A PP4 Holoenzyme Balances Physiological and Oncogenic Nuclear Factor-Kappa B Signaling in T Lymphocytes

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SUMMARY

Signal transduction to nuclear factor-kappa B (NF-κB) involves multiple kinases and phosphorylated target proteins, but little is known about signal termination by dephosphorylation. By RNAi screening, we have identified protein phosphatase 4 regulatory subunit 1 (PP4R1) as a negative regulator of NF-κB activity in T lymphocytes. PP4R1 formed part of a distinct PP4 holoenzyme and bridged the inhibitor of NF-κB kinase (IKK) complex and the phosphatase PP4c, thereby directing PP4c activity to dephosphorylate and inactivate the IKK complex. PP4R1 expression was triggered upon activation and proliferation of primary human T lymphocytes and deficiency for PP4R1 caused sustained and increased IKK activity, T cell hyperactivation, and aberrant NF-κB signaling in NF-κB-addicted T cell lymphomas. Collectively, our results unravel PP4R1 as a previously unknown activation-associated negative regulator of IKK activity in lymphocytes whose downregulation promotes oncogenic NF-κB signaling in a subgroup of T cell lymphomas.

INTRODUCTION

Signaling to nuclear factor-kappa B (NF-κB) is crucial for T lymphocyte activation, differentiation, and proliferation by regulating a wide variety of target genes such as different cytokines (e.g., interleukin-2 [IL-2], interferon gamma [IFN-γ]), and tumor necrosis factor alpha [TNF-α]), chemokines (e.g., IL-8), and anti-apoptotic molecules (e.g., Bcl-2, c-IAPs) (Bonizzi and Karin, 2004; Ghosh and Karin, 2002; Ruland and Mak, 2003). NF-κB activation in response to T cell receptor (TCR) or TNF receptor 1 (TNFR1) triggering is mediated by a high molecular weight IkB kinase (IKK) complex (Häcker and Karin, 2006; Hayden and Ghosh, 2004). The IKK complex comprises two enzymatic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO) (Häcker and Karin, 2006). IKK activation results in phosphorylation of inhibitory IkB proteins, the IkBs, which interact with NF-κB transcription factors and render them inactive within the cytoplasm. Phosphorylated IkB proteins are rapidly ubiquitylated and targeted for proteasomal degradation. In turn, liberated NF-κB proteins, like RelA (p65), translocate into the nucleus, where they bind to and transactivate κB sites within the promoter region of NF-κB-regulated genes (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009).

IKK activation is a transient event that is subject to several postinductive negative feedback mechanisms ensuring proper signal shaping, control, and termination (Acuto et al., 2008). Prominent examples are the ubiquitin-editing enzymes A20 and the cylindromatosis tumor suppressor (CYLD), which act as negative regulatory control elements of NF-κB. Indeed, defects in negative regulation by A20 in lymphocytes cause enhanced and prolonged IKK phosphorylation, resulting in hyperactivation of cells and promoting lymphoid malignancies (Düwel et al., 2009; Hymowitz and Wertz, 2010).

The NF-κB activating signal cascade in T lymphocytes involves multiple phosphorylation events and relies on a complex phosphoproteome (Häcker and Karin, 2006; Well and Israël, 2006). Several kinases that control NF-κB activation have been identified (Häcker and Karin, 2006). By contrast, only a few phosphatases have been shown to regulate and terminate NF-κB activity in a cell type-, pathway-, or substrate-specific fashion (Chew et al., 2009; Eitelhuber et al., 2011; Li et al., 2006, 2008). PP4R1 (protein phosphatase 4, regulatory subunit 1) was the first noncatalytic, regulatory phosphatase subunit identified as a constitutive interaction partner of the catalytic Ser-Thr phosphatase.
phosphatase subunit PP4c (Kloeker and Wadzinski, 1999). PP4c is a ubiquitously expressed phosphatase that shares about 65% amino acid identity with PP2A catalytic subunits and belongs to the PP2A-type phosphatases (Brewis et al., 1993). Several PP4c-interacting proteins have been identified, including PP4R1, PP4R2, PP4R3a, PP4R3b, and PP4R4, which give rise to a diverse collection of distinct PP4 holoenzymes (Chen et al., 2008; Chowdhury et al., 2008; Gingras et al., 2005; Lee et al., 2010).

Given the crucial role of NF-κB in lymphocyte physiology, it is not surprising that aberrant NF-κB activity has been implicated in a number of lymphoid malignancies (Ruland and Mak, 2003b; Skinner and Mak, 2002). One very prominent example is cutaneous T cell lymphoma (CTCL), a subset of primarily skin-homing non-Hodgkin’s lymphomas (NHLs), which show addiction to constitutively activated NF-κB (Hwang et al., 2008). The majority of CTCL are represented by the entities of Mycosis fungoides (MF; tumorous skin manifestation) and the more aggressive Sézary syndrome (SS; leukemic presentation), the latter of which can arise secondarily from late-stage MF or as de novo SS (Herling et al., 2004; Hwang et al., 2008). MF and SS are slowly progressive malignancies of clonally expanded CD4+ T lymphocytes that are difficult to treat and usually incurable. Although several components of the NF-κB signaling machinery have been previously identified as bona fide oncogenes or tumor suppressors in lymphocytes (Compagno et al., 2009; Lenz et al., 2008; Staudt, 2010), the molecular lesions driving aberrant NF-κB signaling in CTCL remain largely enigmatic (Kiessling et al., 2011; Kim et al., 2000).

