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Cyclophilin A Regulates TCR Signal Strength in CD4+ T Cells via a Proline-Directed Conformational Switch in Itk

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Summary

Cyclophilin A (CypA/Ppia) is a peptidyl-prolyl isomerase (PPIase) that binds the immunosuppressive drug cyclosporine. The resulting complex blocks T cell function by inhibiting the calcium-dependent phosphatase calcineurin. To identify the native function of CypA, long suspected of regulating signal transduction, we generated mice lacking the *Ppia* gene. These animals develop allergic disease, with elevated IgE and tissue infiltration by mast cells and eosinophils, that is driven by CD4+ T helper type II (Th2) cytokines. *Ppia*$^{-/-}$ Th2 cells were hypersensitive to TCR stimulation, a phenotype consistent with increased activity of Itk, a Tec family tyrosine kinase crucial for Th2 responses. CypA bound Itk via the PPIase active site. Mutation of a conformationally heterogeneous proline in the SH2 domain of Itk disrupted interaction with CypA and specifically increased Th2 cytokine production from wild-type CD4+ T cells. Thus, CypA inhibits CD4+ T cell signal transduction in the absence of cyclosporine via a regulatory proline residue in Itk.

Introduction

Peptidyl-prolyl isomerases (PPIases) catalyze the *cis-trans* interconversion of peptide bonds N-terminal to proline, an activity that has been extensively studied in vitro by using model peptides or denatured protein substrates (Fischer et al., 1984). Three structurally distinct PPIase families have been identified: the cyclophilins, the FKBPs, and the parvulins (Gothel and Marahiel, 1999). In parallel with the distribution of Hsp70 family members, PPIases are found in all eubacteria, in a few archaebacteria, and in all eukaryotes, consistent with a global role in the folding of nascent proteins in vivo (Hartl and Hayer-Hartl, 2002). PPIases also bind folded proteins, raising the possibility that the function of mature proteins is regulated via catalysis of peptidyl-prolyl isomerization. Alternatively, PPIases may regulate protein-protein interactions or allosteric changes in a non-catalytic fashion via proline-directed binding.

Cyclophilin A (CypA), the prototypical member of the cyclophilin family, is expressed in the cytoplasm and nucleus of most metazoan cell types (Gothel and Marahiel, 1999). It is a globular, eight-stranded β barrel with a solvent-exposed hydrophobic pocket that is the binding site for proline-containing peptides as well as the enzymatic active site (Ke et al., 1991; Mikol et al., 1993). The mouse genome encodes at least 15 cyclophilin family members, most of which are distinguished from CypA by terminal extensions bearing motifs for subcellular targeting or intermolecular interaction. Several studies suggest that CypA influences protein trafficking in cells—in some cases as a constituent of complexes containing chaperone proteins (Ansari et al., 2002; Brown et al., 2001; Huang et al., 2002; Uittenbogaard et al., 1998).

CypA was discovered due to its subnanomolar affinity for cyclosporine (Handschumacher et al., 1984), the immunosuppressant that revolutionized organ transplantation. Although cyclosporine binds the cyclophilin hydrophobic pocket and disrupts PPIase activity, this effect is thought irrelevant for immunosuppression. Rather, the cyclosporine-cyclophilin complex forms a composite surface that binds and inhibits calcineurin (Friedman and Weissman, 1991; Liu et al., 1991), a serine-threonine phosphatase required for cytokine induction in response to TCR stimulation. Here, we show that *Ppia*$^{-/-}$ mice develop an allergic condition associated with increased Th2 cytokine production. CypA was found to interact biochemically and functionally with Itk, a tyrosine kinase required for Th2 function (Fowell et al., 1999), via a conformationally heterogeneous proline in the Itk SH2 domain.

Results

CypA Is Not Essential in Mammals

Mice homozygous for a null allele of *Ppia*, the gene encoding CypA, were generated in a mixed MF1 × 129 genetic background (Figure 1A). Of 694 pups assessed 3 weeks after birth, 27% were *Ppia*$^{+/+}$, 52% *Ppia*$^{+/-}$, and 21% *Ppia*$^{-/-}$, indicating a slight loss of viability in association with the *Ppia*$^{-/-}$ genotype ($p < 0.05$). Viability was compromised further after backcrossing ten generations into the 129 strain (33% *Ppia*$^{+/+}$, 52% *Ppia*$^{+/-}$, and 15% *Ppia*$^{-/-}$; $n = 513$). In either background, however, adult *Ppia*$^{-/-}$ animals appeared robust and suffered no obvious decrease in life span. Thus, despite high-level expression in all tissues (Ryffel et al., 1991), CypA is not essential in mammals.

Allergic Pathology in *Ppia*$^{-/-}$ Mice

30% of *Ppia*$^{-/-}$ mice spontaneously developed swelling and erythema of the eyelid (Figure 1B). Blepharitis first appeared 3 months after birth, generally worsened with

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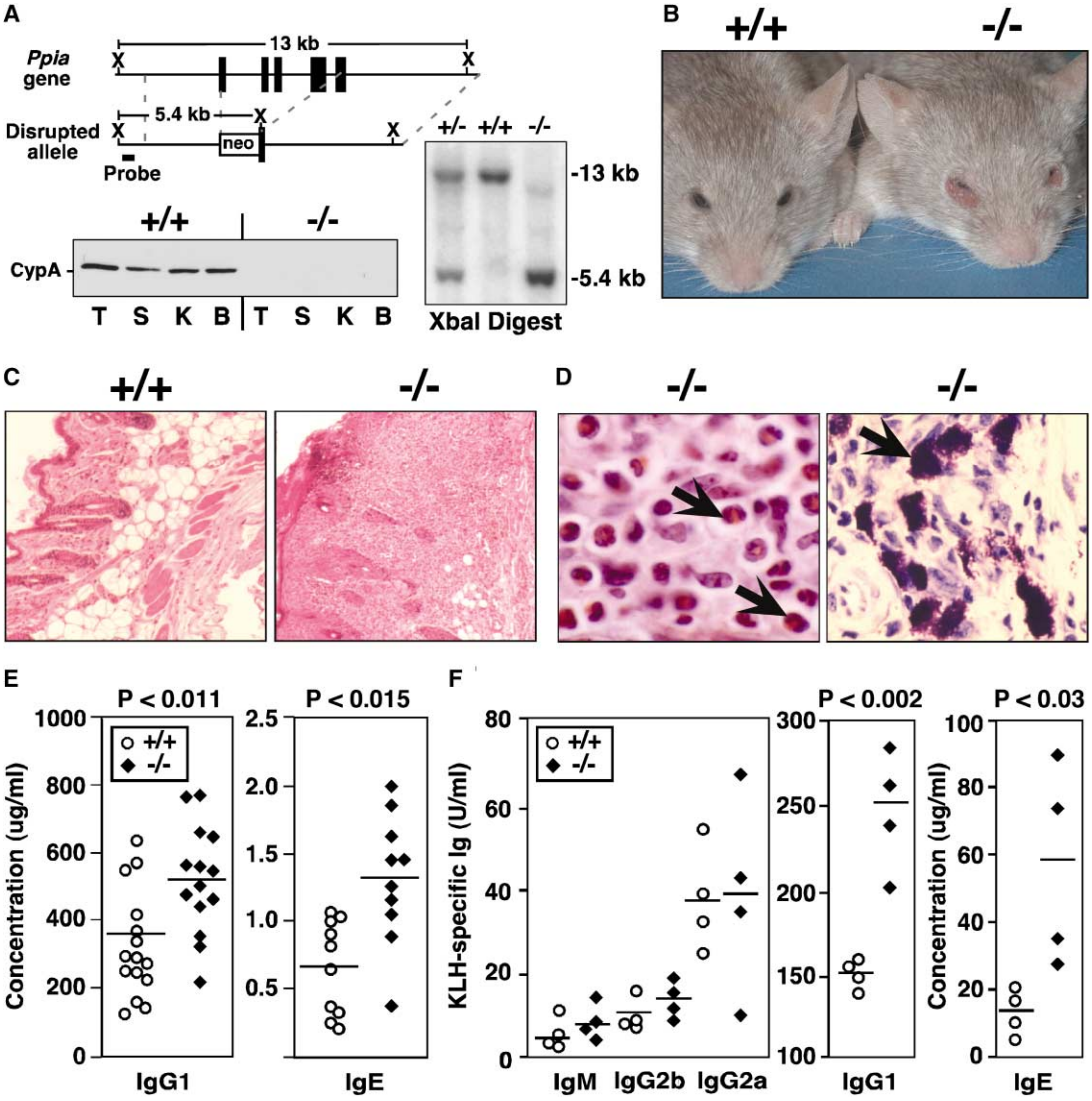

Figure 1. Allergic Blepharitis in *Ppia*$^{-/-}$ Mice

(A) *Ppia* structure showing all five exons and the disrupted allele. X, XbaI. Southern blot of genomic DNA (right) hybridized with probe shown in schematic. Immunoblot (bottom left) of thymus, spleen, kidney, and brain lysates (as indicated) probed with anti-CypA antibody.

