FK506-mediated inhibition of PPIase decreased the ability of T cells to adhere to fibronectin-coated surfaces and migrate toward the stromal cell-derived factor 1 α , suggesting that CsA/FK506 interferes with the PPIase-mediated, CrkII-dependent mechanisms that regulate selected effector T cell functions [66].

In the present study, we found that CypA association with ZAP70 peaks at about 1 min post-TCR stimulation, when Lck-mediated phosphorylation of ZAP70 is near maximum, suggesting that CypA regulates predominantly the phosphorylated and not the non-phosphorylated ZAP70. The results also suggest that CypA-mediated regulation of ZAP70 in vivo occurs in TCR-stimulated, and not in resting T cells and that CsA prevents the TCR engagement-dependent formation of CypA-ZAP70 complexes. Notably, CypA associates with the interdomain B region of the ZAP70 regulatory domain, a region that mediates the association of critical ZAP70-binding partners, such as CrkII and Vav, and also harbors key tyrosine residues, which upon phosphorylation, mediate positive or negative effects on the ZAP70 kinase catalytic activity [20, 22, 28–30]. The early event which promotes ZAP70 association with activated TCR is phosphorylation of the CD3 chain ITAMs, predominantly those of the CD3 ζ . We found that CypA association with ZAP70 enables its recruitment to the vicinity of the activated TCR within the IS. In agreement, the time kinetic of the CypA-ZAP70 association paralleled that of the ZAP70 recruitment to the

IS of TCR-stimulated T cells. Furthermore, CsA was found to inhibit CypA association with ZAP70 and prevent the colocalization of CypA with ZAP70 at the cell membrane. The results suggest that catalytically active CypA plays a role in the conformational regulation of ZAP70, perhaps by isomerization of ZAP70, although conformational constraints imposed on ZAP70 due to its association with CypA might also affect ZAP70 activity by modulating its ability to undergo phosphorylation and/or interaction with binding partners and substrate proteins.

The finding showing that cell treatment with CsA augments the phosphorylation of LAT, an immediate substrate of ZAP70 in TCR-stimulated T cells, is reminiscent of the observation made in studies of the regulation of another CypA-interacting T cell PTK, the Itk [41]. In both cases, the inclusion of CsA at a time point in which CypA binds its target PTK (either Itk or ZAP70) augments the PTK-induced phosphorylation of its respective primary substrates (PLC γ 1 and LAT, respectively). These observations highlight the notion that CypA functions as a negative regulator of PTKs in the early phases of T cell activation.

Studies in Jurkat T cells that express the ZAP70 activity-biosensor, ROZA-XL, revealed that CsA increases ZAP70 activity at 1 min post-TCR-stimulation of T cells, indirectly implying that CypA is a negative regulator of the ZAP70 catalytic activity and that CsA can reverse the effect of CypA on ZAP70. This *in vivo* assay is based on the ability of active ZAP70 to phosphorylate a Tyr-containing LAT epitope in the biosensor protein, an epitope whose phosphorylation directly correlates with ZAP70 activity. Recent studies demonstrated that Lck-mediated phosphorylation of ZAP70 is a crucial step for ZAP70 bridging to LAT and the present study further demonstrates that ZAP70 phosphorylation by Lck is required for ZAP70 interaction with CypA [67]. Notably, CsA appears to inhibit ZAP70 activity at 10 min post-TCR stimulation, when CypA association with ZAP70 is negligible. We suspect that this inhibition reflects the effect of CsA on CypA-mediated isomerization of CrkII, a ZAP70-binding partner that associates with ZAP70 at a slower time kinetic and that its association with ZAP70 can still be observed at 10 min post-TCR stimulation [66]. The data demonstrated that ZAP70 undergoes activation at 1 min post-TCR stimulation, an effect that is augmented upon cell treatment with CsA, apparently representing the CsA-mediated inhibition of CypA.