Here, we report the identification of PP4R1 by a small interfering RNA (siRNA) screen as a negative regulator of TCR and TNFR1-dependent NF-κB activity in T lymphocytes. PP4R1 expression was triggered by T cell activation and proliferation but lost in CTCL. PP4R1 bound to the inhibitor of NF-κB kinases (IKKγ and IKKβ) and the catalytic subunit PP4c and thereby directed PP4c phosphatase activity to the IKK complex. Consequently, PP4R1 silencing caused sustained and increased IKK activity and T cell hyperactivation. Furthermore, deficiency for PP4R1 in CTCL resulted in constitutive IKK-NF-κB signaling and thus formed an important molecular event maintaining the malignant phenotype of a subset of CTCL cells. Our findings show that PP4R1 is a regulator of IKK activity and a suppressor of oncogenic NF-κB signaling in a subgroup of T cell lymphomas.

RESULTS

RNAi Screen Identifies PP4R1 as a Regulator of NF-κB
To systematically uncover phosphatases that are involved in the regulation of NF-κB signaling in T lymphocytes, we performed an RNAi-based screen using a subgenomic siRNA library. Therefore, we engineered a Jurkat reporter T cell line that secretes Gaussia luciferase in an NF-κB-dependent fashion (G-Luc Jurkat; see Figures S1A–S1F available online). Primary RNAi screening identified two PP4R1-targeting siRNAs that augmented NF-κB reporter activity in response to TCR and to TNFR1 stimulation (Figure 1A; Tables S1 and S2). In Jurkat T cells, various independent siRNAs against PP4R1 led to a detectable knockdown of PP4R1 and enhanced PMA+ionomycin-induced upregulation and secretion of several NF-κB-regulated cytokines (Figure 1B; Figures S1G–S1L). Knockdown of PP4R1 elevated TCR+CD28-induced (Figure 1C) or PMA- or TNFR1-induced (Figure 1D) NF-κB reporter activity similarly to knockdown of the known negative regulator CYLD. Furthermore, PP4R1 silencing enhanced expression of the NF-κB target genes A20 and IkxBα (data not shown) and of IL-2 and IL-8 (Figure 1E). The fact that PP4R1 impinges upon both TCR- and TNFR1-induced NF-κB signaling indicated that PP4R1 acts on a common level in canonical NF-κB signaling in T lymphocytes.

PP4R1 Expression Is Triggered by Lymphocyte Activation and Expansion In Vivo
To evaluate the physiological relevance of our findings, we monitored PP4R1 expression in primary human peripheral blood T lymphocytes. Surprisingly, PP4R1 was not detectable in resting T cells. However, PP4R1 expression increased upon T cell activation and in vitro proliferation, whereas the expression of the PP4 catalytic subunit PP4c remained constant (Figure S3A) and tested these cells for NF-κB activity (Figure 2A). The activation-associated pattern of PP4R1 expression in human T lymphocytes was highly reproducible and equally observed for T cells that had been prestimulated in vitro either by the polyclonal mitogen PHA or by CD3+CD28 crosslinking using agonistic antibodies (Figure 2B).

PP4R1 expression generally followed a pattern of lymphocyte activation and differentiation. In fact, we observed a strong PP4R1 positivity for activated centroblastoid B cells (Figures S2A and S2B) and interfollicular T cells (Figures S2C and S2D) in lymph node biopsies, whereas intrafollicular and perifollicular T cells lacked PP4R1 expression (Figures S2F–S2L). As observed in Jurkat T cells, PP4R1 knockdown in prestimulated, expanded human T lymphocytes increased secretion of the NF-κB-dependent cytokines IL-2, TNF-α, and IFN-γ (Figure 2C). Accordingly, knockdown of PP4R1 in prestimulated, expanded human T lymphocytes drastically increased both basal and stimulation-dependent NF-κB activity (Figure 2D), whereas ectopic PP4R1 almost completely blunted NF-κB activation in T cells (Figure 2E).

PP4R1 SpecificallySuppresses NF-κB Activation
To achieve sustained gene silencing, we generated Jurkat T cells stably expressing small hairpin RNAs (shRNAs) targeting PP4R1 (Figure S3A) and tested these cells for NF-κB, AP-1, and NF-AT signaling by transient transfection of reporter genes. As expected, NF-κB activity was significantly elevated in PP4R1-silenced cells, whereas AP-1 and NF-AT activation remained unaffected (Figure 3A; Figure S3B). Importantly, enhanced NF-κB activity of stably PP4R1-silenced cells was lost when PP4R1 expression was transiently reconstituted (Figure 3B). Moreover, exogenous PP4R1 decreased both basal and TCR+CD28-induced NF-κB activity (Figures 3B and 3C, left), whereas AP-1 and NF-AT activation were not altered by overexpression of PP4R1 (Figure 3C). In addition, suppression of TCR+CD28-dependent NF-κB activity by ectopically expressed PP4R1 occurred in a dose-dependent manner (Figure S3C). In line with these findings, stably PP4R1-silenced Jurkat T cells displayed enhanced PMA+ionomycin-triggered expression of multiple NF-κB target genes (Figure 3D) and increased secretion of IL-2, IL-8, and IFN-γ upon stimulation (Figure 3E; Figure S3D).
Collectively, these results show that PP4R1 selectively suppresses NF-κB signaling in T cells.