(B) Eyelid inflammation in *Ppia*$^{-/-}$ mice.

(C) Hematoxylin/eosin-stained section showing cellular infiltrates in *Ppia*$^{-/-}$ eyelid (40×).

(D) *Ppia*$^{-/-}$ eyelid stained with congo red (left; 600×; arrows, eosinophils) or toluidine blue (right, 400×; arrow, mast cell).

(E) Concentrations of total serum IgG1 and IgE in unimmunized mice.

(F) Relative antigen-specific antibody concentrations in sera from mice immunized with KLH. Concentration of total IgE after KLH immunization.

age, and was never observed in *Ppia*$^{+/+}$ or *Ppia*$^{+/-}$ mice caged with affected animals. Histologic examination revealed dense infiltrates of mononuclear cells, eosinophils, and mast cells in the subepithelial stroma of the conjunctivae and skin (Figures 1C and 1D). Eosinophilic infiltrates were sometimes observed in the heart, liver, pancreas, and bone marrow of animals with severe blepharitis (data not shown). A strikingly similar allergic condition has been reported in transgenic mice overexpressing IL-4 (Tepper et al., 1990) and in animals lacking the calcineurin-regulated transcription factors NFATp/1/c2 and NFAT4/c3 (Ranger et al., 1998b), which likewise overproduce IL-4.

Via effects on class switching, IL-4 specifically increases production of IgG1 and IgE immunoglobulin isotypes (Snapper et al., 1988). Total serum IgG1 and IgE were both significantly elevated in *Ppia*$^{-/-}$ mice (Figure 1E). Other isotypes were normal (see Supplemental Figure S1A online at http://www.immunity.com/cgi/content/full/21/2/189/DC1/). The ability of *Ppia*$^{-/-}$ mice to raise specific antibodies was tested by immunizing with KLH or TNP-Ficoll. In both cases, antigen-specific IgG1 was elevated (Figure 1F and Supplemental Figure S1B). Total IgE after immunization was also significantly greater in *Ppia*$^{-/-}$ mice (Figure 1F). To distinguish between an intrinsic B cell abnormality and secondary effects on B cells due to increased IL-4 production, B cells were purified and stimulated in isolation with IL-4

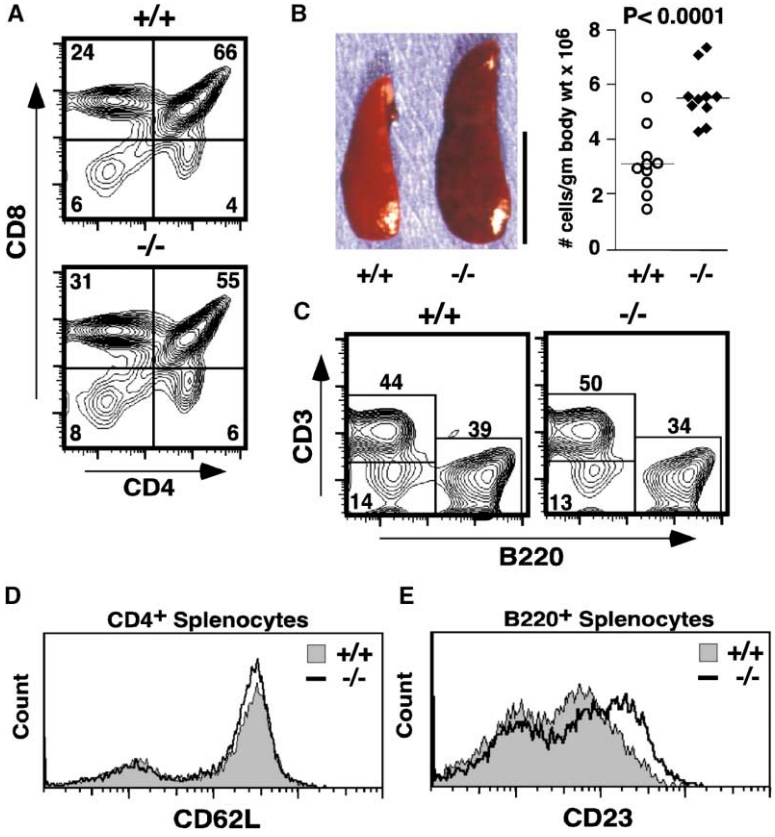

Figure 2. Immune System Abnormalities in *Ppia*$^{-/-}$ Mice

(A) Flow cytometric analysis of thymocytes showing increased percentage of single-positive CD4$^+$ and CD8$^+$ T cells in *Ppia*$^{-/-}$ mice.
(B) Spleens from 10-week-old *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ siblings. Size bar, 1 cm. Number of splenocytes from 10- to 12-week-old mice normalized for body weight.
(C) Flow cytometric analysis of splenocytes showing a slightly increased percentage of CD3$^+$ cells in adult *Ppia*$^{-/-}$ mice.
(D) CD62L expression on CD4$^+$ splenocytes.
(E) Expression of the low-affinity IgE receptor (CD23) on B220$^+$ splenocytes.

and other factors. *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ B cells secreted similar amounts of all antibody isotypes (Supplemental Figure S1C). Together with the allergic pathology described above, these findings suggest that IL-4 is elevated in these mice.

Thymocyte Development and Splenomegaly in *Ppia*$^{-/-}$ Mice

Cell yield from *Ppia*$^{-/-}$ thymus was normal. Flow cytometric analysis of thymocytes from *Ppia*$^{-/-}$ mice revealed increased percentages of both CD4 and CD8 single-positive cells (Figure 2A). Expression of TCRβ, the activation markers CD25 and CD69, or CD5, a marker that is modulated in direct proportion to TCR signal intensity (Azzam et al. 1998), was not significantly altered on *Ppia*$^{-/-}$ thymocytes (data not shown), indicating that T cell development was not grossly perturbed. *Ppia*$^{-/-}$ mice exhibited splenomegaly, and mean splenocyte yield increased roughly 80% (Figure 2B). Spleen architecture was normal with no evidence of clonal expansion (data not shown). Consistent with the changes in single-positive cells in the thymus, the percentage of T cells among *Ppia*$^{-/-}$ splenocytes was slightly increased (Figures 2C). Among either CD4$^+$ or CD8$^+$ T cells, a minor increase in naive cells was detected as based on expression of CD62L and CD45RB (Figure 2D) with no apparent difference in activation state as judged by expression of CD25 and CD69 (data not shown). The proportions of immature, transitional, and mature B cells were unaltered based on expression of surface IgM and IgD (Supplemental Figure S1D). However, *Ppia*$^{-/-}$ B cells expressed higher levels of CD23 (Figure 2E), which is upregulated in response to IL-4 (Erb et al., 1994; Waldschmidt et al., 1989).

Increased IL-2 Expression by Naive *Ppia*$^{-/-}$ CD4$^+$ T Cells

Since CD4$^+$ helper T cells are a major source of IL-4 in vivo, CD4$^+$ T cell function was assessed in detail. In response to immobilized anti-CD3 and anti-CD28 antibodies, proliferation of naive *Ppia*$^{-/-}$ CD4$^+$ T cells was slightly increased (Figure 3A) and *Ppia*$^{-/-}$ cells produced 3- to 5-fold more IL-2 than *Ppia*$^{+/+}$ cells (Figure 3B). In contrast, IL-2 secretion was normal when proximal TCR signaling events were bypassed by stimulation with PMA and ionomycin. Expression of CD25, the inducible component of the high affinity IL-2 receptor, was normal on *Ppia*$^{-/-}$ cells (data not shown), suggesting that increased IL-2 in the supernatant was not due to decreased consumption.

