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This Corrigendum relates to Biol. Open 2013, 2:1264–1269.

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Par-complex aPKC and Par3 cross-talk with innate immunity NF-κB pathway in epithelial cells

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Biology Open 2, 1264–1269
doi: 10.1242/bio.20135918
Received 20th June 2013
Accepted 20th August 2013

Summary
Components of the Par-complex, atypical PKC and Par3, have been found to be downregulated upon activation of NF-κB in intestinal epithelial cells. To determine their possible role in pro-inflammatory responses we transduced Caco-2 human colon carcinoma cells with constitutively active (ca) PKCα or anti-Par3 shRNA-expressing lentiviral particles. Contrary to previous reports in other cell types, ca-PKCα did not activate, but rather decreased, baseline NF-κB activity in a luminiscence reporter assay. An identical observation applied to a PB1 domain deletion PKCα, which fails to localize to the tight-junction. Conversely, as expected, the same ca-PKCα activated NF-κB in non-polarized HEK293 cells. Likewise, knockdown of Par3 increased NF-κB activity and, surprisingly, greatly enhanced its response to TNFα, as shown by transcription of IL-8, GRO-1, GRO-2 and GRO-3.

We conclude that aPKC and Par3 are inhibitors of the canonical NF-κB activation pathway, although perhaps acting through independent pathways, and may be involved in pro-inflammatory responses.

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Key words: Epithelial polarity, Par-complex, Atypical PKC

Introduction
Epithelial apico-basal polarity is controlled by signaling complexes such as the apical aPKC-Par6-Par3 (Par-complex, where atypical PKC comprises PKCζ and PKCδ isoforms). Recently, we found a steep down-regulation of aPKC and Par3 (Bazooka in Drosophila, not to be confused with Protease Activated Receptors) downstream of a common effector of innate immunity NF-κB, in human intestinal epithelial cells. It was demonstrated in Caco-2 cells (human colon carcinoma) in culture and in an animal model of colitis (Mashukova et al., 2011), as well as a negative correlation of aPKC expression with inflammation in enterocytes from Inflammatory Bowel Disease (IBD) patients (Wald et al., 2011). This effect on the Par-complex downstream of a pathway, previously thought to be independent, is important because chronic NF-κB activation is part of the mechanism that contributes to barrier (i.e. tight junction) opening in IBD (Rogler et al., 1998; Wullaert et al., 2011; Xavier and Podolsky, 2007). The functional implication is that the Par-complex is key to the organization of tight junctions (Wang and Margolis, 2007). The aPKC-Par6-Par3 complex is localized to tight junctions and the apical domain in polarized epithelia. This exquisite localization contrasts with a broad cytoplasmic and nuclear localization in non-polarized cells (Stross et al., 2009). The Par-complex is linked by Par6 (Joberty et al., 2000), which binds aPKC through an N-terminal PB1 domain and Par3 through a PDZ domain. The PB1-mediated Par6 binding is essential for localization and activation of aPKC (Graybill et al., 2012). An interaction between aPKC and Par3 through the kinase domain is dynamic, as Par3 direct phosphorylation in Ser827 by aPKC results in Par3 activation and dissociation from Par-αPKC (Mccaffrey and Macara, 2009). Par3 is a scaffolding protein with multiple interactions (Tepass, 2012). However, to our knowledge, no connection with innate immunity pathways has been described for Par3 so far.

The PKCζ and PKCδ, knock-out mice highlighted a role of aPKC activating NF-κB (Frey et al., 2006; Martin et al., 2002; Sajan et al., 2009a; Sajan et al., 2009b; Win and Acevedo-Duncan, 2008; reviewed by Díaz-Meco and Moscat, 2012). The mechanisms for NF-κB activation by aPKC involve IκKβ phosphorylation with the ensuing 1κB degradation (Win and Acevedo-Duncan, 2008) as well as direct phosphorylation and activation of relA(p65) by aPKC (Duran et al., 2003). Bearing in mind the effects of pro-inflammatory stimuli on the Par-complex proteins aPKC and Par3 in intestinal cells, we asked whether these proteins may, in turn, participate in the control of epithelial pro-inflammatory responses. The results showed an antagonistic effect of Par3-aPKC with pro-inflammatory signaling in epithelial cells. Such a role is contrary to the predicted function for aPKC.