### PP4R1 Interacts with IKKα/β and PP4c

The fact that PP4R1 attenuates both TCR- and TNFR1-induced NF-κB signaling suggested that PP4R1 operates on a common level within the canonical NF-κB pathway. A central point of convergence in canonical NF-κB signaling is the heterotrimeric NEMO-dependent IKK complex (Hayden and Ghosh, 2008). To investigate whether PP4R1 targets the IKK complex, we first tested for a potential physical interaction between both components. Indeed, we found coprecipitation of endogenous PP4R1 with the IKK complex following anti-IKKg (NEMO) immunoprecipitation in Jurkat T cells and prestimulated, primary human T cells (Figures 4A and 4B). The IKK-specific association of PP4R1 peaked at around 45 min after TCR+CD28 stimulation and thereby sharply followed the peak of IKK phosphorylation (Figure 4A). To further analyze which components of the IKK complex, IKKα, IKKβ, or IKKγ (NEMO), interact with PP4R1, we used expression of tagged proteins in HEK293T cells. We found a specific interaction of hemagglutinin (HA)-tagged PP4R1 only with FLAG-tagged IKKα or IKKβ but only a minor association with the structural subunit NEMO—most likely due to complex formation between exogenous NEMO and endogenous IKKα or IKKβ (Figure 4C; Figure S4A). The use of in vitro translated proteins confirmed this result (Figure S4B) and suggested a direct interaction of PP4R1 with IKKα and IKKβ, respectively.

PP4R1 lacks intrinsic phospholytic activity but has been reported to interact with the human PP4 catalytic subunit PP4c (Chen et al., 2008; Gingras et al., 2005; Kloeker and Wadzinski, 1999). We confirmed stable complex formation between PP4R1 and PP4c in HEK293T cells (Figure 4D) and further demonstrated a stable interaction between endogenous proteins in Jurkat T cells as well as in primary human T cells but no binding of the structurally related phosphatase PP2Ac (Figures S4C and S4D). The interaction between PP4R1 and PP4c was constitutive and did not change upon TCR stimulation. The dual binding of PP4R1 to PP4c or IKKs prompted us to investigate whether PP4R1-bound PP4c similarly associates with the IKK complex. Indeed, similar to PP4R1, we found PP4c to preassociate with the endogenous IKK complex in Jurkat T cells (Figure 4E; Figure S4E). Several distinct PP4 modules with different regulatory subunits have been described (Brewis et al., 1993; Chen et al., 2008; Chowdhury et al., 2008; Lee et al., 2010; Zhang et al., 2005) and give rise to the possibility that PP4R1 selectively directs PP4c toward the IKK complex. To further substantiate this assumption, we used PP4R1-silenced
cells to test for the presence of PP4c at the IKK complex. Absence of PP4R1 abrogated basal and stimulation-induced IKK-association of PP4c (Figure 4F), which confirms that PP4R1 directs the IKK-specific localization of PP4c in T cells. Furthermore, we could not observe any interaction between recombinant PP4R1 or PP4c with RelA (Figure S4 F).

In accordance with the existence of several distinct PP4c holoenzymes, PP4c was found to be localized both in the nucleus and cytosol, whereas PP4R1 was predominantly confined to the cytosolic compartment (Figure S4 G).

Partial disruption of PP2A homologous heat repeats (Chen et al., 2008; Kloeker and Wadzinski, 1999) within PP4R1 prevented binding of PP4R1 to PP4c and partially abolished binding to IKKα and IKKβ (Figure 4G; Figures S4H and S4I), suggesting a multimeric PP4R1-dependent complex with distinct interfaces between PP4R1 and PP4c and IKKα and IKKβ, respectively. PP4R1 truncation mutants failed to suppress NF-κB activation (Figures S4J–S4L), demonstrating that binding of PP4R1 to PP4c is mandatory for PP4R1-mediated NF-κB suppression.

Our data suggest that PP4R1 selectively directs PP4c phosphatase activity toward the IKK complex to catalyze IKK dephosphorylation and inactivation. To further address this point, we analyzed activation loop phosphorylation of IKKα and IKKβ in PP4R1-deficient compared to control-transfected cells. We observed increased and sustained IKK phosphorylation, which peaked 25 min after TCR+CD28 stimulation (Figure 5 A).