Ppia$^{-/-}$ Memory/Effector CD4$^+$ T Cells Generated In Vivo Overproduce IL-4

To determine if in vivo capacity to produce IL-4 is elevated in *Ppia*$^{-/-}$ mice, memory/effector CD4$^+$ T cells were enriched directly from spleen and immediately stimulated to induce cytokine production (Figure 3C). While *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ cells secreted roughly the same amount of IFN-γ, *Ppia*$^{-/-}$ cells produced larger amounts of IL-4. To assess antigen-specific responses, CD4$^+$ T cells were isolated after immunization with KLH or DNP-KLH. In response to antigen, *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$

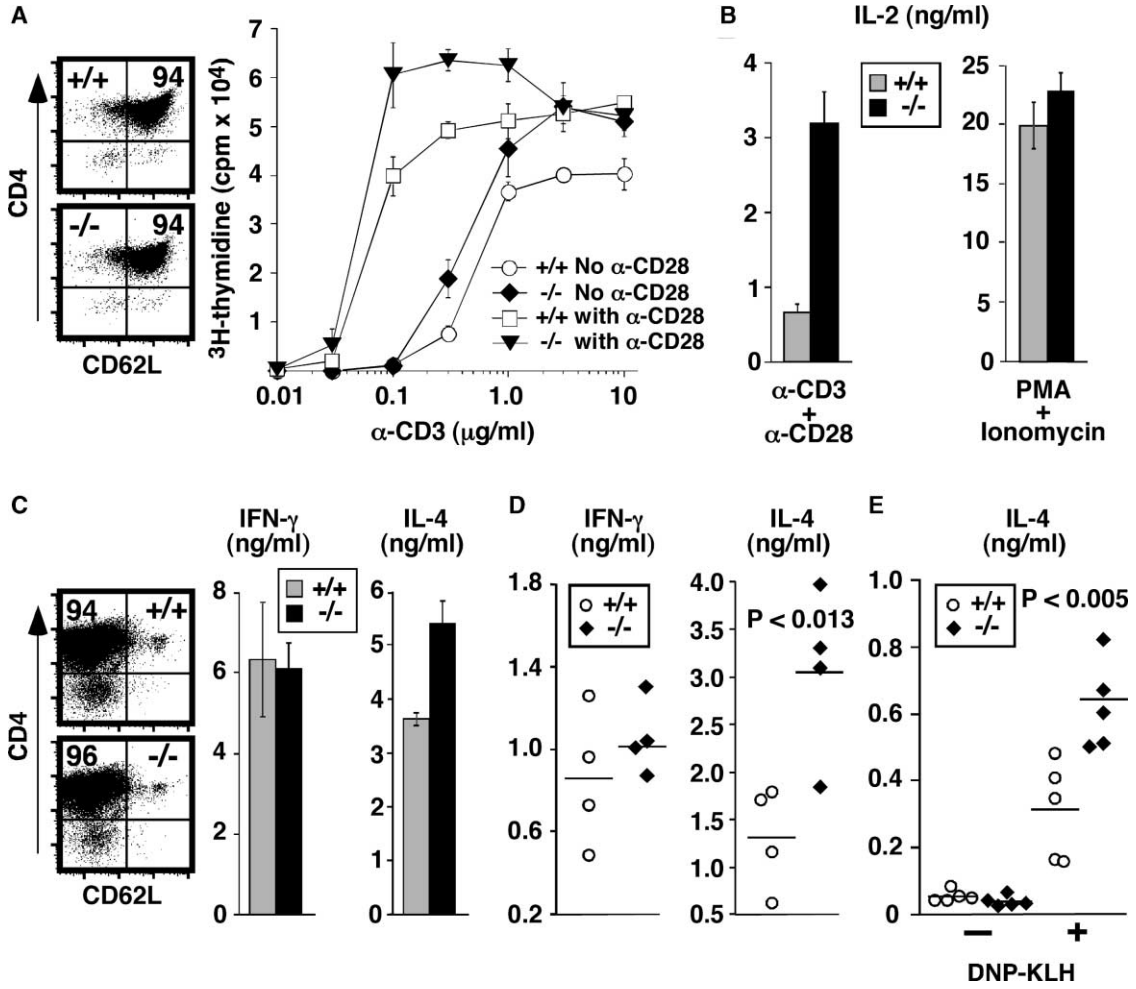

Figure 3. Increased IL-2 and IL-4 Production by *Ppia*$^{-/-}$ CD4^{+} T Cells

(A) Histograms show naive CD4^{+} T cells (CD62L^{Hi}) isolated from spleen. Graph shows proliferative response of these cells to plates coated with the indicated antibodies.

(B) IL-2 concentration in supernatant 48 hr after stimulation of naive CD4^{+} T cells.

(C) Memory/effector CD4^{+} T cells (CD62L^{Lo}) isolated from spleen were stimulated for 2 days on plates coated with anti-CD3 antibody. Bar graphs show concentrations of IFN-γ and IL-4 in supernatant.

(D) CD4^{+} T cells isolated from mice immunized with KLH were stimulated in vitro with antigen and APCs. IFN-γ and IL-4 concentrations in supernatant are shown.

(E) IL-4 concentrations as in (D) except that the antigen was DNP-KLH.

cells secreted similar amounts of IFN-γ, but *Ppia*$^{-/-}$ cells produced more IL-4 (Figures 3D and 3E).

In Vitro-Differentiated *Ppia*$^{-/-}$ CD4^{+} T Cells Overexpress Th2 Cytokines

To see if increased IL-4 production is intrinsic to *Ppia*$^{-/-}$ CD4^{+} T cells, in vitro response to factors that promote T helper cell differentiation was evaluated. Naive CD4^{+} cells were stimulated with anti-CD3 and anti-CD28 antibodies under unbiased conditions or those promoting either Th1 or Th2 differentiation. Cells were harvested after 1 week and stimulated again with anti-CD3 antibody to elicit effector cytokine production. Regardless of culture conditions, *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ cells secreted similar amounts of IFN-γ (Figure 4A). *Ppia*$^{-/-}$ cells differentiated under unbiased or Th2 conditions produced at least three times more IL-4 (Figure 4A). Global expression profiles of Th2-differentiated *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ cells were compared by using Affymetrix GeneChips; other than Th2 cytokines, no differentially regulated genes were identified (data not shown).

To determine if the absence of CypA is sufficient to explain increased IL-4 production, CypA expression in *Ppia*$^{-/-}$ cells was reconstituted by retroviral transduction (Figure 4B). Naïve CD4^{+} T cells were stimulated under Th2-inducing conditions and infected with either a retroviral vector that expresses CypA and GFP or a control vector expressing GFP alone. Transduction efficiency with either vector was >95% (Supplemental Figure S2A). Immunoblot analysis confirmed that CypA expression was restored in *Ppia*$^{-/-}$ cells by the CypA-encoding vector (Figure 4B). As expected, after infection with the GFP control vector, *Ppia*$^{-/-}$ cells produced more IL-4 than *Ppia*$^{+/+}$ cells. In contrast, IL-4 secretion

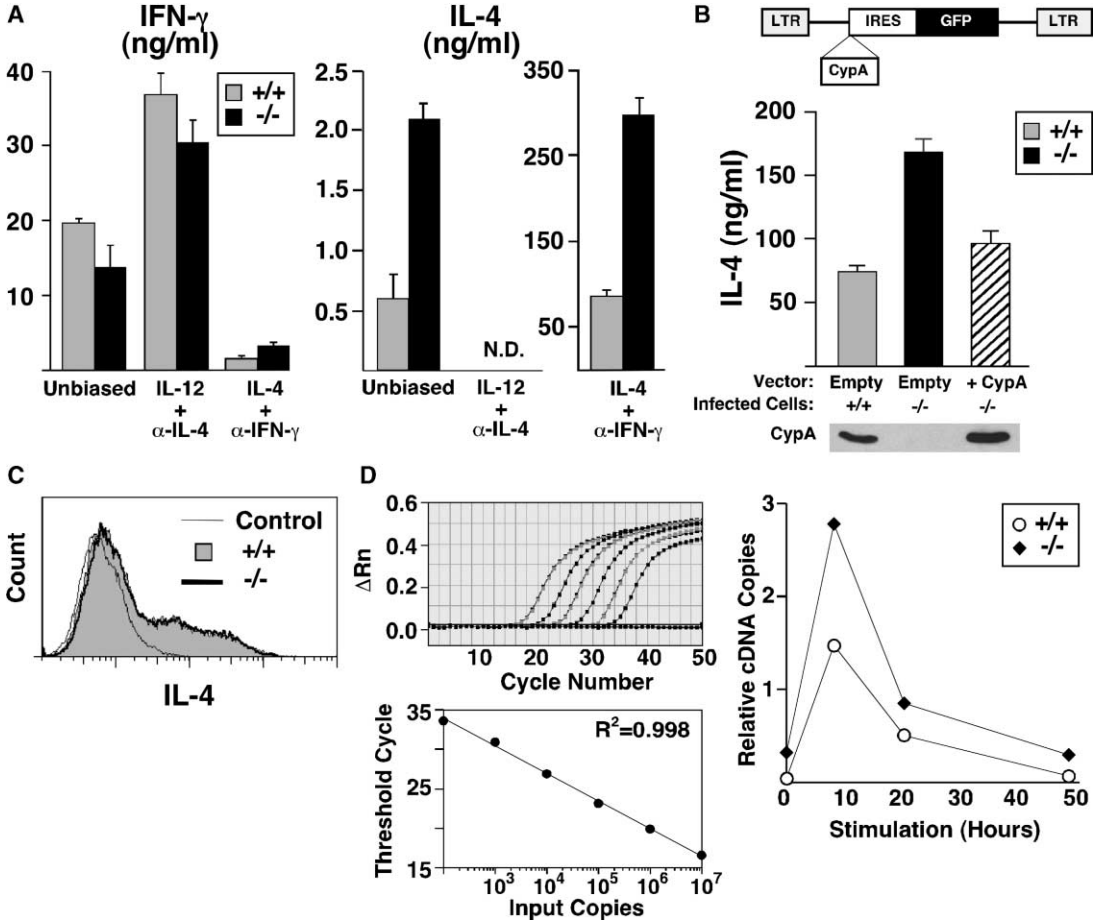

Figure 4. Increased IL-4 Expression Is Intrinsic to *Ppia*$^{-/-}$ CD4^{+} T Cells

(A) Naive CD4^{+} T cells were stimulated for 7 days under the indicated conditions, harvested, and stimulated again. After 48 hr, IL-4 and IFN-γ concentrations in culture supernatant were determined. ND, not detected. Results shown are representative of five independent experiments.