Complexes of CsA-CypA are known to interact with and inhibit the cytoplasmic phosphatase, calcineurin, and thereby its primary target, the NFAT transcription factors [55, 68]. More recent studies reported the recruitment of calcineurin to the TCR signaling complex, where it reverses inhibitory phosphorylation on Lck and indirectly promotes the activation of ZAP70 [69]. It is possible therefore that some of the *in vivo* effects of CsA on ZAP70 that were

observed in the present studies reflect the CsA-CypA-mediated inhibition of calcineurin, which prevent the activation of Lck, and its downstream protein, ZAP70. *In vitro* kinase assay of ZAP70 performed in the absence or presence of enzymatically active human recombinant CypA demonstrated that CypA induces a time- and concentration-dependent reduction in the extent of ZAP70 autophosphorylation as well as substrate phosphorylation (cfb3). The inclusion of CsA in the preincubation step reversed the effect of CypA on ZAP70. The possibility that the inhibitory effect of rCypA on ZAP70 catalytic activity is non-specific, reflecting the presence of contaminating interferences in the commercially obtained rCypA, was refuted by findings showing that CsA, which specifically inhibits rCypA, annuls the inhibitory effect of CypA on ZAP70. The results suggest that CypA, which associates with ZAP70 in TCR-engaged T cells, can impose its effect on ZAP70 activity via a direct mechanism. We hypothesize that CypA modulates ZAP70 activity by isomerization of ZAP70 and/or physical interaction with a ZAP70 motif that alters the catalytic activity of ZAP70 and/or accessibility to substrates or ATP. The observation that Pin1, in contrast to CypA, does not affect ZAP70 catalytic activity suggests a selectivity in PPIases towards ZAP70.

Our data support the hypothesis that maximal phosphorylation of ZAP70 at ~60-s post-TCR stimulation involves the concomitant association of ZAP70 with CypA which can modulate the catalytic activity of ZAP70 (Fig. 6). Under the assays conditions used, CypA was found to inhibit ZAP70 activity. However, CypA can interconvert both *cis* and *trans* isomers and the preferred direction of ZAP70 isomerization under physiological conditions might be determined by the phosphorylation status of ZAP70 or its association with selected binding proteins. The results suggest that CypA functions as a physiological regulator of ZAP70 that might contribute to the amplitude, duration, and fine-tuning of the T cell activation response.

Materials and methods

Reagents and antibodies

Sandimmune (CsA; 50 mg/ml) was from Novartis Pharma AG (Basel, Switzerland). Aprotinin, leupeptin, Triton X-100, and TPA were from Sigma-Aldrich (St. Louis, MO, USA). Protein A, G-, A/G- agarose beads, electrochemiluminescence (ECL), and nitrocellulose membranes were from Amersham Pharmacia Biotech (Uppsala, Sweden). RPMI-1640, Pen/Strep, and FBS were from Biological Industries (Beit Haemek, Israel). AEBSF was from ICN Biomedicals (Aurora, OH). A mouse anti-ZAP70 (clone 1E7.2) was from BioLegend (San Diego, CA) and a mouse anti-phosphotyrosine (p-Tyr) mAb (4G10) was from Upstate