Of note, PP4R1 did not affect phosphorylation of ERK1 or ERK2 (Figures 5 A and 5B). Next we directly measured kinase activity of the endogenous IKK complex in the presence or absence of PP4R1. TCR+CD28- or PMA+ionomycin-induced IKK (auto-) phosphorylation as well as phosphorylation of its recombinant substrate GST:IkBα were strongly enhanced and prolonged in PP4R1-silenced Jurkat T cells (Figures 5 B and 5C; Figures S5 A and S5B). We further investigated PP4R1-mediated inhibition of IKK activity in a reverse experiment by co-expression of HA:PP4R1 and FLAG:IKKβ in Jurkat T cells. Ectopic PP4R1 profoundly suppressed TCR+CD28-induced IKKβ kinase activity (Figure S5C). Consistent with increased IKK phosphorylation, PP4R1-silenced cells displayed a more rapid, enhanced, and sustained nuclear translocation of p65 IkBα.
PP4R1 did not affect NF-κB activity induced by dominant active IKKβ (IKKβSS/IKKβEE) (Mercurio et al., 1997) or by overexpression of RelA, whereas IKKβ wild-type (WT)- or Carma1-mediated NF-κB activity were largely abrogated (Figure 5D), implying that PP4R1 directly controls activation loop dephosphorylation of IKKβ. In line with a direct role of PP4R1 in controlling the dephosphorylation of the IKK complex, PMA+ionomycin-induced interaction of the signal adapter proteins Carma1 and Bcl10 was not altered by silencing of PP4R1 expression (Figure 5E). Moreover, ectopic expression of PP4R1 in epithelial cells did equally suppress PMA- as well as TNF- or IL-1-triggered NF-κB activation (Figure 5F). In concert, these results provide evidence for PP4R1 as a generic and direct negative regulator of IKK activity that may operate in different receptor systems in lymphoid and non-lymphoid cells.

**Figure 3. PP4R1 Is a Pathway-Specific Negative Regulator of NF-κB target Genes in T Cells**

(A) Normalized activity of NF-κB-, AP-1-, or NF-AT-specific reporter genes upon TCR/CD28 or PMA/ionomycin stimulation in Jurkat T cells stably expressing PP4R1 shRNA (shPP4R1#65) or nontargeting control shRNA (shCtrl). *p = 0.043 for anti-CD3/28 and ***p = 0.0007 or PMA/iono. Asterisks indicate statistical significance relative to control cells with *p < 0.05, **p < 0.001, ***p < 0.0001.

(B) TCR/CD28-dependent NF-κB reporter activity of Jurkat T cells stably expressing shPP4R1 or shCtrl with or without ectopic expression of PP4R1.

(C) Jurkat T cells transiently overexpressing HA-tagged PP4R1 were stimulated as indicated, and NF-κB-, AP-1-, or NF-AT-specific luciferase activity was measured. Expression of exogenous PP4R1 was analyzed by IB.

(D) Gene expression in shPP4R1 compared to shCtrl Jurkat T cells upon PMA/ionomycin stimulation (1 hr) was examined by a qPCR gene expression array.

(E) IL-2 and IL-8 secretion of Jurkat T cells stably expressing shPP4R1#63, #65, or shCtrl upon TCR/CD28 stimulation. Results represent at least three independent experiments. Data are presented as mean ± SD of duplicate (A–C) or triplicate (D–E) measurements. *p = 0.036, 0.032, 0.012 and 0.012 for IL-2; **p = 0.005, 0.003, 0.0057 and 0.0053 for IL-8, and, *p = 0.017 and **p = 0.002 for INF-γ, respectively.
PP4R1 Directs PP4c to Dephosphorylate IKK

Next, we asked whether dephosphorylation of IKKα and IKKβ involves both PP4R1 and the enzymatic activity of PP4c. Therefore, we established a phosphatase activity assay using recombinant proteins. Concomitant expression of PP4R1 and PP4c caused a synergistic increase in phosphatase activity (Figure 6A). Furthermore, we observed a strong but transient increase in PP4R1-associated phosphatase activity in Jurkat T cells and primary T lymphocytes upon TCR+CD28 stimulation (Figures S6A and S6B). Expression of increasing amounts of PP4R1 together with PP4c, but not of PP4R1 or PP4c alone, completely blunted IKK phosphorylation on S180, 181 and S176, 177 (Figure 6B; Figure S6C). This cooperative PP4R1-dependent effect was most pronounced for PP4c because co-overexpression of either PP2Ac or PP1c together with PP4R1 reduced IKK phosphorylation less efficiently than concomitant expression of PP4R1 and PP4c (Figure S6D). Moreover, PP4R1-PP4c-dependent dephosphorylation was highly specific for IKKα and IKKβ as phospho-substrates because phosphorylation of RelA was not affected (Figure 6C). Importantly, all PP4R1 truncation mutants—that do not interact with endogenous PP4c—failed to mediate IKK dephosphorylation (Figure 6D). Vice versa, overexpression of a dominant-negative catalytically inactive mutant of PP4c was sufficient to overcome PP4R1-mediated IKK dephosphorylation (Figure 6E), again proving that PP4R1/PP4c forms an inseparable functional unit in counterbalancing IKK-NF-κB signaling. In further support of this assumption, stimulation-induced NF-κB activity in PP4R1-silenced Jurkat cells was not increased by administration of ocadic acid (OA), a potent inhibitor of PP2A-like phosphatases, whereas in control-transduced cells, stimulation-dependent NF-κB activity increased in a dose-dependent manner (Figure 6F). These data indicate that the well-established effect of OA on NF-κB signaling is based, at least in part, on inhibition of the PP4R1/PP4c holoenzyme in T cells. To finally test for a direct dephosphorylation of IKKα and IKKβ by PP4R1/PP4c, we purified phosphorylated IKK proteins and subjected them to an in vitro phosphatase assay. Again, presence of recombinant PP4R1 or PP4c led to dephosphorylation of IKKα and IKKβ (Figure S6E), indicating that IKK proteins serve as direct substrates of the PP4R1/PP4c phosphatase complex. Collectively, these results demonstrate that (1) PP4R1 potentiates PP4c phosphatase activity, (2) PP4R1 directs PP4c to specifically dephosphorylate the IKK complex, and (3)
PP4R1-associated phosphatase activity is further enhanced by TCR stimulation.