(B) Increased IL-4 is attributable to the lack of *Ppia*. Naive CD4^{+} T cells were stimulated under Th2-biasing conditions and infected with a control retroviral vector or a vector expressing CypA (top). Cells were harvested and stimulated again, and IL-4 was measured. Immunoblot of infected cell lysates probed with anti-CypA antibodies (bottom).

(C) Th2-differentiated cells produced and stimulated as in (A) were analyzed by flow cytometry for cell-associated IL-4.

(D) IL-4 mRNA from Th2 effector cells 8, 20, and 48 hr poststimulation was quantified by real-time RT-PCR and normalized for HPRT cDNA (right). Product amplification (upper) and standard curve (lower) are shown for purified IL-4 template.

by *Ppia*$^{-/-}$ cells transduced with the CypA vector was similar to that from *Ppia*$^{+/+}$ cells infected with the control vector. Thus, greater IL-4 production by *Ppia*$^{-/-}$ CD4^{+} cells can be attributed to the absence of CypA expression.

No significant differences in proliferation or apoptosis of *Ppia*$^{-/-}$ CD4^{+} effector T cells were detected (data not shown). Intracellular staining for IL-4 indicated that *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ cells differentiated into Th2 effectors with similar efficiencies (Figure 4C). The steady-state level of IL-4 mRNA was higher in *Ppia*$^{-/-}$ cells than in *Ppia*$^{+/+}$ cells (Figure 4D). These results indicate that greater IL-4 secretion by *Ppia*$^{-/-}$ CD4^{+} T cells is not due to increased numbers of producer cells but, rather, to higher expression in cells competent to produce this cytokine.

Histologic examination of inflamed tissues from *Ppia*$^{-/-}$ mice revealed large numbers of mast cells (Figure 1D), another potential source of elevated IL-4. Mast cells were generated from bone marrow by in vitro culture with IL-3. The rates of proliferation and differentiation, as monitored by the cell surface expression of Fcε-Receptor and c-Kit, were not altered by CypA deficiency (Supplemental Figure S2B). IL-2 and IL-4 production by *Ppia*$^{-/-}$ mast cells stimulated by crosslinking the Fcε-Receptor was normal (Supplemental Figure S2C). These results indicate that the allergic pathology in *Ppia*$^{-/-}$ mice likely results from increased IL-4 production by CD4^{+} T cells.

Ppia$^{-/-}$ Memory Th2 Cells Are Hypersensitive to TCR Stimulation

To generate Th2 memory cells, Th2-differentiated effector cells were cultured without TCR stimulation or cytokine addition for 7–14 days (Figure 5A). Cell size and CD25 expression decreased, but high-level CD44 expression was maintained, as is characteristic of unstimulated memory cells (Hu et al., 2001). CD3 and CD28

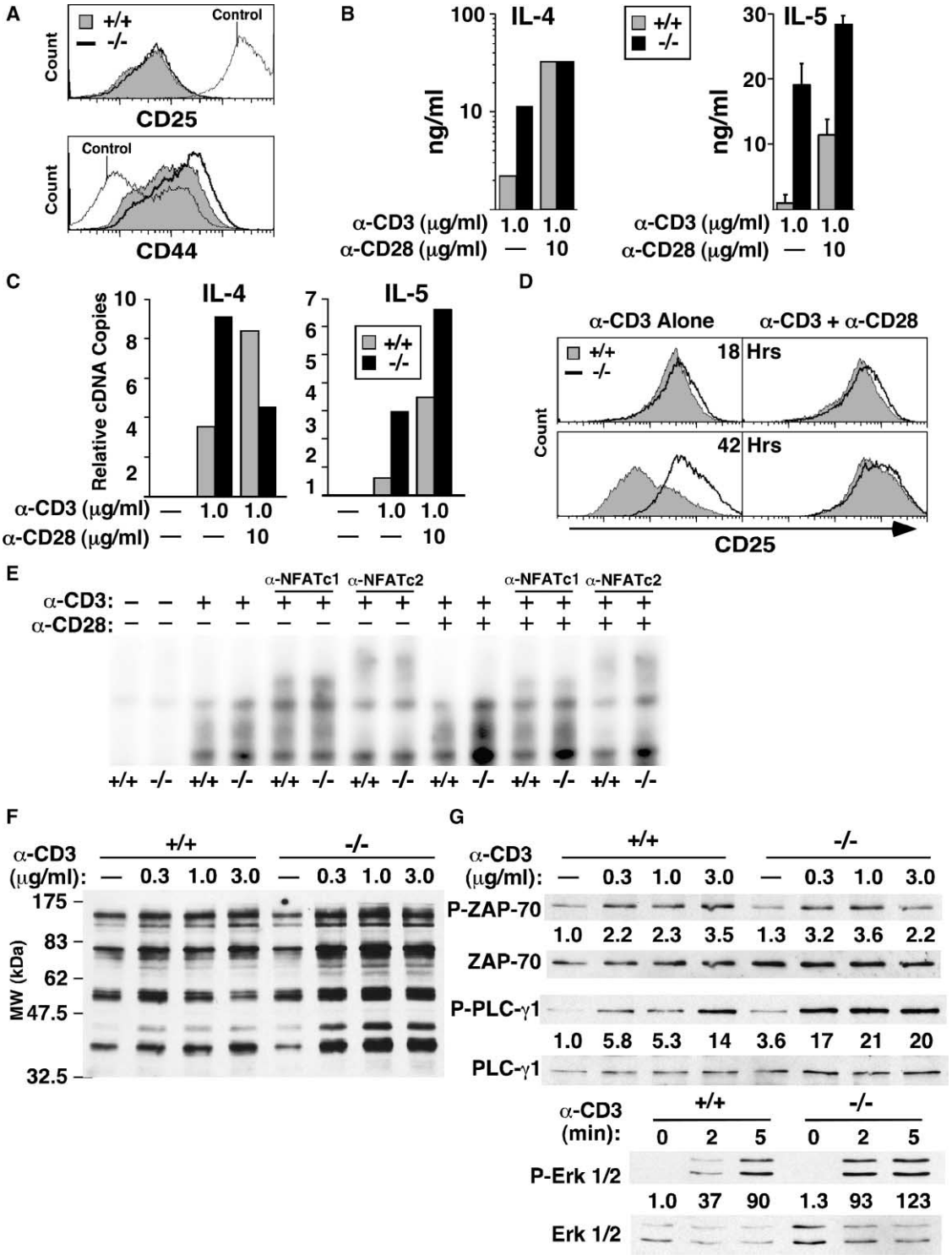

Figure 5. *Ppia*$^{-/-}$ Th2 Memory Cells Are Hypersensitive to TCR Stimulation

(A) Flow cytometric analysis of CD25 and CD44 expression on rested Th2 cells. Control for CD25 is unrested Th2 effector cells. Control for CD44 is CD4^{+} T cells directly from spleen.

(B) IL-4 and IL-5 concentrations in supernatant 48 hr after stimulation of memory Th2 cells on plates coated with the indicated antibodies.

(C) IL-4 and IL-5 mRNA from memory Th2 cells stimulated as indicated for 7 hr and measured as in (4D).

(D) Flow cytometric analysis of CD25 expression on memory Th2 cells stimulated on plates coated with anti-CD3 (1.0 μg/ml) with or without anti-CD28 (10 μg/ml) for the indicated duration.

(E) Electrophoretic mobility-shift assays for NFAT DNA binding activity using nuclear extracts from cells stimulated for 16 hr on plates coated with anti-CD3 (1.0 μg/ml) alone or anti-CD3 (1.0 μg/ml) and anti-CD28 (10 μg/ml). Supershifts were performed with the indicated NFAT antibodies.