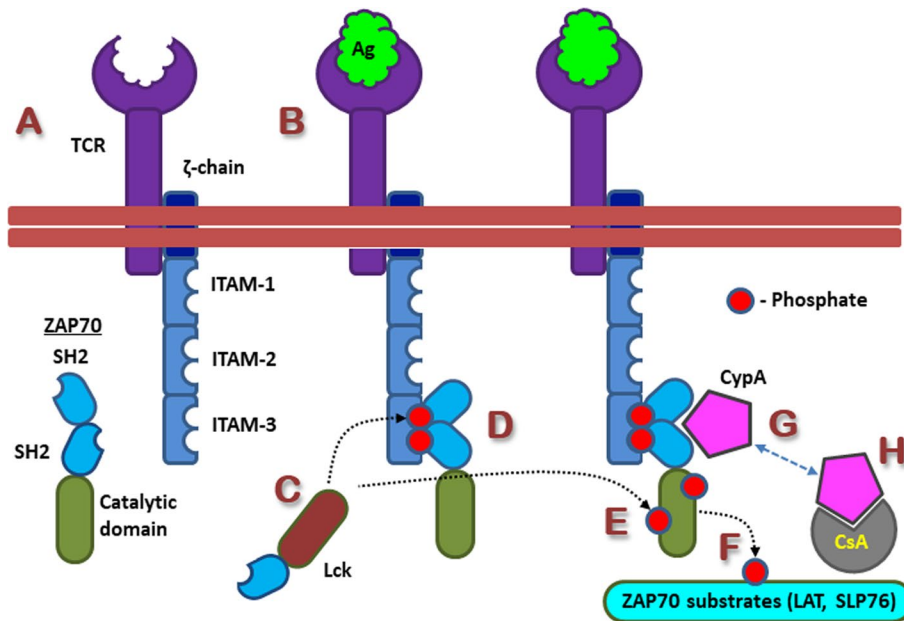


Fig. 6 A schematic model of ZAP70 regulation by CypA. Resting T lymphocytes (A) can be activated by the engagement of their antigen receptors (TCR) with a peptide antigen (Ag) presented on MHC molecules on the surface of an antigen-presenting cell (B). CD4- or CD8-associated Lck is then recruited to the liganded TCR (C) and phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of the CD3 chains, predominantly CD3 ζ . The phosphorylated ITAMs serve as docking sites for the SH2 domains of the cytosolic, inactive, ZAP70 tyrosine kinase (D). Lck-mediated phosphorylation of ZAP70 (E) upregulates the catalytic activity of ZAP70 which then undergoes autophosphorylation

and transphosphorylate TCR downstream effector molecules, including LAT and SLP76. Cyclophilin A (CypA) binding to phosphorylated ZAP70 (G) inhibits ZAP70 catalytic activity, either by steric hindrance of ATP and/or substrate binding, or by modulation of its conformation, leading to downregulation of phosphorylation of downstream substrates (F). Cyclosporin A (CsA) interaction with CypA reverses the effect of CypA on ZAP70 (H), either by reducing the affinity of CypA to ZAP70 and outcompeting CypA binding to ZAP70 or by inhibition of the CypA catalytic activity and reversing the effect of CypA on ZAP70 enzymatic activity

Biotechnology (Lake Placid, NY). Mouse anti-GST, mouse anti-cyclophilin A (CypA), mouse anti-phospho-CD3 ζ (pCD3 ζ ; clone C145.9A), rabbit anti-Lck, rabbit anti-CypA, and goat anti-CD3 ϵ (clone M-20) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti- β actin (clone AC-15) and mouse anti-MBP (clone M-1891) mAbs were from Sigma-Aldrich. Mouse anti-human CD3 ϵ (OKT3) and c-Myc mAbs (clone 9E10) [70] were prepared as previously described. Rabbit anti-CypA, rabbit anti-LAT, rabbit anti-Pin1, rabbit anti-IgG isotype control, goat polyclonal anti-mouse IgG, and anti-hamster IgG Abs were from Abcam (Cambridge, UK). Mouse anti-PKC alpha (PKC α) mAbs were from BD Transduction Laboratories (Lexington, KY). FITC-conjugated goat Abs directed against mouse IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit immunoglobulin Abs were from Amersham Pharmacia Biotech. Alexa FluorTM 488, 546- and 633-conjugated rabbit and goat anti-mouse Abs, and Alexa FluorTM 633-conjugated phalloidin were from Thermo Scientific, Inc. (Waltham, MA). GST-tagged human recombinant ZAP70 was from R&D Systems, Inc.

(Minneapolis, MN). Human recombinant Cyclophilin A (rCypA), human recombinant Pin1 (rPin1), and CytoPainter Cell Staining Reagent were from Abcam (Cambridge, UK). [γ -³²P] ATP was from Perkin Elmer (Waltham, MA).

