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Review

PKCζ at the crossroad of NF-κB and Jak1/Stat6 signaling pathways

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Abstract

The atypical protein kinase C (PKC) isoforms (aPKC) have been implicated in the regulation of a number of essential signaling events. Early studies using dominant-negative mutants suggested that they are important intermediaries in the activation of the canonical nuclear factor (NF)-κB pathway. More recent data using knockout mice genetically demonstrate that in fact the PKCζ isoform is essential for the adequate activation of this cascade both upstream and downstream the IκB kinase complex. In this review, we summarize the mechanistic details whereby the aPKC pathway regulates important cellular functions and how this is achieved by the ability of these kinases to interact with different protein regulators and adapters, as well as to impinge in NF-κB-independent signaling cascades such as the Janus kinase-1/signal transducer and activator of transcription 6 system, which plays a critical role in T-cell-mediated hepatitis and asthma.
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Abbreviations: BCR, B-cell receptor; CBP, CREB-binding protein; COPD, chronic obstructive pulmonary disease; CREB, cyclic AMP-binding protein; Dif, dorsal-related immunity factor; EGF, epidermal growth factor; ERK, extracellular responsive kinase; IFN-γ, interferon-γ; Ig, immunoglobulin; IκB, inhibitor of κB; IKK, IκB kinase; IL, interleukin; Jak1, Janus kinase-1; JNK, Jun kinase; KID, kinase-inducible transactivation domain; LPS, lipopolysaccharide; LT, lymphotoxin; MEK, MAPK/ERK kinase; MSK-1, mitogen- and stress-activated protein kinase 1; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; Par-4, prostate androgen responsive-4; Par-6, partitioning defective-6; PI-PLC, phosphoinositide-specific phospholipase C; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RANK-L, receptor activator of NF-κB-ligand; RIP, receptor-interacting protein; Stat6, signal transducer and activator of transcription 6; TCR, T-cell receptor; Th, T helper; TNFα, tumor necrosis factor α; TRAF, TNF receptor-associated factor; UBA, ubiquitin associated; XIAP, X-linked mammalian inhibitor of apoptosis protein

Introduction

Cell signaling is an exciting field of research because it not only will give us a full understanding of important biological processes in health and disease but also is serving to identify novel and more selective therapeutic targets to design the medicines of the future. The mechanisms regulating specificity and crosstalk, two important aspects of cell signal transduction, need to be investigated if we want to know how a cell works under the control of complex activating/repressing networks. Specificity is especially relevant when we are dealing with kinases, such as the atypical protein kinase C (aPKC), which are tremendously promiscuous in the selection of their substrates.[1] Therefore, for these enzymes to function efficiently and selectively, there must be ways to constrain their activity and, more importantly, their ability to specifically target substrates at a given time and cell location during activation. The second concept, crosstalk, is likewise important since cells are confronted with complex biological situations, such as embryonic development or inflammation, in which a single kinase may be required to perform a variety of functions by targeting different substrates. Unraveling the mechanisms underlying both processes at a molecular level is of paramount importance to understand how information flows through the different pathways, and how cells are able to cope with complex biological or pathophysiological conditions. A detailed understanding of these phenomena will certainly offer new venues to design better and more selective drugs for the treatment of complex diseases such as cancer and inflammation.

The Atypical and the Other PKCs

The protein kinase C family of isozymes is formed by a large number of members that are grouped in three subfamilies (Figure 1), depending on their structure and mechanism of regulation.[2] All the isoforms have relatively well-conserved kinase domains at their C-terminus and a clearly divergent regulatory region at the N-terminus. The topology of the regulatory domain serves to ascribe a given PKC isotype to a particular subfamily. Thus, the classical isoforms have a phospholipid and a Ca^{2+}-binding region and a double zinc-finger domain that have been shown to account for the ability of these PKC subtypes to be regulated by Ca^{2+} and lipid second messengers, such as diacylglycerol, which are

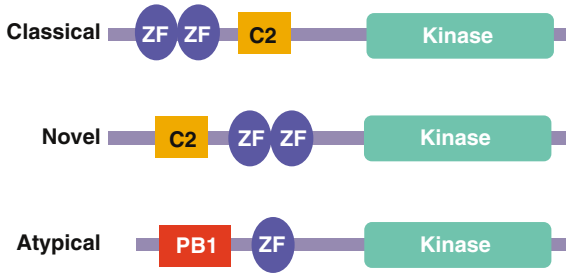

Figure 1 Schematic representation of the different PKC subfamilies. C2, C2 domain; PB1, PB1 dimerization domain; ZF, zinc-finger

generated as a consequence of the activation of the phosphoinositide-specific phospholipase Cs (PI-PLC).[2] Genetic evidence of a functional interaction between PI-PLC and the classical PKC isotypes has been obtained in studies of B-cell activation in the immune system. Thus, the genetic ablation of PLC-γ2 produces defects in B-cell function similar to those induced as a consequence of the genetic inactivation of PKC-β.[3,4] On the other hand, genetic and biochemical studies have functionally linked a novel PKC isoform, PKCθ, as a *bona fide* downstream target of PLC activation in mature T cells.[5] The so-called novel PKC subfamily is composed of members that, in contrast to the classical isotypes, are insensitive to Ca^{2+} but, like the classical, can be activated by phorbol esters which are stable pharmacological analogs of diacylglycerol.[6] The reason why phorbol esters activate the classical and novel PKCs may be due to the fact that their regulatory domains harbor a double zinc-finger motif.[7] The atypical PKCs are insensitive to Ca^{2+} and diacylglycerol probably because they lack the Ca^{2+}-binding region and have only a single zinc-finger domain, which suggests that their mechanism of activation must be necessarily different from that of the other PKC isotypes. Two different isotypes belong to the atypical subfamily: PKCζ and PKCλ/ι. They are highly related especially in their catalytic domain, but must clearly play different roles based on the different phenotypes of the respective knockout mice. Thus, PKC$\lambda/\iota-/-$ mice are embryonic lethal at very early stages probably due to defects in cell polarity (Diaz-Meco and Moscat, unpublished observations), whereas the PKC$\zeta-/-$ mice are born in Mendelian ratios, indicating that, whereas PKCλ/ι plays a role in the developing embryo, the PKCζ isotype does not seem involved in such a function.[8]