(F) Phosphotyrosine immunoblot of lysates from memory Th2 cells incubated for 5 min with magnetic beads coated with the indicated concentrations of anti-CD3 antibody.

(G) Lysates from (F) probed in immunoblots with the indicated antibodies. Numbers indicate relative signal intensity of the phosphorylated proteins.

expression were similar on *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ cells (data not shown), but CD44 expression was consistently higher on *Ppia*$^{-/-}$ cells, perhaps reflecting a more differentiated state. *Ppia*$^{-/-}$ Th2 memory cells secreted 5-fold more IL-4 and 10-fold more IL-5 than *Ppia*$^{+/+}$ cells when stimulated with anti-CD3 alone, but addition of anti-CD28 normalized IL-4 and lessened the difference for IL-5 (Figure 5B). Differences in secretion were reflected in mRNA levels for these cytokines (Figure 5C). IL-13 mRNA was also elevated with a pattern resembling that of IL-5 (data not shown). CD25 upregulation after stimulation with anti-CD3 was the same on *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ cells 18 hr after stimulation (Figure 5D). By 42 hr, CD25 expression had been downregulated on *Ppia*$^{+/+}$ cells but remained high on *Ppia*$^{-/-}$ cells, indicating that signaling events were prolonged in *Ppia*$^{-/-}$ cells. No differences in CD25 expression were observed when the stimulus was increased by including anti-CD28 (Figure 5D) or by using higher concentrations of anti-CD3 (Supplemental Figure S2D).

Electrophoretic mobility shift assays with nuclear extracts from memory Th2 cells stimulated with anti-CD3 showed that NFAT DNA binding activity was modestly increased in *Ppia*$^{-/-}$ cells (Figure 5E). Supershift analysis showed that the increase was due to more DNA bound NFATc/2/c1, a key regulator of IL-4 expression (Ranger et al., 1998a, 1998b; Yoshida et al., 1998). Increased NFAT DNA binding activity in extracts from *Ppia*$^{-/-}$ cells was also observed after stimulation with anti-CD3 and anti-CD28 (Figure 5E), but this was due to more NFATp/1/c2 in the complexes. NFκB DNA binding activity was the same in extracts from *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ cells regardless of the stimulus used (Supplemental Figure S2E). No difference in NFAT binding activity was seen when cells were stimulated with PMA and ionomycin (Supplemental Figure S2F) or with a titration of ionomycin to modulate the magnitude of calcium release (Supplemental Figure S2G). These results indicate that the function of molecules upstream of calcineurin in the TCR signaling pathway are altered in *Ppia*$^{-/-}$ cells.

TCR engagement initiates signaling cascades controlled in part by tyrosine kinases (Weiss and Littman, 1994). Prior to stimulation, tyrosine phosphorylated proteins in *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ cells were similar, but after TCR stimulation significantly greater phosphorylation of several proteins was observed in *Ppia*$^{-/-}$ cells (Figure 5F). Phosphorylation of ZAP-70, a tyrosine kinase regulated by TCR-proximal events, was similar in *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ cells (Figure 5G). Phosphorylation of PLC-γ, which is controlled by factors downstream of ZAP-70, was 3- to 4-fold higher in *Ppia*$^{-/-}$ cells than in *Ppia*$^{+/+}$ cells (Figure 5G). Phosphorylation of ERK-MAPK was also increased in *Ppia*$^{-/-}$ cells (Figure 5G). These results demonstrate that *Ppia*$^{-/-}$ memory Th2 cells are hypersensitive to TCR stimulation and suggest that CypA regulates a component of TCR signaling that promotes activation of PLC-γ.

A Conformationally Heterogenous Proline in the SH2 Domain of Itk Is Required for Interaction with CypA

The hypersensitivity of *Ppia*$^{-/-}$ CD4^{+} T cells to stimulation with anti-CD3, but not with PMA and ionomycin (Figure 3B; Supplemental Figures S2F and S2G), suggested that CypA represses a factor linking TCR engagement to protein kinase C and calcineurin. Attempts to detect an interaction between CypA and several signaling molecules, including calcineurin and NFATs, were unsuccessful (data not shown). The nonreceptor tyrosine kinase Itk became a prime candidate for CypA-mediated repression when it was discovered that the mature, folded SH2 domain of Itk undergoes a profound conformational change in response to peptidyl-prolyl isomerization at Proline 287 and that CypA inhibits Itk autokinase activity (Brazin et al., 2002; Mallis et al., 2002). The hypothesis that CypA represses Itk activity is consistent with increased activation of PLC-γ and ERK MAPK in *Ppia*$^{-/-}$ Th2 cells since both signaling molecules are activated by Itk (Liu et al., 1998; Miller and Berg, 2002a).

To test for interaction between CypA and Itk, retroviral vectors were generated that express either wild-type Itk or an Itk mutant, P287G, which is locked in the *trans* conformation and does not undergo isomerization (Brazin et al., 2002). Equal amounts of Itk-WT and Itk-P287G were recovered by immunoprecipitation from cell lysates by using a Myc epitope tag fused to the C terminus (Figure 6A). Endogenous CypA copurified with Itk-WT, but not with Itk-P287G, indicating that Proline 287 was necessary for the interaction. Cyclosporine disrupted CypA's interaction with Itk (Figure 6B), and the CyPA R55A mutant failed to interact (data not shown), demonstrating that the PPIase active site of CypA mediates binding to Itk.

CypA Regulates Itk Protein-Protein Interactions

Itk possesses SH3, SH2, and kinase domains (Lewis et al., 2001; Miller and Berg, 2002b), but unlike Src, it lacks an autoinhibitory tyrosine phosphorylation site in the carboxy-terminal tail (Xu et al., 1997). Alternative regulatory mechanisms were sought, and the Itk SH3 domain was found to associate intermolecularly with the Itk SH2 domain (Brazin et al., 2000). This would promote Itk dimerization, block association with other signaling molecules, and, by analogy with the downregulated form of Src, decrease Itk activity. P287, the residue in the Itk SH2 domain required for interaction with CypA (Figure 6A), is preceded by a peptide bond that spontaneously undergoes peptidyl-prolyl *cis-trans* isomerization (Brazin et al., 2002; Mallis et al., 2002). Conformer-specific binding to the Itk SH2 domain is observed since the Itk SH3 domain preferentially binds the *cis* conformer, while the *trans* SH2 conformer is preferred by tyrosine-phosphopeptides (Breheny et al., 2003). If CypA interaction with P287 favored the *cis* conformer it would promote Itk dimerization and downregulate its activity.

To determine if Itk protein-protein interactions in cells are altered by CypA, we first examined Itk dimerization. Itk-Myc was pulled down with Itk-GST, but not with GST alone (Figure 6C), showing that full-length Itk self-associates. When cells were treated with cyclosporine to block CypA, the amount of Itk-GST recovered on glutathione beads was unchanged, but the associated Itk-Myc was decreased 2-fold (Figure 6D), consistent with CypA favoring Itk dimerization. Next we examined interaction between Itk-GST and SLP-76, a known ligand for the Itk SH2 domain (Lewis et al., 2001; Miller and