Animals, cell lines, and culture conditions

Spleen and thymus cells were obtained from 8-wk-old C57BL/6 J female mice that were housed under controlled conditions with 12 h light/dark cycle and free access to food and water. This study was approved in advance by the Ben-Gurion University Institutional Animal Care and Use Committee and conducted in accordance with the Israeli Animal Welfare Act following the guidelines of the Guide for Care and Use of Laboratory Animals (National Research Council 1996). The Animal ethical clearance protocol used for this research is IL-44-08-2017.

Cell lines used include the Jurkat human T cell line (clone E6-1; ATCC[®] TIB-152TM), Jurkat TAg T cells which express the Simian Vacuolating Virus 40 (SV40) T antigen, CypA-deficient Jurkat T cell line (NIH HIV Reagent Program, ARP-10095), ZAP70-deficient Jurkat T cell

line, P116, Lck-deficient Jurkat T cell line, JCaM1.6, TCR-deficient Jurkat T cell line, CH7C17, which was transfected with the influenza hemagglutinin (HA^{307–319}) peptide-specific, DR1-restricted HA1.7 TCR, and the EBV-transformed human B cell line, LG2, which expresses the HLA-DR1 and B7.1 surface receptors. Cells were maintained at a logarithmic growth phase in complete RPMI (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (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-Aldrich)). Cells were grown in 75-cm² growth-area tissue culture flasks (Cellstar, Greiner, Germany) in a humidified atmosphere of 95% air and 5% CO₂, at 37 °C. Jurkat and Jurkat-derived subline cells were stimulated with 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 the indicated time intervals at 37 °C. Mouse T cells were stimulated with anti-CD3ε mAbs (2C11 mAbs by 30-min incubation on ice plus crosslinking with a secondary Ab (goat anti-hamster IgG) for the indicated time intervals at 37 °C.

DNA constructs

A Myc-tagged full-length ZAP70 in pSXR expression vector was prepared and its sequence was verified, as described [30, 71]. GST-tagged human cyclophilin A (hCypA) in pEF expression vector was a gift of Michael Emerman (Fred Hutchinson Cancer Research Center, Seattle, WA) [72]. GST-tagged human ZAP70 SH2N and SH2C domains was a gift from Tony Pawson (Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON). GST-tagged human ZAP70 interdomain B was a gift from Joseph B. Bolen (Bristol-Myers Squibb). The fluorescence resonance energy transfer (FRET)-based biosensor plasmids which are designed to detect ZAP70 activity (ROZA)-XL and ROZA-XL-YF (ROZA-XL in which LAT Tyr191 was replaced by Phe) were gifts of Annemarie Lellouch (INSERM U1067, Marseille, France) [48] (Addgene plasmid # 64194; <http://n2t.net/addgene:64194>; RRID: Addgene_64194).

Nucleofection of Jurkat T cells

For FRET analysis, Jurkat T cells (15 × 10⁶/group) were nucleofected with ROZA-XL or ROZA-XL-YF plasmids (described in Fig. S7) using Bio-Rad Gene Pulser Xcell electroporation system. Briefly, cells were suspended in pre-warmed (37 °C) Mirus Ingenio® electroporation solution, mixed with indicated plasmids (10 µg) in sterile Bio-Rad cuvettes (0.4 cm gap). The cuvettes were then placed in the electroporator and pulsed using the exponential decay pulsing protocol. After electroporation, cells were carefully

transferred to a culture medium-containing tissue culture dish and were incubated at 37 °C for 48 h prior to CsA treatment followed by cell activation and FACS analysis. For protein binding studies, Jurkat T cells (20 × 10⁶/group) were nucleofected with the indicated plasmids (10 µg/cuvette) using the above-mentioned electroporation protocol. At 48 h post-nucleofection, cells were either stimulated or left untreated and subsequently subjected to immunoprecipitation. Unless otherwise indicated, all nucleofection experiments were carried out in triplicates using 3 separate dishes for each point.