Results
Expression of constitutively active PKCα fails to induce NF-κB activation in intestinal epithelial cells
To test the hypothesis that Par-complex aPKC regulates NF-κB activity in epithelia, we prepared two C-terminal V5-tagged constitutively active (ca) constructs as follows. A120E-PKCα is a...
ca-mutant with a non-functional internal inhibitory pseudosubstrate (Spitaler et al., 2000). Second, we also deleted the entire PB1 domain (aa 29–108), in the same ca-A120E background (hereafter referred to as ΔPB1 mutant). This mutant was meant to differentiate the scaffolding functions of the PB1 interaction (i.e. Par6 binding) from its kinase regulatory function. In other words, the ΔPB1-A120E-PKC\( \alpha \) is active but unable to bind Par6. A120E-PKC\( \alpha \) stably transduced with lentivirus in Caco-2 cells, localized normally to tight junctions and the apical domain, just like the endogenous aPKC. The ΔPB1-A120E-PKC\( \alpha \) mutant, conversely, showed a diffuse cytoplasmic and nuclear distribution (Fig. 1A). This nuclear redistribution was to be expected since PKC\( \alpha \) contains nuclear localization signals (Perander et al., 2001). It confirmed that aPKC localization to the tight junctions and the apical domain is strongly dependent on its PB1 domain interactions.

First, we transduced A120E-PKC\( \alpha \) under a tetracyclin-inducible promoter. Doxycycline induced the expression of A120E-PKC\( \alpha \) at levels similar to those of the endogenous aPKC as determined by comparing phosphorylation of the turn domain (pT555) (Fig. 1B, higher band, arrow shows the V5-tagged mutant; we used anti-pT555 antibodies because they recognize the active conformation of both PKC\( \alpha \) and PKC\( \beta \), i.e. total active aPKC). However, A120E-PKC\( \alpha \) failed to activate NF-κB as determined by IκB levels and a luciferase reporter assay (Fig. 1B–D): IκB significantly increased rather than decreasing (Fig. 1C), and A120E caused no increase in luciferase expression (Fig. 1D). TNFα stimulation was used as a positive control (Fig. 1D). Then, similar experiments were also conducted in Caco-2 cells stably transduced with either LacZ (control), A120E-PKC\( \alpha \), or ΔPB1-A120E-PKC\( \alpha \). Expression of both ca-mutants resulted in increased steady-state levels of IκB and relA(p65), and no changes in IKK\( \alpha \) phosphorylation (Fig. 1E). Furthermore, both ca mutants failed to stimulate NF-κB as indicated by the luminescence reporter (Fig. 1F). Instead, there were significant decreases in of luciferase expression (65 and 90%) in the ca-PKC\( \alpha \)-expressing cells as compared to LacZ-expressing cells. This suggests a steep decrease in the baseline activity of NF-κB. Conversely, ca-PKC\( \alpha \) expression failed to affect the response to TNFα as these responses were not statistically different form each other (Fig. 1F). These results were further confirmed by IL-8 mRNA transcription, which did not change in A120E-PKC\( \alpha \)-expressing cells (Fig. 1G).

In addition, we also determined RelA(p65) nuclear translocation in A120E-PKC\( \alpha \)-expressing Caco-2 cells. In TNFα-stimulated cells RelA signal appeared in the nucleus and cytoplasm (signal ratios near 1), while in non-stimulated cells or in A120E-PKC\( \alpha \)-expressing cells the nuclei remained mostly negative for relA, with nuclear/cytoplasmic ratios around 0.2. These low ratios were not statistically different from each other (Fig. 2). In summary, ca-aPKC failed to cause RelA nuclear translocation.