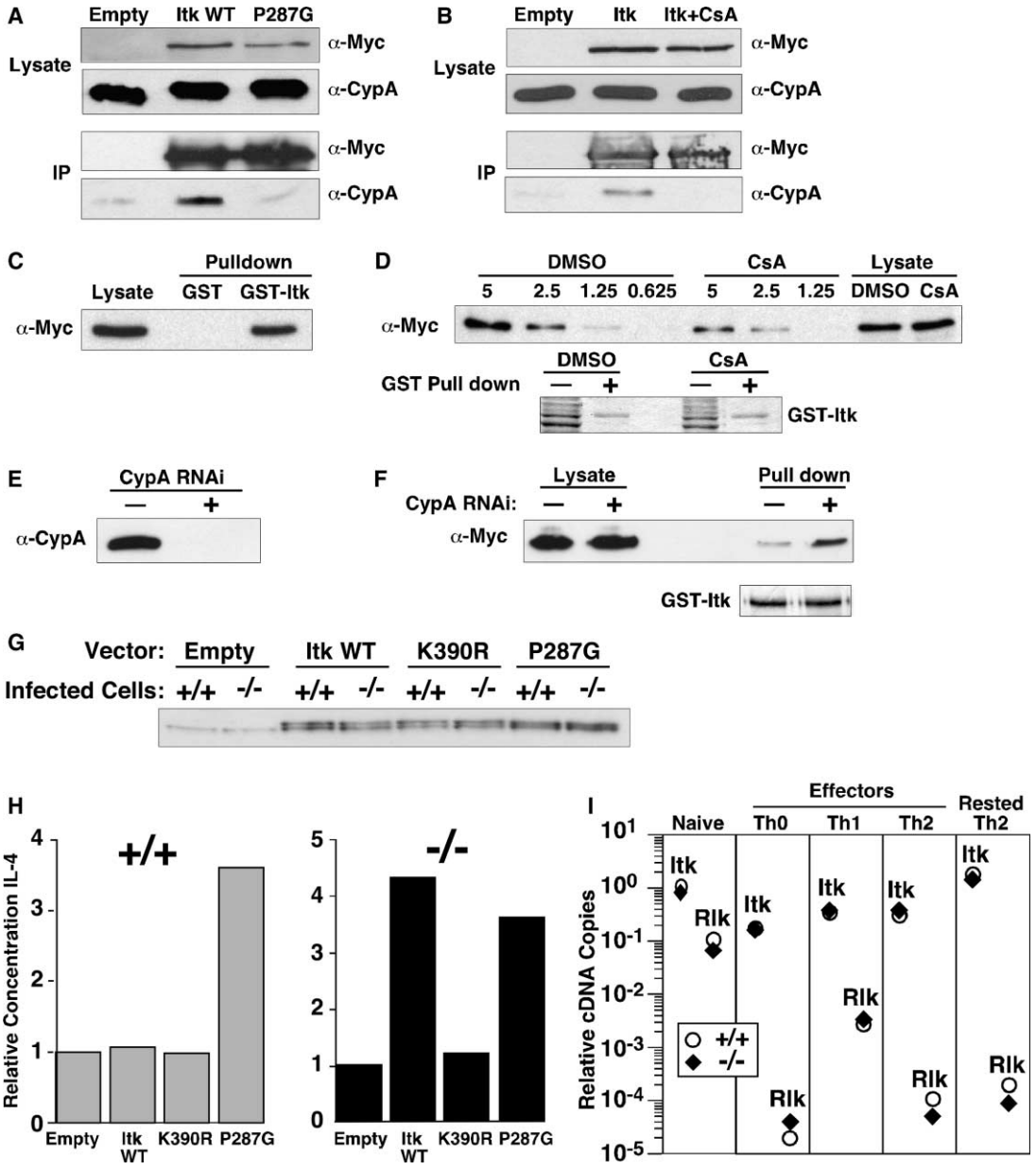

Figure 6. CypA Regulates Itk

(A) Wild-type Itk and Itk-P287G were expressed in 293T cells and immunoprecipitated by using an antibody against the Myc-epitope. Cell lysates and immunoprecipitates were probed in immunoblots with the indicated antibodies.

(B) Itk was immunoprecipitated and immunoblotted as in (A) in the presence of 10 μM cyclosporine (CsA), as indicated.

(C) GST or Itk-GST pullouts from 293T cells coexpressing Itk-Myc immunoblotted with anti-Myc antibody.

(D) Itk-GST pulldowns from 293T cells coexpressing Itk-Myc. Cells were treated with 10 μM cyclosporine (CsA) as indicated. Anti-Myc immunoblot (top) of the indicated sample volumes (in μl). Coomassie gel (bottom) shows Itk-GST pull out (+) and total lysates (−).

(E) Anti-CypA immunoblot of HeLa cells transduced with control shRNA vector (−) or with shRNA vector targeting CypA (+).

(F) Itk-GST pullout from HeLa cells (control or with CypA-knockdown) transfected with Myc-SLP-76. Anti-Myc immunoblot (top) and Coomassie gel to visualize the efficiency of Itk-GST pullout.

(G) Itk immunoblot of $CD4^+$ T cells stimulated under unbiased conditions and transduced with retroviral vectors expressing the indicated cDNAs.

(H) IL-4 concentration in supernatant 2 days after secondary stimulation of the cells in (G).

(I) Quantitation of Itk and Rlk mRNAs (relative to HPRT) in the indicated $CD4^+$ T cell populations by real-time RT-PCR.

Berg, 2002b), by using Hela cells stably expressing shRNA molecules that knock down CypA expression (Figure 6E). Equal amounts of Itk-GST were recovered from control and CypA-depleted cells, but the amount of Myc-tagged SLP-76 associated with Itk-GST was higher in extracts from CypA-depleted cells (Figure 6F), showing that CypA inhibits Itk interaction with TCR signaling partners.

CypA Inhibits IL-4 Induction by Itk

To test the functional relevance of CypA's interaction with Itk, naive $CD4^+$ T cells were stimulated and infected

with Itk-expressing retroviral vectors. Transduced cells were cultured for 1 week, washed, and stimulated again to induce effector cytokine production. Regardless of the vector used, transduction efficiency was >95% (Supplemental Figure S3A), and all Myc-tagged Itk derivatives accumulated to similar levels, reaching concentrations slightly greater than those of endogenous Itk (Figure 6G). In *Ppia*$^{+/+}$ cells, transduction of Itk-WT had no effect on IL-4 synthesis (Figure 6H). Strikingly, transduction of Itk-P287G boosted IL-4 production 3- to 4-fold. In *Ppia*$^{-/-}$ cells, transduction of either Itk-WT or Itk-P287G resulted in a greater than 4-fold increase in IL-4 secretion (Figure 6H). Itk kinase activity was required for this effect, since transduction of Itk-K390R, a kinase inactive mutant (Heyeck et al., 1997), did not increase IL-4 expression. None of the Itk vectors significantly altered IFN-γ production by either *Ppia*$^{+/+}$ or *Ppia*$^{-/-}$ cells (Supplemental Figure S3B). These results demonstrate that CypA limits the ability of Itk to induce IL-4 expression.

Differerential Expression of Itk and Rlk

The absence of CypA, or of Itk (Fowell et al., 1999), most profoundly affects Th2 function. This might be explained by regulated expression of Itk or other Tec family kinases in CD4$^+$ T cell subsets. Disruption of CypA expression had no significant effect on Tec kinase mRNA levels (Figure 6I). The quantity of Itk mRNA in naive CD4$^+$ T cells and in all effector or Th2 memory populations was similar (Figure 6I). The expression of Rlk, a Tec family kinase that functionally overlaps with Itk (Schaeffer et al., 1999, 2000, 2001; Sommers et al., 1999), decreased with stimulation of naive CD4$^+$ T cells. In unbiased or Th2 cells the level was reduced at least 1000-fold. In contrast, in Th1 cells Rlk mRNA decreased roughly 10-fold. Higher Rlk expression in Th1 cells as compared to Th2 cells provides a possible explanation for the unique importance of Itk in Th2 function.

Discussion

CypA Regulates TCR Signaling in the Absence of Cyclosporine

Here, we report that *Ppia*$^{-/-}$ mice develop allergic pathology with elevated Th2 cytokine production. The etiologic link between allergy and hyperactive Th2 CD4$^+$ T cells is well established (Herrick and Bottomly, 2003). Expression of many cytokines, in particular the Th2 cytokine IL-4, is controlled by the NFAT transcription factors (Rao et al., 1997). Given that NFAT activity is dependent upon calcineurin, and therefore cyclosporine-sensitive, and that CypA was discovered as a cyclosporine binding protein (Handschumacher et al., 1984), the phenotype of *Ppia*$^{-/-}$ mice is particularly pleasing.

Cyclosporine inhibits calcineurin but only as a complex with CypA (Friedman and Weissman, 1991; Liu et al., 1991). This unusual property of cyclosporine, as a molecular glue bridging two cellular proteins that don't normally interact, provoked speculation that cyclosporine mimics an endogenous CypA ligand and that CypA regulates signal transduction in the absence of cyclosporine (Schreiber and Crabtree, 1992). The putative endogenous ligand of CypA has yet to be discovered, but the striking similarity of the pathology in *Ppia*$^{-/-}$ mice with that of IL-4 and NFAT transgenic animals (Ranger et al., 1998b; Tepper et al., 1990) and the increased NFAT DNA binding activity in *Ppia*$^{-/-}$ mice indicates that CypA does indeed dampen signals originating from the TCR. More specifically, our finding that CypA inhibits Itk, a nonreceptor tyrosine kinase that couples TCR stimulation with capacitative calcium flux (Liu et al., 1998), reveals CypA's endogenous function as a calcineurin regulator, with the clarification that CypA acts indirectly at a point upstream in the signal transduction pathway.