aPKC Cellular Functions in Cell Growth and Apoptosis

The initial studies to address the role of the aPKCs in cell function focused on the impact that their inhibition may have in the control of cell proliferation, and relied on experiments in which cells were microinjected with inhibitor peptides based on the sequence of the aPKC kinase's pseudosubstrate.[9] Those studies led to propose that the aPKCs were important players in the control of cell growth and survival. Subsequent transfection experiments using dominant-negative mutants of these kinases supported this notion and indicated that the target of their action was the nuclear factor (NF)-κB pathway.[10] This was considered to be of potential great relevance as NF-κB is critically involved in cell survival and differentiation, two important cellular parameters in immunity and inflammation.[11] However, both strategies, the pseudosubstrate peptides and the dominant-negative mutants, are not totally specific when one intends to establish the selective role played by PKCζ *versus* PKCλ/ι, because the pseudosubstrate sequences are identical in both isoforms and the dominant-negative mutant experiments may be impinging pathways other than those physiologically relevant for each atypical PKC isotype, a caveat of all such overexpression studies. The first independent indication that the aPKCs may in fact be relevant in apoptosis came from a completely unbiased study aimed at identifying potentially relevant regulators of these PKCs by searching for interacting protein partners using the two-hybrid system in yeast.[12] This strategy led to the identification of the proapoptotic protein prostate androgen responsive-4 (Par-4) as an atypical PKC-binding protein. This protein binds both aPKCs and inhibits their enzymatic activity.[12] This is very interesting because Par-4 had been previously identified by Sells and co-workers in screenings designed to isolate genes involved in apoptosis.[13] Importantly, the overexpression of Par-4 in cell model systems inhibits NF-κB activation and activates apoptosis.[14] This is consistent with, and constitutes an independent confirmation of, the proposed role of the atypical PKCs in NF-κB activation. The genetic inactivation of Par-4 in mice allowed the generation of Par-4$-/-$ embryo fibroblasts, and experiments, which confirmed the role of Par-4 as a naturally occurring negative regulator of NF-κB and positive inducer of apoptosis.[15] Thus, Par-4$-/-$ embryonic fibroblasts show enhanced NF-κB transcriptional activity and elevated levels of the NF-κB target and antiapoptotic protein, X-linked mammalian inhibitor of apoptosis protein (XIAP). Interestingly, the upregulation of XIAP by NF-κB was reported to downregulate Jun kinase (JNK) and the subsequent apoptotic response.[16] Consistently, JNK activation was severely inhibited in Par-4$-/-$ cells in keeping with the fact that they have enhanced NF-κB activity and XIAP levels.[15,17] Considering the link between decreased apoptosis and tumor progression, Par-4 should be important from the point of view of cancer and its potential therapeutics. In this regard, in good agreement with this notion, tumor cells and tissues display dramatically reduced levels of Par-4, suggesting that Par-4 expression is a roadblock for tumors to develop.[18] The functional relevance of this finding is highlighted by observations that restoration of Par-4 levels in Ras-transformed cells to the values of the parental nontransformed cells makes the tumor cells more sensitive to the action of anticancer drugs both *in vitro* and *in vivo*.[18] This suggests that Par-4 behaves as a tumor suppressor gene. Proof of this assertion is the fact that Par-4$-/-$ mice are prone to spontaneously develop tumors with the endometrium and the prostate particularly sensitive to the development of proliferative lesions.[19] Consistent with these observations, it has been shown recently that PKCι contributes to poor prognosis in ovarian cancer.[20] Interestingly, like in the embryonic fibroblasts, the loss of Par-4 leads to enhanced expression of XIAP in both the uterus and prostate,[19] validating *in vivo* the Par-4/PKCζ/NF-κB/XIAP axis as a critical controller of tumor development.

Genetic Inactivation of PKCζ Reveals its Role in the Control of RelA Transcriptional Activity

The genetic inactivation of RelA and IκB kinase (IKKβ), two major mediators of NF-κB activation, is lethal due to tumor necrosis factor (TNF)α-dependent liver apoptosis during gestation.[11] However, PKCζ gene-deficient mice do not display liver toxicity, although NF-κB and IKK activation is impaired in lung extracts from mice injected with TNFα and lipopolysaccharide (LPS).[8] The lung results suggest that PKCζ may be upstream of IKK in this pathway. However, the analysis of this cascade in embryonic fibroblasts reveals the surprising observation that, whereas the expression of at least some NF-κB-dependent genes is inhibited in PKCζ−/− cells, the activation of IKK or the nuclear translocation of NF-κB is not affected.[8] Also, apoptosis in response to TNFα is increased in PKCζ−/− fibroblasts, which is a clear hallmark of NF-κB deficiency.[8] Therefore, PKCζ is required for NF-κB transcriptional activity, but not for the initial steps of the activation of this pathway. This is in line with recent evidences indicating that the regulation of NF-κB takes place at different steps of the pathway and not only through the regulation of IKK. In this regard, the phosphorylation of the RelA subunit of the NF-κB complex has received considerable attention. The pioneer work of Ghosh *et al*.[21] identified Ser276 as a critical residue for the control of the transcriptional activity due to its ability to recruit, when phosphorylated, the transcriptional cofactor cyclic AMP-binding protein (CREB)-binding protein (CBP). The initial work of Ghosh suggested that Ser276 is a protein kinase A (PKA)-targeted residue, but TNFα or the other cytokines implicated in NF-κB activation do not appear to be potent activators of this kinase. Studies have indicated that Ser276 may be constitutively phosphorylated at least in some cells,[22] whereas recent reports suggest that mitogen- and stress-activated protein kinase 1 (MSK-1) may be the Ser276 kinase activated by TNFα in cell culture experiments.[23] However, more *in vivo* studies should be carried out to firmly confirm these initial cellular observations. Other studies suggest that Ser536 is phosphorylated by the IKK complex, but this site seems to negatively regulate the activity of RelA by modulating its proteasomal degradation, playing a role in the resolution of inflammation by the IKKα subunit of the IKK complex.[24]