CTCL Cells Lack PP4R1

Constitutive NF-κB activity is central in the survival of CTCL cells, and besides a skin homing-phenotype, CTCL tumor cells exhibit a strongly activated gene expression signature (i.e., HLA-DR, CD25, IFN-γ) in a site and stage-dependent manner (Swerdlow et al., 2008). Thus, we asked whether dysregulation of NF-κB in these cells correlates with reduced PP4R1 levels. Indeed, we found significant downregulation of PP4R1 transcription as well as protein expression in primary tumor cells derived from peripheral blood of CTCL patients (Figure 7 A; Figures S7 A and S7B). Moreover, and in contrast to reactive nonmalignant T cells in lymph node biopsies (Figures S2 C and S2D) and in skin biopsies from psoriasis patients (Figure 7 B, top panel), lack of PP4R1 expression was also observed for subepidermal, skin-infiltrating tumor cells in diagnostic biopsies from eight CTCL patients, with generally lower PP4R1 expression in the four SS patients compared to the four MF patients (Figure 7 B, bottom panel; Table S3). We also observed defective PP4R1 expression in the two CTCL cell lines Hut78 and HH, both originating from primary Sézary cells (Figure 7C; Figure S7C). Strikingly, HH cells completely lacked PP4R1 expression, whereas Hut78 cells showed reduced PP4R1 expression. Moreover, these cell lines exhibited a specific NF-κB target gene expression signature that was most pronounced for PP4R1-deficient HH cells compared to Jurkat T cells (Figure 7D; Table S4). Given constitutive NF-κB signaling, we next analyzed basal IKK activity in multiple CTCL cell lines and found that loss of PP4R1 expression in HH cells correlated with constitutive IKKb kinase activity (Figure 7 E), as well as with constitutive IKK and IkBα phosphorylation, respectively (Figure 7F). Forced PP4R1 re-expression in HH cells reversed IKK activation and constitutive NF-κB signaling (Figure 7G; Figure S7D), suggesting that PP4R1 deficiency is a causative factor for constitutive IKK activation in these cells.

Figure 5. PP4R1 Negatively Regulates IKK Complex Activation in T Cells by Governing IKK Dephosphorylation
(A and B) Immunoblotting (IB) of lysates from siCtrl or siPP4R1-transfected (A) or stably silenced Jurkat T cells (B) stimulated with anti-CD3+CD28 antibodies (0.5 μg/ml) or PMA+ionomycin. Endogenous IKK activities of shRNA expressing Jurkat T cells were determined by NEMO-immunoprecipitation and in vitro kinase assay (KA).
(C) Phosphorylation of endogenous IKKα and IKKβ was quantified using total IKK levels for normalization.
(D) HEK293T cells were transfected with the indicated expression vectors and NF-κB activity was measured. *p = 0.038 and ***p = 0.0009.
(E) PP4R1-targeting or control shRNA expressing Jurkat T cells were stimulated with PMA+ionomycin and anti-Bcl10 immunoprecipitations were IB using the indicated antibodies. Filled and open arrowheads depict specific proteins and IgG heavy or light chains (IgGH/L), respectively.
(F) HEK293T cells with or without ectopic PP4R1 were stimulated as indicated and NF-κB activity was measured. **p = 0.003 and *p = 0.015 or 0.028 for PMA or IL-1, respectively. Asterisks indicate statistical significance relative to control cells with *p < 0.05, **p < 0.001, ***p < 0.0001. Data are presented as mean ± SD of triplicate measurements.
To analyze whether reconstitution of PP4R1 alters the malignant phenotype of CTCL cells, we cloned the complementary DNA (cDNA) of PP4R1 into a GFP-coexpressing retroviral vector to transduce four different CTCL cell lines. In contrast to PP4R1-sufficient SeAx and MyLa cells, the abundance of positively transduced Hut78 and most prominently HH cells was dramatically decreased by forced expression of exogenous PP4R1 (Figure 7H; Figure S7E). This effect was highly specific for PP4R1 expression because overexpression of PP2Ac or PP4c did not impact relative cell abundance. Thus, PP4R1 downregulation in CTCL favors constitutive IKK activity and forms an oncogenic condition promoting and/or maintaining the malignant phenotype of a subset of CTCL cells.

DISCUSSION

Multiple kinases and phosphorylated target proteins that induce NF-κB activity have been identified. However, the molecular machinery involved in the termination of antigen receptor-mediated NF-κB activation is only partially understood. Because signal transduction from activated antigen receptors to NF-κB largely relies on phosphorylation events (Hayden and Ghosh, 2004; Ruland and Mak, 2003b), it is generally assumed that phosphatases play a major role in the modulation and termination of NF-κB activity.