Itk Dysregulation and Th2 Hyperactivity in *Ppia*$^{-/-}$ Mice

CypA inhibits Itk autokinase activity in vitro (Brazin et al., 2002). Consistent with CypA functioning as an Itk repressor, the phenotype of *Ppia*$^{-/-}$ mice is in many respects the opposite of that of *Itk*$^{-/-}$ mice. CD4$^+$ T cells from *Itk*$^{-/-}$ mice respond to TCR engagement with decreased activation of PLC-γ and downstream effectors (Lewis et al., 2001; Liu et al., 1998; Miller and Berg, 2002a, 2002b; Schaeffer et al., 1999, 2000). CD4$^+$ T cells from *Ppia*$^{-/-}$ animals respond with increased activation of these molecules. Peripheral T cell function is compromised in *Itk*$^{-/-}$ mice (Liao and Littman, 1995; Liu et al., 1998; Lucas et al., 2002; Schaeffer et al., 1999, 2000), while naive *Ppia*$^{-/-}$ CD4$^+$ T cells produce more IL-2. In vitro assessment of *Itk*$^{-/-}$ CD4$^+$ T cells and in vivo challenge with pathogens have clearly established that Itk is essential for Th2 function (Bachmann et al., 1997; Fowell et al., 1999; Mueller and August, 2003; Schaeffer et al., 2001). Th2 cytokine secretion is elevated in *Ppia*$^{-/-}$ effector/memory CD4$^+$ T cells and *Ppia*$^{-/-}$ animals spontaneously develop allergic pathology.

Our retroviral transduction experiments provide strong evidence that CypA controls Th2 cytokine expression via interaction with Itk. Itk overexpression increased IL-4 production in *Ppia*$^{-/-}$ cells, but not in wild-type cells. Thus, repression of Itk by CypA is dominant, and, in the absence of CypA, Itk is limiting for signal transduction from the TCR. These conclusions are supported by the demonstration that Itk-P287G, a mutant that cannot bind CypA, released Itk from control by CypA and increased IL-4 production even in wild-type cells. No effect of Itk transduction on IFN-γ production was detected, indicating that Itk activity is limiting specifically for IL-4 expression.

Some phenotypes of *Itk*$^{-/-}$ and *Ppia*$^{-/-}$ mice are not reciprocal. Both *Itk*$^{-/-}$ and *Ppia*$^{-/-}$ mice have elevated serum IgE (Bachmann et al., 1997; Fowell et al., 1999; Mueller and August, 2003; Schaeffer et al., 2001). Although *Itk*$^{-/-}$ mice produce less IL-4, other cytokines that regulate IgE production might also be altered (Bachmann et al., 1997; Fowell et al., 1999; Mueller and August, 2003; Schaeffer et al., 2001). In some instances, *Itk*$^{-/-}$ CD4$^+$ T cells produce less IFN-γ (Bachmann et al., 1997; Fowell et al., 1999; Mueller and August, 2003; Schaeffer et al., 2001), but production of this cytokine by *Ppia*$^{-/-}$ CD4$^+$ T cells appears normal.

Why Does Itk Regulate Th2 Function and Not Th1 Function?

Itk and Rlk gene disruptions have additive effects on CD4$^+$ T cell signaling (Schaeffer et al., 1999, 2000). Why

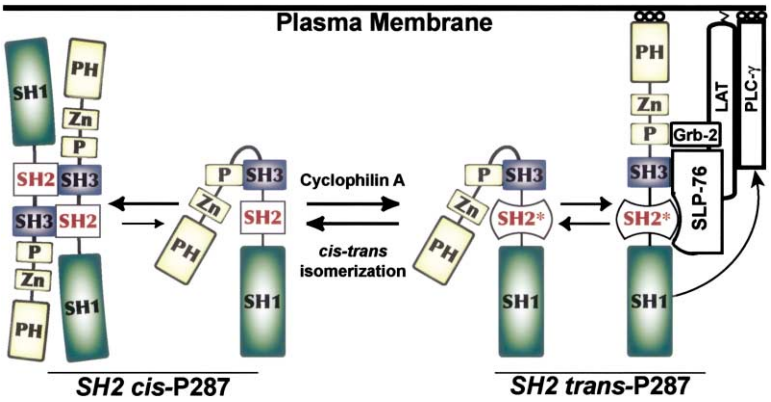

Figure 7. Model for Regulation of Itk by CypA

The imide bond preceding Proline 287 in the Itk SH2 domain spontaneously interconverts between *cis* and *trans* conformers. The self-associated, dimeric form of Itk predominates for the *cis* imide bond containing conformer (left), while the *trans* imide bond within the SH2 domain (denoted SH2*) favors interactions with transiently phosphorylated signaling partners such as Slp-76 (right). Interconversion between the two conformations of Itk likely occurs via the monomeric form of the enzyme (center). The Itk-CypA interaction depends on the conformationally heterogeneous P287 in the SH2 domain and leads to downregulation of Itk.

then is Itk specifically required for Th2 function? In contrast to naive $CD4^+$ T cells and Th1 effector cells, Rlk expression is almost undetectable in Th2 cells (Figure 6E). Similar restriction of Rlk expression to Th0 and Th1 cells has been observed with murine clones and human cells (Hu et al., 1995; Kashiwakura et al., 1999). If Itk and Rlk are functionally redundant then the Th2-specific effects associated with Itk could be explained by the fact that Rlk expression is restricted to Th1 cells.

Several observations suggest that the relationship between Itk and Rlk is more complicated. The Th2 defect in *Itk*$^{-/-}$ mice is paradoxically rescued by concurrent disruption of *Rlk* (Schaeffer et al., 2001), perhaps due to compound effects of the double knockout on TCR signal strength and GATA-3 expression (Schaeffer et al., 2001). Alternatively, it might result from specific, positive effects of Rlk on IFN-γ transcription (Takeba et al., 2002) with secondary inhibition of Th2 cytokine production. Our Itk transduction experiments boosted Th2 cytokine expression with no effect on IFN-γ, regardless of the cytokine milieu during naive $CD4^+$ T cell differentiation, suggesting that Itk possesses specific Th2 stimulatory activity. Perhaps Itk interacts in a unique fashion with molecules such as STAT6 or GATA-3 that have been specifically associated with Th2 function (Murphy and Reiner, 2002). NFATc/2/c1 is a positive regulator of IL-4 whereas other NFAT family members seem to inhibit this activity (Kiani et al., 1997; Ranger et al., 1998a, 1998b; Yoshida et al., 1998). NFATs differ from one another with respect to threshold for activation (Brogdon et al., 2002), raising the possibility that Itk differentially activates NFAT family members, perhaps indirectly via effects on TCR signal intensity or calcium flux. Regulation by CypA is unique to Itk; other Tec kinase family members lack the critical SH2 domain proline 287 (Mallis et al., 2002).

A Novel Mechanism of Tyrosine Kinase Regulation

HIV-1 Capsid is the only mature folded protein directly shown to be a catalytic substrate of CypA (Bosco et al., 2002). CypA forms a stable complex with HIV-1 Capsid, as it does with Itk, and promotes HIV-1 replication (Braaten and Luban, 2001; Luban et al., 1993), but the functional significance for HIV-1 replication of peptidyl-prolyl *cis-trans* isomerization has not been demonstrated. In contrast, Itk is a mature folded protein for which there is reasonable evidence that CypA catalytic activity is biologically relevant. Itk-P287G, a mutant that converts the imide bond into an amide bond, constrains the SH2 domain in the *trans* conformation (Brazin et al., 2002), disrupts association with CypA (Figure 6A), and mimics the phenotype of the *Ppia*$^{-/-}$ mice. Our results suggest that by catalyzing *cis-trans* isomerization of the imide bond preceding P287, CypA controls a molecular switch that regulates Itk activity (Figure 7). In this model, the *trans* Itk conformer associates with tyrosine-phosphorylated peptides (Breheny et al., 2003; Mallis et al., 2002) and, in response to TCR stimulation, would recruit Itk to the signalsome via interaction with transiently activated molecules such as SLP-76 (Lewis et al., 2001; Miller and Berg, 2002b). The *cis* conformer is likely an inactive form of Itk as it precludes interaction with tyrosine-phosphorylated peptides and instead favors Itk homodimers via interaction with the SH3 domain (Breheny et al., 2003; Mallis et al., 2002). With TCR signaling and the sudden increase in concentration of tyrosine phosphorylated peptides, catalysis of peptidyl-prolyl isomerization by CypA would increase the rate by which Itk associates with the signalsome. But, given the fleeting nature of the tyrosine-phosphorylated signaling molecules and the relatively constant levels of Itk, which serve as a sink for the inactive *cis* isoform, the net effect of catalysis by CypA would be to increase the rate by which Itk is transferred into inactivating interactions. This model for the effect of CypA on Itk inactivation is analogous to the model for the effect of the PPIase Pin1 on cdc25 dephosphorylation by the conformer-specific phosphatase PP2A (Yaffe et al., 1997; Zhou et al., 2000). In the case of CypA and Itk the equilibrium between *cis* and *trans* isoforms is determined by level of tyrosine-phosphorylated peptides.