The loss of PKCζ impairs RelA phosphorylation in response to TNFα, which suggest that this kinase is in fact a RelA kinase. Recent data demonstrate that PKCζ directly interacts with NF-κB once this is released from IκBα following IKK action phosphorylates and Ser311.[25] This, like in the case of Ser276 phosphorylation, serves to recruit CBP allowing κB-dependent transcription.[25] Interestingly, PKA-driven phosphorylation of Ser133 in the transcription factor CREB was early shown to mediate the recruitment of CBP. Ghosh *et al*.[26] noted that the amino-acid sequence surrounding Ser133 in CREB and that around Ser276 in RelA show a high degree of homology. This region in CREB, termed kinase-inducible transactivation domain (KID), contacts the KIX domain of CBP.[27] Phosphorylated Ser133 is located in the N-terminus of KID and makes the contacts required for KID-KIX stabilization.[28] Of note, whereas Ser276 (in RelA) and Ser133 (in CREB) lie at different ends of the homologous region, Ser311 in RelA is located at the same end as Ser133 in CREB. The implications of this are unclear and must wait the availability of protein cocrystallization data. In any case, the fact that both Ser276 and Ser311 are required for CBP recruitment and taking into account that they seem to be independently targeted by PKA and PKCζ, respectively, suggests that both sites may regulate independently CBP binding and NF-κB transcription in response to separate pathways. Further *in vivo* studies should address this important question.

The Atypical PKCζ in the *Drosophila* Immune Response

The NF-κB pathway is remarkably conserved in *Drosophila*, controlling not only development but also the innate immune response.[29] Thus, the RelA homologs in *Drosophila*, dorsal-related immunity factor (Dif) and Dorsal, have been shown to be necessary for the synthesis of the antimicrobial peptide drosomycin in response to the activation of the Toll pathway by fungal pathogens. This pathway involves the adapter Tube, the kinase Pelle and the phosphorylation, and subsequent degradation of the inhibitor of κB (IκB) homolog, named Cactus. Pelle resembles IRAK and is required for Cactus degradation and the subsequent release of Dif for its nuclear translocation. However, the kinase directly involved in the activation of Cactus still remains to be discovered. Parallel to this pathway, there is another one in *Drosophila* that involves the kinase dTAK1, which acts upstream the *Drosophila* IKK complex and that serves to control the degradation of Relish. Relish is the fly homolog of NF-κB1/NF-κB2 and is required for the synthesis of diptericin in response to bacterial infection. Interestingly, knocking down PKCζ with RNAi in *Drosophila* cells dramatically inhibits drosomycin expression but not that of diptericin, indicating that PKCζ is located specifically in the Toll antifungal pathway.[30] Remarkably, PKCζ downmodulation does not affect Cactus or Relish degradation, but it does inhibit the drosomycin promoter activity of a luciferase reporter plasmid. Therefore, in *Drosophila*, like in mammalian cells, PKCζ is necessary for NF-κB transcriptional activity, but not for the control of the nuclear translocation of RelA or Dorsal/Dif. In this regard, PKCζ is capable of phosphorylating directly Dif, which suggests a high degree of conservation of PKCζ in the NF-κB pathways.[30] Notably, recent data confirmed these observations by direct injection of double-stranded RNA of *Drosophila* PKCζ and Ref(2)P into *Drosophila* adult flies.[31]

PKCζ and the Adaptive Immune System

The phenotypic analysis of PKCζ−/− mice reveals significant alterations in the development of secondary lymphoid organs in young animals.[8] Thus, although the overall structure of the spleen of these mice was preserved, there was a detectable defect in the marginal zone with smaller B-cell follicles in the white pulp. In addition to these alterations, there were also defects in peripheral and mesenteric lymph nodes and in Peyer's Patches showing impaired segregation between the