The IKK complex forms a central signaling hub in canonical NF-κB signaling that couples and integrates various receptor signals to induce NF-κB transcriptional activity (Häcker and Karin, 2006). Here we provide several lines of evidence that the PP4R1-PP4c phosphatase complex is a direct negative regulator of IKK phosphorylation and activation in T cells. (1) PP4R1 physically interacts with the native IKK complex in activated T lymphocytes and this association increases following IKK activation. (2) Knockdown of PP4R1 enhances and prolongs IKK activation, whereas ectopic PP4R1 largely abrogates IKKβ kinase activity. Conversely, enhanced basal and
induced IKK hyperactivation in PP4R1-silenced T cells or PP4R1-deficient CTCL cells was rescued by re-expression of ectopic PP4R1. Although PP4R1-silenced cells display already increased basal IKK phosphorylation and NF-κB activity, PP4R1 seems to be primarily involved in the shutdown of stimulation-dependent IKK phosphorylation and kinase activity. (3) PP4R1 recruits the catalytic subunit PP4c to the IKK complex, and absence of PP4R1 prevents association of PP4c and IKK proteins. (4) The PP4R1-PP4c complex mediates dephosphorylation of activation loop serine residues within IKKα and IKKβ in vitro, and PP4R1-associates phosphatase activity further increases upon TCR+CD28 stimulation following the peak of IKK activity. (5) Constitutively active IKKβ (IKKβSS/ IKKβEE) was sufficient to bypass PP4R1-mediated NF-κB inhibition, and PP4R1 deficiency had no effect on CBM complex assembly upstream of IKK activation, as well as on MAPK activation. In addition, PP4R1-silencing caused enhanced NF-κB activity in response to TCR+CD28, PMA, or TNF-R1 stimulation. In conclusion, PP4R1 is a regulator of canonical NF-κB signaling, which directly acts on the level of the IKK complex in T cells.

We have shown that PP4R1 is upregulated in proliferating and prestimulated T cells, indicating a molecular mechanism of activation-induced feedback inhibition limiting T cell activation upon TCR restimulation. The physiological relevance of this finding was demonstrated by enhanced cytokine secretion of primed T cells lacking PP4R1, whereas, conversely, ectopic PP4R1 blunted basal and stimulation-dependent NF-κB activity in expanding T lymphocytes in vitro. The fact that resting T cells lack PP4R1 expression, however, already suggests alternative mechanisms of IKK control and dephosphorylation in T lymphocytes. Nonetheless, it is tempting to speculate that—through differential expression of phosphatases—resting versus preactivated T lymphocytes differ in the kinetics and strength of IKK activation and, therefore, exhibit differential sensitivities toward TCR stimulation.

Negative regulation of the NF-κB activating signaling apparatus in lymphocytes is mediated by several control elements
that operate on distinct levels within the NF-κB pathway (Acuto et al., 2008; Hymowitz and Wertz, 2010; Vallabhapurapu and Karin, 2009). The role of phosphatases in NF-κB signaling is not yet precisely defined. Recently, several studies demonstrated the selective involvement of different phosphatase entities in NF-κB inhibition and dephosphorylation of specific phosphosubstrates, such as PP2Ac, PP1, and WIP1 (Chew et al., 2008; Eitelhuber et al., 2011; Li et al., 2006, 2008). However, in part contradictory results among different approaches already suggested cell type- and pathway-specific patterns of expression and/or activity of individual phosphatase components (Li et al., 2006, 2008). Indeed, in contrast to a previous study (Li et al., 2008), knockdown of PP1c did not affect IKK phosphorylation and activity in T cells (data not shown). Furthermore, given the potential phosphosubstrate specificity of individual phosphatase modules, our data do not contradict previous findings but rather imply a nonredundant role of different phosphatase complexes in dephosphorylating distinct target proteins, such as Carma1, TRAF proteins, RelA, or the IKK complex itself.

PP4R1 is a noncatalytic, regulatory subunit that stably and specifically associates with the PP4 catalytically active subunit PP4c, but not with the structurally-related subunit PP2Ac (Gingras et al., 2005; Kloeker and Wadzinski, 1999). Besides PP4R1, a number of different PP4 regulatory proteins have been described that give rise to a wide collection of PP4 holoenzymes with different subcellular distributions, substrate specificities, and molecular functions, such as DNA damage repair or microtubule organization (Brewis et al., 1993; Chowdhury et al., 2008; Gingras et al., 2005; Lee et al., 2010). Consistent with the pleiotropic roles of PP4c, the genetic ablation of PP4c results in embryonic lethality of mice, and T cell-specific PP4c gene deletion causes abnormal thymic development and pre-TCR signaling (Shui et al., 2007). The molecular functions of different PP4R subunits are largely elusive and have been best defined for ternary PP4c-PP4R2-PP4R3 complexes that have been recently implicated in DNA damage repair (Chowdhury et al., 2008; Lee et al., 2010). In accordance with the concept of modular PP4c activity, we have identified a distinct PP4c-PP4R1 complex as a negative regulator of NF-κB signaling in T lymphocytes.

The fact that previous studies have rather linked PP4c to positive regulation of NF-κB activity (Li et al., 2006; Yeh et al., 2004) may be explained by ambivalent signaling functions of PP4c that are most likely determined by the coaction of different regulatory subunits; for example, PP4c has been shown to directly target NF-κB subunits downstream of the IKK complex (Yeh et al., 2004). Pleiotropic and counteracting functions of PP4c might mask and superimpose separate PP4c signaling roles and, therefore, complicate its functional analysis.