Preparation of cell lysates Immunoprecipitation, and GST pulldown

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 Na₃VO₄, 50 mM NaF, 10 mg/ml each of leupeptin and aprotinin, 2 mM AEBSF, and 1% Triton X-100), followed by a 30-min incubation on ice. Lysates were spun down at 13,000 × g for 30 min at 4 °C and the nuclear-free supernatants were subjected to immunoprecipitation.

Immunoprecipitation was performed by pre-adsorption of primary Abs to protein A-, G-, or protein A/G-coupled beads, based on the differential affinities of the proteins to specific Abs, for 2 h at 4 °C. Excess Abs were removed by 3 washes in lysis buffer and Ab-coated beads were incubated with cell lysates for 16 h at 4 °C. Immune complexes were precipitated by centrifugation followed by extensive washing in 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.

Bead-immobilized GST pulldown assay was performed as previously described [30]. Briefly, bead-adsorbed GST or GST fusion proteins (10 µg/sample) were incubated with cell lysates at 4 °C on a rotator for 4 h. The beads which include the bound proteins were eluted by boiling in 2 × Laemmli SDS sample buffer and subjected to SDS-PAGE under reducing conditions, followed by immunoblotting.

Electrophoresis and Immunoblotting

Whole-cell lysates and Ab immunoprecipitates were resolved by electrophoresis either on 8%, 10%, or 12.5% polyacrylamide gels using Bio-Rad Mini-PROTEAN II cells. Proteins from the gel were electroblotted onto nitrocellulose membranes (Schleicher and Schuell) at 100 V for 1 h, using BioRad Mini Trans-Blot transfer cells. After 1 h of blocking with 3% BSA in TBST at 37 °C, the nitrocellulose membranes were incubated in the presence of the indicated primary Abs, followed by incubation with HRP-conjugated secondary Abs. Immunoreactive protein bands

were visualized using an ECL reagent and autoradiography. Whenever required, nitrocellulose membranes were stripped by incubation in stripping buffer (100 mM 2-ME, 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 TBST).

Cell treatment with Cyclosporin A (CsA)

CsA (Sandimmun, 50 mg/ml in oil solution) was diluted in RPMI 1640 culture medium before each experiment. For immunoprecipitation assays, spleen and thymus cells from C57BL/6 J mice and Jurkat T cells (50×10^6 /group) were treated with CsA (0.5 μ M) followed by cell stimulation, lysis, and subsequent immunoprecipitation studies. For immunofluorescent cell staining, spleen and thymus cells from C57BL/6 J mice and Jurkat T cells (2×10^6 /group) were treated with CsA and processed for immunofluorescence as described below. For FRET analysis, ROZA-XL/ROZA-XL-YF-transfected Jurkat T cells (15×10^6 /group) cultured for 48 h were treated in the presence or absence of CsA followed by cell stimulation and flow cytometry as described below.

Fluorescent cell staining and confocal microscopy

C57BL/6 J mouse spleen and thymus cells were treated with anti-CD3 ϵ mAbs (2C11; 30-min incubation on ice) and human Jurkat T cells (2×10^6 /group) were treated with anti-CD3 ϵ mAbs (OKT3; 30-min incubation on ice). Cells were stimulated by crosslinking with a secondary Ab (goat anti-hamster IgG, 1:200 and goat anti-mouse IgG, 1:200, respectively) for 1 min at 37 °C. Cells were plated on poly-L-lysine-coated chamber slides and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT). Cells were washed twice, permeabilized with 0.1% triton-X-100 for 10 min, and blocked with Ab Diluent blocking solution (GBI Labs) for 1 h at RT. Cells were reacted with mouse anti-ZAP70 mAbs and rabbit anti-CypA mAbs diluted in blocking buffer overnight at 4 °C. After washing with PBS, the cells were incubated with Alexa Fluor 546-conjugated anti-mouse Ig and Alexa Fluor 488-conjugated anti-rabbit Ig secondary Abs for 1 h in the dark at RT. In parallel, a control experiment was performed by staining OKT3-stimulated cells with labeled Alexa FluorTM546-conjugated goat anti-mouse secondary Abs and ruled out the possible basal, non-specific staining. Cells were then counterstained with the nuclear stain DAPI diluted in Tris-HCl (pH 7.5) at RT for 5 min. Cells were also stained for tyrosine-phosphorylated proteins using mouse anti-pTyr mAbs (4G10) and a secondary FITC-conjugated anti-mouse Ig mAbs plus counterstain with DAPI. Similarly, in Jurkat T cells and in ZAP70-deficient P116 cells, after the fixation and