T- and B-cell zones and a decrease in the follicular dendritic cells.[8] This phenotype is complex and reminiscent of those of mutant mice for the TNFα receptor-1 (TNFR-1) and the lymphotoxin β-receptor (LTβ-R). In fact, the activation of κB-dependent genes is impaired in PKC$\zeta-/-$ fibroblasts not only when activated by TNFα or interleukin (IL)-1 but also in response to LT-β.[8] In this regard, other KO mice for NF-κB family members display defects in the development of secondary lymphoid tissues. Thus, p52$-/-$ mice as well as IKKα mice genetically engineered to express a kinase-dead mutant of IKKα, or knockout mice for the IKKα upstream kinase NIK, all show altered splenic architecture as well as impaired B-cell responses.[32] The p52$-/-$ mice show reduced formation of follicular dendritic cells, and alterations in the germinal centers and the marginal zone.[33] On the other hand, studies on p50$-/-$ mice also reveal the role of this protein in the development of marginal zone B-lymphocytes.[32,34] The deletion of the gene encoding for receptor activator of NF-κB-ligand (RANK-L) demonstrates the role of this cytokine in the development of lymph nodes, and Peyer's Patches, whereas signaling through the LT-β receptor is likewise necessary for the development of these secondary lymphoid organs as well as for a normal splenic architecture.[35] The TNFR-1$-/-$ mice, although have normal lymph nodes, are unable to form Peyer's Patches properly.[35,36] On the other hand, the double TNFα receptor/RelA knockout mice have a more profound phenotype characterized by lack of lymph nodes, Peyer's Patches, and organized spleen microarchitecture.[37] The congruence of phenotypes of the PKC$\zeta-/-$ mice and mice genetically deficient in TNFR family and NF-κB components suggests that PKCζ plays a role in signal transduction in one or more of these pathways, that is, TNFR-1, LTβ-R, and RANK. However, it should be emphasized that the loss of PKCζ does not lead to a total blockade in the development of the secondary lymphoid organs but just a delay, because as the mice age the defects in lymph nodes disappear and those of the Peyer's Patches become much less dramatic in adult mice.

The development of secondary lymphoid organs is controlled by dynamic interactions between stromal and hematopoietic cells. Interestingly, young PKC$\zeta-/-$ mice displaying defects in these organs also show a reduced percentage of mature B cells in peripheral and mesenteric lymph nodes, whereas in Peyer's Patches of these mutant mice the content of immature B cells was significantly increased.[8] Potentially relevant for this observation is the fact that B cells from PKC$\zeta-/-$ mice display increased spontaneous apoptosis and reduced proliferative responses when challenged through the B-cell receptor (BCR), but not by other activators such as CD40 or LPS.[38] This is specific for B cells, since the activation of T cells through the T-cell receptor (TCR) is not affected by the loss of PKCζ.[38] These observations are in keeping with the fact that these mice show a small, although reproducible, inhibition of serum immunoglobulin (Ig)M production when challenged with a T-independent antigen.[38] Surprisingly, the response to a T-dependent challenge was also impaired in the PKC$\zeta-/-$ mice.[38] Since T cells respond normally to an anti-CD3 stimulation, this observation suggests that PKCζ may be required for some kind of T-cell function but not directly downstream of the TCR signaling cascade. An interesting aspect of the role of the atypical PKCs in the control of adaptive immune response was discovered when the phenotype of the Par-4$-/-$ mouse was analyzed.[15] The loss of Par-4 leads to hyperactivation of both atypical PKCs and, therefore, its analysis will shed light on the role of both PKCs *in vivo*. The first striking observation was that these mice have a dramatically enlarged spleen due to a general increase in the number of splenocytes, without detectable alterations in the percentages of B cells or in CD4$+$ or CD8$+$ T cells.[17] In addition, the proportions of different subpopulations of B cells were not affected by the loss of Par-4, indicating that it may be a negative regulator of B- and T-cell proliferation, but not of the maturation programs of these cells. Interestingly, and consistent with the data from the PKC$\zeta-/-$ mice, the Par-4-deficient B cells hyperproliferate when challenged through the BCR, indicating that Par-4 is a *bona fide* negative regulator of PKCζ in B cells, since the B-cell phenotype of both mice is just opposite.[17] Because the loss of PKCζ does not affect T-cell proliferation, the observation that Par-4-deficient cells hyperproliferate in response to a TCR-dependent challenge was a surprise. However, since Par-4 targets both PKCζ and PKCλ/ι, these observations may suggest that PKCλ/ι, but not PKCζ, is critically involved in TCR signaling. This would account for the T-cell phenotype of Par-4$-/-$ mice. Consistent with this notion, Par-4 is induced during long-term incubation of T lymphocytes with anti-CD3 plus anti-CD28 and its loss leads to the hyperactivation of NF-κB, in keeping with the hypothesis that the aPKCs play a key role in the activation of this important transcription factor.[17] How PKCλ/ι regulates NF-κB in T cells in the context of other more established NF-κB activators and adapters, including PKCθ, is unclear and must wait until conditional PKCλ/ι knockout mice can be investigated. In any case, the loss of Par-4 promotes the hyperproliferation of T cells, probably – although not yet proven – through the stimulation of PKCλ/ι and NF-κB.

PKCζ in Th2 Polarization and IL-4 Signaling

Another unanticipated discovery from the analysis of the Par-4-deficient mice was the potential implication of the aPKCs in the control of T-cell polarization programs. It is well established that naïve CD4$+$ T cells can differentiate in response to antigen stimulation into two distinct subsets of effector cells, Th1 and Th2, which display distinct cytokine profiles and immune-regulatory functions.[39] Th1 cells produce interferon-γ (IFN-γ) and IL-2, and are essential for cell-mediated immune responses against intracellular pathogens.[39] Th2 cells produce a different set of cytokines, including IL-4, IL-5, IL-10, and IL-13, and are important in the control of humoral immunity and allergy.[40] The investigation of the signaling cascades that control Th2 differentiation and function is of paramount importance because it may lead to the identification of novel therapeutic targets for asthma and other allergic diseases.[41]

The differentiation program for Th2 cells is controlled essentially by the GATA-3 transcription factor, whose induction is required and sufficient for Th2 polarization.[42] In addition, other transcription factors such as c-Maf, nuclear

factor of activated T cells (NF-AT)c1, and NF-κB also contribute to this differentiation process, although evidence exists that, at least in the case of NF-κB, its actions are mediated by GATA-3 induction.[42] In contrast, Th1 polarization is controlled by T-bet.[42] A number of regulators and adapters have been genetically implicated in the Th2 differentiation process. Of special relevance for this review is PKCθ, since it is a member of the large family of PKCs, whose deletion in mice suggests its role in Th2 function *in vitro* and *in vivo*.[43–45] However, in contrast to PKCζ, PKCθ has been show to play a broader role in T-cell activation, including that of naïve T cells.[5] Thus, several reports demonstrate that PKCθ is critical for mature T-cell proliferation most likely acting upstream of the adapters Bcl-10 and Malt1.[46,47] However, further studies are required to totally establish the mechanism of action of this important PKC.