CTCL represents a clinically heterogeneous group of primarily skin-residing lymphomas among which MF and SS are the most common entities. SS is the prototypic variant of an aggressive skin-homing leukemia that often transformed from a MF stage (Hwang et al., 2008). A growing body of evidence has established a key role of NF-κB in CTCL progression and survival. Recent reports demonstrated constitutive activation of the canonical NF-κB pathway in the CTCL cell lines SeAx, MyLa, Hut78, and HH, as well as in malignant peripheral blood T lymphocytes from patients with SS (so called Sézary cells) (Sors et al., 2006). Consistently, we observed an NF-κB-specific target gene expression signature for the CTCL lines SeAx, Hut78, and HH in comparison to Jurkat T cells. Constitutive activation of the NF-κB pathway does not seem to be a paraneoplastic event but is indispensible for the continued proliferation and survival of CTCL cells in vitro and in vivo (Kesseling et al., 2009). The dependence of CTCL cells on chronic NF-κB signaling for survival reflects a general phenomenon of oncogenesis, known as oncogenic addiction (Staudt, 2010). The NF-κB-addicted malignant phenotype has also been reported for other lymphoid malignancies, such as ABC-DLBCL or Hodgkin’s lymphoma for which a number of dominant active, oncogenic mutations for components of the BCR-induced NF-κB pathway as well as inactivating mutations of the negative regulator A20 have been identified, causing constitutive, ligand-independent NF-κB activity (Compagno et al., 2009; Lenz et al., 2008; Staudt, 2010). In contrast, the molecular etiology underlying constitutive NF-κB signaling in CTCL cells remains largely unknown (Kim et al., 2000).

Here we report significant downregulation of PP4R1 expression in a fraction of CTCL cell lines and primary CTCL cells from SS patients, indicating a role of PP4R1 as a suppressor of oncogenic NF-κB in CTCL. Indeed, PP4R1 deficiency correlated with constitutive IKKβ activity in PP4R1-deficient CTCL cells. Forced re-expression of PP4R1 in HH cells reversed constitutive IKK activation and NF-κB signaling, indicating that PP4R1 deficiency may promote IKK hyperactivation in these cells. Moreover, reconstitution of PP4R1 expression decreased the abundance of HH cells, demonstrating that PP4R1 deficiency unleashes IKK activity and promotes oncogenic NF-κB signaling. However, we do not exclude the possibility that PP4R1 deficiency is a necessary rather than sufficient prerequisite for dysregulated IKK/NF-κB signaling in these cell and additional molecular lesions may contribute to the aberrant NF-κB signaling phenotype (Kesseling et al., 2011; Kim et al., 2000). Moreover, the observed effect of PP4R1 re-expression on CTCL abundance may be attributed to pleiotropic functions culminating in increased cell death, decreased proliferative capacity, and/or metabolic disadvantages compared to PP4R1-deficient cells.

Collectively, our results provide genetic, biochemical, and functional evidence for PP4R1 as a physiological suppressor of the NF-κB pathway in T lymphocytes. PP4R1 acts as a regulator of IKK activity, and PP4R1 deficiency results in deregulated IKK/NF-κB signaling as well as T cell hyperactivation and is required for the maintenance of the malignant phenotype of a subset of CTCL cells.

**EXPERIMENTAL PROCEDURES**

**siRNA Library and NF-κB Reporter Cells**

For RNAi screening, a subgenomic, commercially available siRNA library targeting 298 known or putative phosphatase genes was used (Applied Biosystems). The library contained three single, independent siRNA oligos per gene, yielding a total of 894 phosphatase-specific siRNAs. Each individual siRNA was rearrayed in a 96-well plate format and transfected in duplicate. G-Luc Jurkat cells were generated by stably integrating a G-Luciferase reporter cassette under the control of 8 × NF-κB sites into the Jurkat T cell clone J16-145.

**RNAi Screening Procedure**

Large scale siRNA transfection of G-Luc Jurkat cells was performed by nucleofection using a 96-well shuttle system (Lonza) according to the manufactures’
instructions. For each 96-well transfection plate, a panel of positive and negative control siRNAs encompassing oligos specific for CYLD, RelA, Carma1, and TNF-R1 as well as nontargeting siRNA and mock transfections (H2O) were included. Seventy-two hr posttransfection cells were either left untreated or were stimulated using anti-CD3 (OKT3)/anti-CD28/goat anti-mouse antibodies (0.1 μg/ml each) or recombinant TNF-α (20 ng/ml). Five hr poststimulation Gausia activity was measured in the cell culture supernatant using a 96-well injection luminometer (Berthold Detection Systems) and normalized to cell viability using the Celltiter-Glo assay (Promega). All measurements were performed in duplicate.

**Retroviral and Lentiviral Transductions**

Retroviral supernatants were prepared by transfection of pMX IRES-GFP expression vectors into amphotropic Phoenix cells (Swift et al., 2001). For production of lentiviral particles, HEK293T cells, pretreated with 25 μg chloroquine for 1 hr, were transfected with TRC-derived shRNA vectors and a media using 10–30 μg plasmid (Lonza). For nucleofection, 2 to 5 × 10⁶ cells were transfected with 1 μg puromycin. The percentage of phosphatase-reconstituted cells was normalized by flow cytometry. For a single assay, cells were transduced and analyzed in triplicate. The toxicity of ectopic phosphatase expression was examined by retroviral resistance (1 ml of viral supernatant. Stable integrants were selected by puromycin.