permeabilization, cells were immunoreacted with mouse anti-pCD3 ζ and rabbit anti-CypA followed by the incubation with Alexa Fluor 546-conjugated anti-mouse Ig and Alexa Fluor 488-conjugated anti-rabbit Ig secondary Abs and counterstaining with DAPI. For the triple staining procedure in Jurkat T cells which involved the CD3 receptor, the cells were stained using the primary Abs: mouse anti-ZAP70, rabbit anti-CypA, and goat anti-CD3 ϵ , followed by incubation with the respective secondary antibodies: Alexa Fluor 633-conjugated anti-mouse Ig, Alexa Fluor 488-conjugated anti-rabbit Ig, and Alexa Fluor 546-conjugated anti-goat Ig, plus counterstaining with DAPI. The coverslips were mounted on slides using DAKO mounting medium and imaged by Olympus FluoView FV1000 laser-scanning confocal microscope. Immunofluorescence images represent single 2-D confocal sections. The extent of colocalization of CypA and ZAP70 was quantified using the JACoP ImageJ plugin [73].

Conjugate formation assay and immunofluorescence

Immunological synapse studies were performed as previously described [74]. Briefly, Jurkat-CH7C17 T cells, which express the influenza hemagglutinin (HA³⁰⁷⁻³¹⁹)-specific TCR, were incubated with peptide-loaded antigen-presenting cells (APCs), LG2, at a ratio of 1:2 at 37 °C for 5 min. APCs were pre-loaded with 200 μ g/ml of HA³⁰⁷⁻³¹⁹ peptide, or an inactive HA peptide, in which Lys316 was replaced by Glu (K316E), for 3 h at 37 °C. To enable the discrimination between the two cell types, LG2 cells were prestained using CytoPainter Cell Staining Reagent prior to the cell mixing. Following 5 min of co-incubation, the cells were fixed, permeabilized, and analyzed by confocal microscopy. Immunofluorescence staining was performed using the primary Abs: mouse anti-ZAP70 and rabbit anti-CypA, followed by incubation with Alexa Fluor 633-conjugated phalloidin and: Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 546-conjugated anti-rabbit IgG. CytoPainter was detected at 405 nm (Olympus FluoView FV1000 laser-scanning confocal microscope).

Fluorescence resonance energy transfer analysis by FACS

Fluorescence resonance energy transfer (FRET) analysis of live cells was performed by flow cytometry as previously described [65]. Briefly, Jurkat T cells (15×10^6 /group) transfected with ROZA-XL or ROZA-XL-YF plasmids were cultured for 48 h and then split into two identical groups that were cultured for an additional 24 h in the presence or absence of CsA. After washing with PBS (without Ca²⁺ and Mg²⁺), cells (2×10^6 /group) were placed in Eppendorf