Interestingly, the induction of GATA-3 during Th2 polarization depends on signals emanating from the TCR and the IL-4 signaling cascade.[42] Genetic evidences demonstrate the critical role played by this latter pathway, which involves the transcription factor signal transducer and activator of transcription 6 (Stat6), *in vitro* and *in vivo*.[42] However, it should be borned in mind that IL-4 is a Th2-secreted cytokine and, paradoxically, is required for the Th2 polarization process. Therefore, IL-4 is required for the generation of the cells that are at the same time its producer. This apparent contradiction can be resolved if it is taken into account that there are sources other than the Th2 cells for the production of IL-4 and that, in fact, Th2 differentiation can be induced through IL-4-independent pathways such as those regulated by Notch ligands. In any event, it is clear that Th2 responses are dramatically reduced in Stat6$-/-$ mice, which reinforces the notion that the IL-4 signaling cascade is at least important for Th2 differentiation not only *in vitro* but also *in vivo*.[42] The first indication that PKCζ could be involved in Th2 polarization came from the studies on the Par-4$-/-$ mice. Thus, when CD4$+$ T cells were incubated during a prolonged period of time in the presence of anti-CD3 plus anti-CD28, it was apparent that secretion of IFN-γ, a hallmark of Th1 polarization, was not affected in the Par-4$-/-$ cell cultures, but that of IL-4 was significantly increased, suggesting that an aPKC could be involved in IL-4 production by T cells and consequently in Th2 polarization.[17] If this were the case, these observations could explain at least in part the inability of PKCζ-deficient mice to mount an optimal immune response when challenged with a T-dependent antigen, which was inconsistent with the fact that PKC$\zeta-/-$ naïve T cells did not show proliferative defects.[38] More importantly, serum IgE levels were dramatically reduced in PKC$\zeta-/-$ mice, suggesting a potential defect in IL-4 function, since this is an important cytokine also for B-cell Ig isotype switching to IgE.[38] Therefore, it seems plausible that PKCζ may play an important role during Th2 differentiation. In fact, when CD4$+$ T cells were induced to differentiate *in vitro*, it became apparent that the loss of PKCζ produced a dramatic inhibition in Th2 differentiation markers such as the secretion of IL-4 and other Th2 cytokines or the production of GATA-3.[48] Interestingly, PKCζ is induced during Th2, but not Th1, polarization and the activation of Stat6 phosphorylation and nuclear translocation was also robustly inhibited in the PKC$\zeta-/-$ cells.[48] Biochemical evidence locates PKCζ downstream the IL-4 receptor (IL-4R), but not as a direct target of TCR-derived signaling pathways, suggesting that PKCζ is a novel essential element in IL-4 signal transduction.[48] This pathway has been investigated extensively and consists of the tyrosine kinase Janus kinase-1 (Jak1), which phosphorylates the IL-4R α-chain, creating a docking site for the SH2 domain of Stat6 that is then recruited and phosphorylated by the receptor-bound Jak1. Once tyrosine-phosphorylated, Stat6 dimerizes and translocates to the nucleus. Importantly, the activation of Jak1 by IL-4 addition is severely impaired in PKC$\zeta-/-$ cells, indicating that this kinase is required for an optimal activation of the pathway.[48] The residue phosphorylated by PKCζ in Jak1 as well as the precise mechanism whereby this phosphorylation event controls Jak1 activation remain to be determined, but it is clear that there are additional cytokines that activate Jak1 independently of PKCζ and that PKCζ is not required for the stimulation of other Jak1-like tyrosine kinases, such as Jak3 or Tyk2. The structural constraints that account for this specificity have not been revealed yet, but suffice to say that PKCζ is recruited to the IL-4R α complex most likely through its ability to interact with Jak1, although whether other still unidentified components of this complex may confer specificity to the PKCζ recruitment to the IL-4R must await further research. The precise mechanism whereby PKCζ regulates Jak1 needs to be clarified. This will require mapping the PKCζ phosphorylation sites in Jak1, as well as cell transfection studies to determine the impact that mutation of these putative phosphorylation sites has in the formation of the Jak1/IL-4Rα complex.