**Retroviral Reconstitution Assays**

The toxicity of ectopic phosphatase expression was examined by retroviral reconstitution of the CTCL lines SeAx, MyLa, Hut78, and HH. For the reconstitution assay, CTCL cell lines were retrovirally transduced with pMX-IRES-GFP vectors either expressing GFP alone (ctrl) or coexpressing WT human PP4R1, PP4c, or PP2Ac. The frequency of GFP-positive cells was monitored over time by flow cytometry. For a single assay, cells were transduced and analyzed in triplicate. The percentage of phosphatase-reconstituted cells was normalized to that of control-transduced cells at the same day. In addition, the ratio of GFP-positive cells at various time points was normalized to the initial ratio at day 3–5 posttransduction.

**Reverse Transcriptase PCR and Quantitative Real-Time PCR**

Total cellular RNA was isolated using the RNAqueous®-Micro Kit (Applied Biosystems). Total RNA (0.5–1.0 μg) was reverse-transcribed with a reverse transcription (RT)-PCR kit (Applied Biosystems). Quantitative RT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression was analyzed using the 7.500 Real-Time PCR Systems and Sequence Detection Software version 2.0.1 (Applied Biosystems). For some experiments, Universal Probe Libray (UPL) assays were designed and quantitative RT-PCR was performed using the ProbesMaster Kit and the LightCycler® 480 System (Roche). Relative expression was determined from cycle threshold (Ct) values and was normalized using hypoxanthine phosphoribosyltransferase 1 (HPRT1) and/or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference genes. Sequences of cPCR primers are available upon request.

**Cell Culture, Transfections, and Reporter Assays**

Human peripheral T cells were prepared and cultivated as described previously (Peter et al., 1997). For transient reporter assays, 1 × 10⁶ Jurkat T cells or 2 × 10⁶ 293T cells were cotransfected with p3×AP-1-Luc and pfos-LacZ or pGL8×NF-κB-fos and pfos-LacZ as described previously (Arnold et al., 2001). Transfection efficiency was normalized to LacZ expression. Values represent mean and SD of triplicate measurements. HEK293T cells were transfected by Ca²⁺-phosphate coprecipitation using 3–10 μg of plasmid DNA. After 48 hr, cells were lysed in lysis buffer (30 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, and 1% Triton X-100, Serva’s, and 10% glycerol). For transient reporter assays, 1 × 10⁶ Jurkat T cells were transfected by electroporation (250V, 950 μF) in 400 μl of IMDM media using 10–30 μg plasmid DNA. For siRNA and cDNA transfections, primary human T cells or Jurkat T cells were transfected by nucleasefection (Lonza). For nucleasefection, 2 to 5 × 10⁶ cells were transfected with 1 μg siRNA.

**Immunoprecipitation, Immunoblotting, and In Vitro Kinase Assays**

Cells were lysed using 1% NP-40 in 20 mM Tris pH 7.4 and 150 mM NaCl supplemented with protease and phosphatase inhibitors. Two micromgs of antibody coupled to Protein A sepharose was used to immunoprecipitate proteins from cell lysates for 2–18 hr at 4°C. Proteins were resolved by SDS-PAGE, transferred to Hybrid nitrocellulose membrane (Amersham Pharmacia Biotech) and processed according to the manufacturer’s protocol. In vitro kinase assays were performed as described previously (Brenner et al., 2005). Horseradish peroxidase (HRPO)-conjugated antibodies (Abs) were purchased from Southern Biotechnology Associates. The Abs used were anti-PP4R1, and anti-PP4a (all from Bethyl), anti-PP2Ac (Thermo Scientific), anti-FLAG (M2, Sigma), anti-MA (SF10, Roche), anti-Erk, anti-p-β-actin (Sigma), anti-iKKα; (FL-419), and anti-PP1c (4033) (all from Santa Cruz), anti-phospho-ERK (BD Biosciences), anti-Carm1 (ProSci), anti-iKKβ (Imgenex), anti-iKKα, and anti-phospho-iKαβ (Cell Signaling).

**Patient Data**

CTCL was diagnosed according to the WHO-EORTC classification of cutaneous lymphomas and the criteria of the International Society of Cutaneous Lymphomas. This study includes patients with MF and SS seen at Mannheim University Medical Centre and the University Hospital Cologne and was performed in accordance with the ethical guidelines of the German Cancer Research Center (DKFZ, Heidelberg) and with the provisions of the Helsinki protocol. Patients signed the institutional consent forms for use of tissue for research. In total, 32 CTCL patients were included for analysis of PP4R1 expression by qPCR (n = 12), immunoblot analysis (n = 12), or IHC analysis of skin biopsies (n = 8).

**Statistical Analysis**

Mean and SD for all quantitative measurements are representative of triplicate measurements. In order to examine statistical significance the Student’s t test (independent two-sample t test, unequal variance) was used. A p value < 0.05 was considered as statistically significant. Overall, the level of statistical significance was defined as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.07.014.

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