tubes and resuspended in phenol red-free RPMI 1640 plus 1 mM HEPES buffer. Following the addition of anti-CD3ε mAbs (OKT3) and crosslinking with a goat anti-mouse IgG secondary Ab for the specified time intervals at 37 °C, the cell activation was immediately terminated by the addition of cold PBS (without Ca²⁺ and Mg²⁺), and cell pellets were resuspended in FACS buffer (2% FBS, 1 mM EDTA, and PBS (without Ca²⁺ and Mg²⁺)) followed by live data acquisition on a FACS Canto II device (BD Biosciences). Excitation of CFP was at 405 nm and emission was detected simultaneously in the CFP and YFP emission windows. A shift in the ratio of YFP/CFP emission intensities reflects the change in FRET efficiency. Due to the unique structure of ROZA-XL, higher values of FRET efficiency were observed in resting cells, where ZAP70 was inactive, while ZAP70 activation resulted in reduced FRET efficiency values (see ref. [48]). The data were analyzed using FlowJo v10.7 software.

In vitro kinase assays

ZAP70 kinase assay: Jurkat T cells were activated using OKT3 mAbs for 1 min at 37 °C. ZAP70 was immunoprecipitated from whole-cell lysates using protein G-agarose bead-immobilized mouse anti-ZAP70 Abs (Biolegend), and the immunoprecipitates were washed four times with Triton X-100-containing lysis buffer and once with a ZAP70 kinase buffer (1 M Tris, 3 M NaCl, 100 mM MnCl₂). Samples were pre-incubated in the absence or presence of the indicated amounts of catalytically active recombinant human CypA in kinase buffer for indicated time intervals at 37 °C. The samples were resuspended in a 100 μl kinase reaction mixture containing kinase buffer plus [γ -³²P]-ATP (5 μCi) and incubated for 10 min at 37 °C with gentle shaking. Reactions were terminated by the addition of 5 × sample buffer and boiling for 5 min, followed by SDS-PAGE on 10% acrylamide gels under reducing conditions. Samples were then transferred to nitrocellulose membranes that were developed by autoradiography.

PKCα kinase assay: Jurkat T cells were activated using PMA for 30 min at 37 °C. PKCα was immunoprecipitated from whole-cell lysates using protein A/G-agarose bead-immobilized mouse anti-PKCα Abs (BD Transduction Laboratories), and the immunoprecipitates were washed four times with Triton X-100-containing lysis buffer and once with a PKC kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, and 0.1 mM EGTA). Samples were pre-incubated in the absence or presence of the indicated amounts of catalytically active recombinant human Pin1 in kinase buffer for indicated time intervals at 37 °C. The samples were then resuspended in a 100 μl kinase reaction mixture containing kinase buffer and 50 μg/μl phosphatidylserine, 0.3 mM CaCl₂, 100 nM PMA, [γ -³²P]-ATP (5 μCi) and MBP (5 μg) as a substrate and incubated for 30 min at 32 °C with

gentle shaking. Reactions were terminated by the addition of 5 × sample buffer and boiling for 5 min, followed by SDS-PAGE on 10% acrylamide gels under reducing conditions. Samples were then transferred to nitrocellulose membranes that were developed by immunoblot and autoradiography.

Statistical analysis

Statistical analyses were carried out using either MS office Excel 365 software Version 2107 or GraphPad Prism software Version 7.00. Microscopy data was quantified using the ImageJ plugin, JACoP, and FRET analysis with FlowJo Version 10.7. Statistical significance of differences between groups of averaged data points were assessed using Student's unpaired *t*-test.

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Author contributions NI conceived the study, designed experiments, and supervised the project; NPA, AKA, AM, JS, and PRN carried out the experiments; ZS, AB, and NI analyzed the data; EL provided essential tools for the study; NPA, AKA, and NI wrote the manuscript. All authors commented on the previous versions of the manuscript. All authors have read and approved the final manuscript and have given consent for publishing the work.

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Data availability The data that support the findings of this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethics approval This study was approved in advance by the Ben-Gurion University Institutional Animal Care and Use Committee and conducted in accordance with the Israeli Animal Welfare Act following the guidelines of the Guide for Care and Use of Laboratory Animals (National Research Council 1996). The Animal ethical clearance protocol used for this research is IL-44-08-2017.

Consent to participate Not Applicable as human subjects were not involved in the study.

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