All these observations would have been of limited significance had *in vivo* data not been reported on the role of PKCζ in physiological processes in which IL-4 and Th2 cells have been firmly established. One such process is allergic airway inflammation, or asthma. Compelling evidence demonstrated that the pathology of asthma is associated with aberrant activation of CD4$+$ T cells differentiated along the Th2 lineage.[49] Therefore, if PKCζ is relevant for Th2 differentiation, the induction of asthma in a model of ovalbumin-induced allergic airway disease should be impaired in PKC$\zeta-/-$ mice as compared to WT controls. In fact, the loss of PKCζ inhibits most of the signs of asthma in this model, including the recruitment of eosinophils in the bronchoalveolar fluid, as well as secretion of Th2 cytokines by activated T cells, strongly suggesting that PKCζ can be considered a therapeutic target in asthma.[48] However, it has been shown that PKCζ is required for NF-κB activation in response to TNFα in pulmonary artery endothelial cells,[50] a critical parameter in the expression of leukocyte adhesion molecules. Therefore, it could be argued that this function of PKCζ in lung-resident cells could also contribute to its role during asthma. When Th2 cells from WT mice were adoptively transferred to PKCζ-deficient mice, it was apparent that the requirement for PKCζ in asthma was overcome, indicating that it is the function of PKCζ in Th2 polarization that is critical for allergic airway inflammation, although a potential role of PKCζ in the lung stroma cannot be ruled out in other pulmonary inflammatory pathologies.[48] Actually, the evidence that PKCζ is heavily expressed in lung extracts under resting conditions would be consistent with a putative role of this

kinase in other pulmonary diseases, including chronic obstructive pulmonary disease (COPD) and lung cancer. Future studies should address this important question.

PKCζ at the Crossroad of Inflammatory Pathways in the Liver

The fact that PKCζ is able to play roles in two different signaling cascades (Stat6 and NF-κB) suggests that this kinase may work as a finely tuned switch whose regulation determines the final outcome of a given inflammatory response. This is particularly true in circumstances in which Stat6 and NF-κB play antagonistic roles. This concept is well exemplified in recent studies in which the signaling cascades activated during T-cell-mediated hepatitis in PKCζ and Par-4 KO mice were investigated.[51] This disease model is very interesting from the point of view of cell signal transduction as a whole network of cells and signaling molecules are set in motion in response to an immunological insult and, in addition, this serves as an important model system of a worldwide human health problem. This model is induced in response to the injection of concanavalin A (ConA), which leads to T-cell-mediated hepatitis with features of autoimmune hepatitis. This is important because the immune response is central to the pathogenesis of hepatitis whatever the etiology of the disease. As NF-κB is important in the control of immunity and inflammation, the investigation of the role and function of this pathway in T-cell-mediated hepatitis is the subject of recent important studies. In addition, the liver is a particularly relevant tissue from the point of view of NF-κB activation, since this transcription factor has been shown genetically to be essential in the control of TNFα-induced apoptosis during embryonic development.[11] Actually, both RelA−/− and IKKβ−/− mice die of liver apoptosis during gestation. Is NF-κB also playing a critical role in hepatocyte survival in adult mice subjected to an inflammatory insult? Two recent studies have addressed this important matter using liver-specific IKKβ conditional KO mice and two models of inflammatory-mediated liver damage. The first model relates to the action of LPS, which provokes the release of TNFα that, in the absence of NF-κB, induces lethality as consequence of liver failure. If NF-κB is an important survival factor in TNFα-induced liver damage as the data from the classical studies of RelA−/− and IKKβ−/− mice demonstrate, the prediction is that injection of LPS should induce lethality in the liver-specific conditional IKKβ KO mice as compared to the WT controls. Surprisingly, both studies using these mutant mice show that the loss of IKKβ in the liver does not sensitize this organ to LPS- or TNFα-induced cell death.[52,53] However, liver NF-κB activation is severely inhibited, as is the synthesis of a series of NF-κB-dependent genes. These results suggest that inhibition of NF-κB activation is not sufficient to induce hepatocyte death in response to even high doses of circulating TNFα. However, Luedde *et al*.[53] show that liver-specific IKKγ (Nemo) conditional KO mice experience massive liver apoptosis when injected with TNFα. These apparently paradoxical results could be explained if it is considered that the genetic inactivation of IKKγ leads to a more complete inactivation of NF-κB than that observed in IKKβ conditional KO mice. An alternative explanation to these findings is that NF-κB is important for the prevention of cell death induced by membrane-bound TNFα, which targets both TNFα receptor 1 and 2.[52] In contrast, circulating TNFα, that is produced in LPS-injected mice, only targets receptor 1 and activates JNK less potently than membrane-bound TNFα. This is produced in ConA-injected mice, and activates JNK more potently than the circulating TNFα and therefore is a more powerful inducer of apoptosis in the absence of NF-κB. In agreement with this model, Maeda *et al*.[52] elegantly show that although the liver-specific IKKβ conditional mice are resistant to LPS-induced lethality, they readily undergo massive liver apoptosis and lethality when injected with ConA. Therefore, one may predict that NF-κB will play a beneficial role as a liver protective factor in pathologies such as immune-mediated fulminant hepatic failure. Since PKCζ is important for NF-κB activation, it would be of interest to determine whether these mice will be likewise resistant to LPS or circulating TNFα but sensitive to ConA-induced liver damage. Unexpectedly, ConA injection did not induce lethality or liver apoptosis in PKCζ-deficient mice, despite the fact that NF-κB activation was ablated in the mutant liver.[51] These results were surprising and could only be explained if PKCζ, in addition to being critical for ConA-induced NF-κB, could also be important in a pathway required for liver inflammation and damage provoked by ConA-activated T cells (Figure 2). Interestingly, it has been genetically demonstrated that the IL-4/Stat6 pathway plays an essential role in the synthesis of eotaxin and IL-5, two important mediators of eosinophil recruitment and induction of liver damage.[54] In this regard, it is clear that the loss of PKCζ severely inhibited the synthesis of IL-5 and eotaxin-1 in ConA-injected mice, as well as in primary mouse embryo fibroblasts challenged *in vitro* with IL-4, in agreement with the role of PKCζ in Th2 polarization.[51] Both mediators are essential players in the regulation of T-cell mediated hepatitis at the level of heptocytes and NKT cells (Figure 3). This requirement of PKCζ for the induction of these inflammatory mediators very well correlated with inhibited tyrosine phosphorylation of Jak1 and Stat6. However, when these pathways were analyzed in mice and cells deficient in the PKCζ negative regulator, Par-4, ConA-induced liver injury was enhanced and the activation of the Stat6 pathway was more prominent than in WT mice.[51] Therefore, activation of