Implications of CypA's Th2 Regulatory Function for Suppression of Allograft Rejection by Cyclosporine and for HIV-1 Pathogenesis

Inhibition of calcineurin and T cell cytokine production by the CypA-cyclosporine complex is believed sufficient to account for suppression of transplant rejection by cyclosporine in vivo (Schreiber and Crabtree, 1992). This gain in CypA function presumably prevents expansion of alloreactive T cells. However, our finding of Th2 hyperactivity in *Ppia*$^{-/-}$ mice suggests that additional direct effects of cyclosporine on native CypA function might contribute to acceptance of allografts. By virtue of its

ability to compete with Itk for binding to CypA (Figure 6B), cyclosporine might alter the balance between Th1 and Th2 effector populations. Distinct cytokine profiles expressed by different mouse strains change the character of the host response to allografts (Wang et al., 2003), and prolongation of graft survival by blockade of costimulatory molecules has been associated with a shift toward Th2 cytokines (Hancock et al., 1996; Newell et al., 1999; Sayegh et al., 1995).

Though all HIV-1 isolates encode a Capsid that interacts with CypA, some isolates are not CypA dependent for replication (Braaten et al., 1996), suggesting that selection for the interaction might be related to avoidance of antiviral immunity. $CD4^+$ T cells are required to maintain effective anti-viral CTL activity (Zajac et al., 1998). Among protective $CD4^+$ T cells, Th1 cells dominate, though balanced populations of Th1 and Th2 cells correlate with long-term antiviral protection (Imami et al., 2002; Whitmire et al., 1998). Our data here suggest that if HIV-1 Capsid binding to CypA were to increase Itk signaling in $CD4^+$ T cells, the balance might gradually shift toward a Th2 response, which has been proposed as an explanation for progression to AIDS (Clerici and Shearer, 1993).

Experimental Procedures

Generation of *Ppia*$^{-/-}$ Mice

Ppia$^{+/-}$ ES cell lines (Colgan et al., 2000) were injected into MF1 (Schwartzberg et al., 1989) blastocysts. Germline transmission was obtained in a 129/Ola × MF1 background. Sex-matched, 10-to 12-week-old *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ siblings, backcrossed 10 to 14 generations into 129S6(SvEv), were used in all experiments.

Antibody and Cytokine ELISAs

Concentrations of Ig isotypes, KLH-specific antibodies, and cytokines were determined by using reagents from Southern Biotechnology Associates, Pharmingen, and Calbiochem.

Analysis of Antigen-Specific Responses

Mice were injected intraperitoneally with 100 μg KLH (Calbiochem) mixed with Alum (Pierce), boosted at 21 days, and serum was collected 10 days later. To raise antigen-specific $CD4^+$ T cells, mice were immunized with 100 μg of KLH or DNP-KLH in complete Freund's adjuvant (Calbiochem), boosted 30 and 60 days later with 100 μg of protein in incomplete Freund's adjuvant, and $CD4^+$ spleen cells were isolated 10 days after the second boost. APC were prepared by irradiating splenocytes (2500 rads) depleted of T cells by using anti-Thy 1.2-coated beads (Dynal). Cells were resuspended at 1×10^6/ml in RPMI, 10% fetal bovine serum, 0.2% glucose, 2 mM glutamine, 50 μM β-mercaptoethanol, 100 IU/ml pencillin, and 100 mg/ml streptomycin. Equal numbers of $CD4^+$ T cells and APC were mixed with antigen and cultured for 4 days.

Flow Cytometry

Single-cell suspensions treated with red blood cell lysis buffer (Sigma) were resuspended in staining buffer (PBS, 3% mouse serum, 0.1% sodium azide), and incubated on ice with fluorochrome-conjugated antibodies (Pharmingen or Caltag). Cells were analyzed by using a FACS Calibur and Cellquest software (Becton Dickinson).

$CD4^+$ T Cell Purification and Differentiation

Total $CD4^+$ T cells were isolated by using anti-CD4-coated magnetic beads and eluted with Detachabead (Dynal); purified cells were typically >98% $CD4^+$ as judged by flow cytometry. $CD62L^{hi}$ and $CD62L^{lo}$ cells were isolated from the total $CD4^+$ fraction by using anti-CD62L beads and magnetized columns (Miltenyi). $CD62L^{hi}$ $CD4^+$ T cells in media containing 20 U/ml of IL-2 were stimulated 7–14 days on 24-well plates coated with anti-CD3 and anti-CD28 antibodies (10 μg/ml each). IL-12 (4 ng/ml) plus anti-IL-4 antibody (4 μg/ml) or IL-4 (1000 U/ml) plus anti-IFN-γ antibody (4 μg/ml) were added to promote Th1 and Th2 differentiation, respectively. Cytokines and antibodies were purchased from Pharmingen. To analyze effector responses, cells were harvested, washed, and immediately plated on 24-well plates coated with anti-CD3 and/or anti-CD28 antibodies at the indicated concentrations. Culture supernatant was collected 48 hr later. Memory Th2 cells were generated by culturing Th2 effector cells without stimulatory antibodies or cytokines for 7–14 days.

Analysis of Proliferative Responses

Cells were cultured in 96-well plates for 24 or 48 hr at 2×10^5/well and pulsed with ^{3}H-TTP for 6 hr. ^{3}H-incorporation was measured by using a Top Count (Packard Instruments).

RT-PCR

1×10^6 cells were incubated in ice-cold lysis buffer (50 mM Tris Cl [pH 7.9], 140 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% IGEPAL) for 5 min. Nuclei were pelleted by centrifugation (500 × g for 5 min). Total RNA was purified by using RNeasy columns (Qiagen). Genomic DNA was removed by treating with DNase on the column and again after elution. 50% of each RNA sample was reverse transcribed by using Superscript II (Life Technologies) and random hexameric primers; remaining RNA was mock reverse transcribed. PCR was performed by using an ABI-Prism 7700 sequence detector and Sequence Detection Software (Applied Biosystems). Products were detected with SYBRgreen (Molecular Probes) or gene-specific oligonucleotide molecular beacons (Supplemental Table S1). 50 μl reactions contained 2 U AmpliTaq Gold (Applied Biosystems), 20 pmoles of each primer, 10 pmoles of molecular beacon, and 60 nM ROX as a passive reference. Product copy number was determined by comparison to standard curves generated using amplicon-containing plasmids as template.

Biochemical Analysis

3×10^6 cells were placed at 37°C for 5 min, mixed with magnetic beads (Dynal) coated with either anti-CD3 or isotype control antibody for 5 min, and 5 volumes of ice-cold stop buffer was added (10 mM NaF, 1mM $NaVO_4$ in PBS). Cells were pelleted, resuspended in lysis buffer (1% n-octyl β-D-glucopyranoside, 50 mM Tris Cl [pH 7.5], 75 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 1 mM $NaVO_4$, Roche protease inhibitor cocktail), and incubated on ice for 1 hr. Insoluble material was pelleted and supernatant mixed with 2× SDS loading buffer.

For immunoprecipitation and GST pulldowns, Lipofectin (Invitrogen) was used to transfect HEK 293T cells or HeLa cells with pBOS expression plasmids. 48 hr later cell lysates were prepared by using RIPA buffer and mixed for 1 hr with Protein-G Agarose beads preloaded with anti-Myc monoclonal antibody (9E10) or with G beads. After washing, beads were resuspended in 2× SDS loading buffer.

RNAi knockdown HeLa cells were generated by retroviral transduction of pSUPER shRNA constructs engineered with CypA-specific oligonucleotides (Supplemental Table S1).

Immunoblot analysis was performedby using the following antisera: anti-CypA (Affinity Bioreagents), anti-phospho-tyrosine (clone Py20), anti-Zap-70 (clone 29; Transduction Laboratories), anti-Itk (clone 2F12), anti-phospho-ZAP-70, anti-phospho-ERK1/2 (clone E10), anti-ERK1/2 (Cell Signaling Technology), phosphoPLCγ (Biosource), and anti-PLC-γ (clone E12) and anti-Myc (clone 9E10) from Santa Cruz Biotech.

For electrophoretic mobility-shift assays, 2×10^6 cells were stimulated with plate bound antibodies for 16 hr or with PMA and ionomycin for 4 hr, as described (Schaeffer et al., 1999, 2000, 2001; Sommers et al., 1999).

Retroviral Transduction

Calcium phosphate precipitation was used to transfect a 10 cm plate of 293T cells with 25 μg of pMIG (Van Parijs et al., 1999) and 15 μg of pCL-Eco (Naviaux et al., 1996). At 48 hr culture supernatant was collected, filtered (0.45 μM), and polybrene (Sigma) was added to 8 μg/ml. Two volumes of supernatant was added to T cell cultures

plated 24 hr earlier. After centrifugation at 450 × g for 2 hr, 75% of the culture volume was removed and fresh media added. This process was repeated 24 hr later. Cells were cultured for another 5 days, washed, and plated on wells coated with anti-CD3 antibody (10 μg/ml) to induce cytokine production.

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