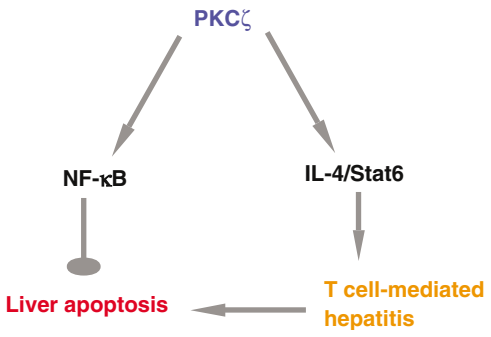

Figure 2 Potential involvement of PKCζ in pathways other than NF-κB in ConA-induced hepatitis. Liver apoptosis is positively promoted by the IL-4/Stat6 pathway and antagonized by NF-κB

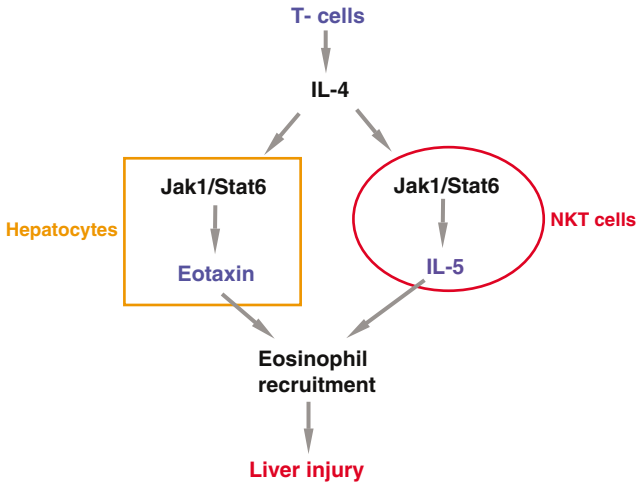

Figure 3 Mechanism of liver injury activated by IL-4. IL-4 activated Jak1/Stat6 in hepatocytes to produce eotaxin, whereas triggers IL-5 production by natural killer T cells (NKT). Both mediators promote liver eosinophil recruitment and damage

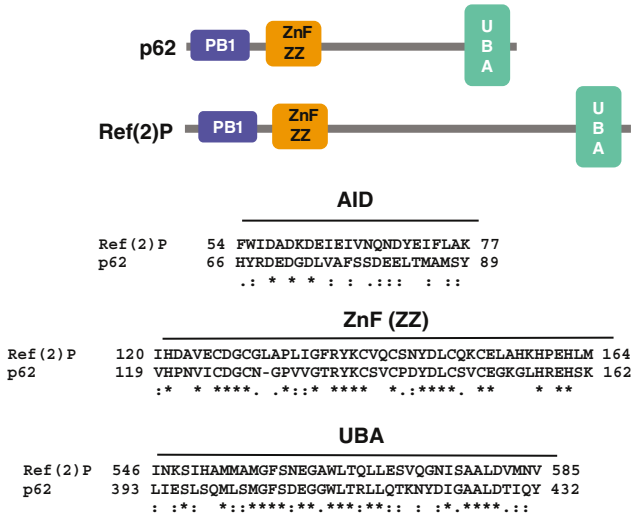

Figure 4 Sequence alignment of the different signaling modules of p62 and Ref(2)P. Overall structures of p62 and its *Drosophila* homolog Ref(2)P are shown. Also, the sequences of the AID region in the PB1 domains, the zinc-finger and the UBA domain alignments are shown. *, identical residues; ., conserved residue; **:** , highly conserved residues

PKCζ is required for ConA-induced hepatitis due to its role in the activation of Jak1, and the fact that NF-κB is not activated is irrelevant under these conditions because liver damage does not occur. However, there are conditions at which PKCζ becomes redundant for the activation of the IL4/Stat6 pathway in liver, such as, for example, when mice were challenged with high doses of ConA. Under these circumstances, Stat6 is activated in the absence of PKCζ and the KO mice display more, not less, liver injury than the WT mice because NF-κB activation is still severely reduced in the PKCζ-deficient liver, but the Stat6 pathway is fully functional.[51] Therefore, PKCζ emerges as a critical control step that determines the final outcome of a biological complex process such as liver inflammation, thanks to its ability to control what in the livers are two antagonistic signaling pathways, and depending on a number of conditions such as the intensity of the signal. These observations reinforce the concept that considerable crosstalk must be established when cells 'have to address' intricated pathophysiological situations such as inflammation, in which a whole collection of signaling networks is set in motion.

Adapters and the Specificity During Cell Signaling

The PKCs are kinases that display little specificity *in vitro* and presumably *in vivo*, unless cells have mechanisms that impose specificity to the kinases' actions. As the aPKCs have been implicated in a relatively diverse series of functions, one hypothesis is that different adapter proteins that would serve to provide the required selectivity must exist.[7] In other words, the aPKCs would be the 'catalytic subunit' of different signaling complexes designed to perform different functions. Two-hybrid screenings in yeast using the regulatory domain of PKCζ as the bait identified p62 as a selective adaptor for the aPKCs.[55,56] p62 interacts with PKCζ and PKCλ/ι, but not with any of the other closely related PKC family members. It is not a substrate and does not seem to significantly affect the intrinsic kinase activity of PKCζ or of PKCλ/ι. However, p62 has a number of domains that strongly suggested its role as a novel scaffold protein.[56] Interestingly, p62 was shown to be required for NF-κB signaling in several systems[57–59] including *Drosophila*, in which a functionally relevant homolog termed Ref(2)P was identified.[30] This protein has an overall structure very similar to that of p62 (Figure 4), suggesting that it may be a critical mediator of NF-κB in *Drosophila* cells in partnership with PKCζ. In fact, the knockdown of Ref(2)P with RNAi in *Drosophila* cells leads to the impairment of Drosomycin expression.[30,31] Therefore, this indicates the remarkable state of conservation for the role of PKCζ in the control of the transcriptional activity of NF-κB downstream of IκB degradation, which reinforces the importance of this kinase in the regulation of NF-κB and the innate immune response.

Several studies demonstrated that p62 harbors at its N-terminal sequence a domain, named PB1,[60] whose crystal structure has been resolved and that accounts for the interaction with PKCζ, which also has an N-terminal PB1 region.[61–64] Therefore, the mechanism whereby PKCζ binds p62 involves homotypic interactions between their respective PB1 regions. Solution structure and crystallographic and mutational analysis has revealed at the atomic level the interactions that govern the binding of the aPKCs to p62. More importantly, PB1 domains have also been identified in other scaffold proteins such as partitioning defective-6 (Par-6) and MAPK/ERK kinase (MEK)5.[7,65] Par-6 has been shown genetically in *Caenorhabditis elegans* and *Drosophila* to be critically implicated in the control of cell polarity.[66,67] Although genetic data have not yet been produced to prove the role of the aPKCs and Par-6 in different aspects of mammalian cell polarity, overexpression analysis indicate that the Par-6/aPKC complex can be implicated in, for example, the control

of epithelial–mesenchymal transition,[68] T-cell[69] and neuronal polarity,[70] or cell polarity in migrating astrocytes[71] among others. On the other hand, the interaction of MEK5 with the aPKCs serves to regulate extracellular responsive kinase (ERK)5, which leads to c-Jun regulation and cell growth in response to certain mitogens such as epidermal growth factor (EGF).[65] Therefore, the formation of aPKC complexes with different adapters and scaffold proteins serves to confer specificity and plasticity to these kinase actions.

The mechanistic link between p62 and NF-κB received further support when it was shown that TNF receptor-associated factor (TRAF)6 interacts with p62.[58] This is particularly relevant because TRAF6 is an important intermediary in the IL-1, NGF, and LPS signaling pathways controlling NF-κB through still not totally clarified mechanisms likely involving K-63 ubiquitination of IKKγ.[72] Interestingly, Ref(2)P interacts not only with *Drosophila* PKCζ but also with the fly homolog of TRAF6, dTRAF2, reinforcing again the conservation of this pathway in flies.[30] In mammalian cells p62 also interacts with receptor-interacting protein (RIP), which mediates NF-κB activation in response to TNFα.[57] Both interactions seem to be physiologically relevant, since down-regulation of p62 levels with antisense constructs leads to a significant reduction of NF-κB activation in IL-1- or TNFα-activated cells. The generation of p62-deficient mice provided a unique opportunity to address the actual role of p62 *in vivo*. Interestingly, TRAF6$-/-$ mice are osteopetrotic due to the critical role that TRAF6 plays in the control of osteoclatogenesis in response to RANK-L,[73,74] which has also been shown genetically to be required for osteoclast differentiation *in vitro* and *in vivo*.[75] Interestingly, mutations in the gene coding for p62 are the cause of the Paget disease of bone (PDB), a genetic disorder characterized by aberrant osteoclastogenic activity.[76] Although the p62-deficient mice are not osteopetrotic, they show a clear impairment in osteoclastogenesis *in vivo* when injected with the calciotropic hormone PTHrP.[77] This suggests that although p62 may not be essential in basal bones for the control of osteoclastogenesis and bone remodeling, under induced conditions it may be important. In fact, p62-deficient osteoclast precursors respond poorly to RANK-L in cell cultures and are unable to produce a sustained NF-κB response.[77] Therefore, p62 emerges as a critical cell-type-specific NF-κB regulator in osteoclasts and a potential novel therapeutic target in osteoporosis and other bone diseases. As the PKCζ mice does not seem to have any alterations in osteoclastogenesis,[77] it is likely that the other aPKC, PKCλ/ι, is the essential aPKC component in this pathway although other possible mediators cannot be ruled out. In this regard, p62 harbors an ubiquitin associated (UBA) domain at its C-terminal region, which suggests its potential involvement in ubiquitin-mediated events, like, for example, the activation of the IKK complex. Ubiquitination of proteins of the NF-κB pathway in a K63-type fashion serves to assemble signaling complexes not linked to proteasomal degradation, whereas ubiquitination with K48-linked chains leads to proteasome degradation and inactivation of signaling cascades.[78,79] Recent data suggest that p62 preferably binds to K63-linked ubiquitinated proteins, which may open the possibility that p62 participates in the activation of the IKK complex through mechanisms other than, or in addition to, those mediated by the aPKCs.[80] Future studies will address this important question. In any case, these observations highlight the importance of specificity during cell signaling through the existence of specific adapters that restrict the kinase's action, but at the same time allows enough crosstalk between pathways, necessary to regulate complex biological situations such as inflammation. If p62 were also implicated in the IL-4/Jak-1 pathway, an important question to be solved would be the precise mechanism whereby two completely different ligands, such as IL-4 and TNFα, can use the same PKCζ cassette to perform their functions. This will require more biochemical and cell biology studies to investigate the cell kinetic and topology of the formation of the different signaling pathways when activated by the distinct stimuli.

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