Constitutively active A120E PKC\( \alpha \) activates NF-κB in mesenchymal HEK cells

To rule out the possibility of unnoticed mutations in the ca-PKC\( \alpha \) constructs as a trivial explanation for the negative results in Caco-2 cells, we decided to confirm the biological activity of our ca-PKC\( \alpha \) mutants. Thus, we transduced human embryonic kidney (HEK293) cells with the same lentiviral vectors used in Figs 1 and 2. In these cells aPKC is known to activate NF-κB (Sanz et al., 1999). Unlike in Caco-2 cells, HEK cells expressing

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**Fig. 1. Expression of constitutively active PKC\( \alpha \) fails to activate NF-κB in intestinal epithelial cells.** (A) Caco-2 cells transduced with lentiviral particles expressing V5-tagged ca-A120E-PKC\( \alpha \), ca-ΔPB1-A120E-PKC\( \alpha \), or LacZ (control) and selected with blasticidin were fixed at 7 days after confluency. Bars, 10 μm. (B–D) Caco-2 cells were transduced with similar lentiviral particles expressing A120E-PKC\( \alpha \) under a tetracycline-inducible promoter. Expression was induced with 2 ng/ml doxycycline (dox). (B) Immunoblot from parallel cultures confluent for 10 days and induced (+) or not (−) for 24 hours. The V5-tagged PKC\( \alpha \) product can be observed as a slightly higher molecular weight band (arrow). (C) Quantification of signal from independent experiments similar to B. Matched samples t test: * \( P < 0.001 \), ** \( P < 0.05 \), n = 7. (D) Experiments similar to those shown in B–C were conducted but the cells were further transduced with lentiviral particles expressing luciferase under a NF-κB-inducible promoter and puromycin resistance. After puromycin selection, cells confluent for 10 days on filters were induced with doxycyclin or not, or stimulated with basolateral TNFα for 24 hours. * \( P < 0.05 \), n = 3. (E) Caco-2 cells were transduced and selected to express LacZ (control), A120E-PKC\( \alpha \) (A120E), or ΔPB1-A120E-PKC\( \alpha \) (ΔPB1) and analyzed by immunoblot. (F) Similar cultures were stimulated or not with TNFα and NF-κB activation was measured by a luciferase transcriptional reporter. * \( P < 0.05 \), ** \( P < 0.025 \), n = 3. (G) Expression levels of IL-8 mRNA were measured by RT-qPCR in LacZ- and A120E-PKC\( \alpha \)-expressing cells and expressed as fold change respect to the non-treated (−) control LacZ sample.
A120E-PKCi showed a diffuse cytoplasmic and nuclear localization with minimal localization to the cell-cell contact (Fig. 3A). HEK293 cells expressed other components of the Par-complex, such as Par3 and Par6 (Fig. 3B), but displayed a mesenchymal phenotype, with expression of vimentin and N-cadherin, instead of keratins or E-cadherin. Both cell types expressed the cognate aPKC PB1 binding proteins, Par6 and p62 (Fig. 3B). Therefore, the lack of localization of A120E-PKCi may be due to the absence of tight junctions in HEK293 cells. Stable expression of the ca-PKCi resulted in a sustained and significant decrease in IxB (Fig. 3C,D). Importantly, expression of A120E-PKCi strongly induced the expression of reporter luciferase (Fig. 3E,F, luc). Altogether, these data indicate that PKCi inhibits the NF-κB pathway in Caco-2 epithelial cells, an opposite effect to its action in other cells, including HEK293. This inhibition is independent of PB1 scaffolding interactions and PKCi subcellular localization.

Par3 inhibits NF-κB activation downstream of the TNFR pathway

Because Par3 is activated by aPKC (St Johnston and Ahringer, 2010), and it is also down-regulated upon pro-inflammatory stimulation (Mashukova et al., 2011), we expected that Par3 knockdown (kd) would have an opposite effect and rescue ca-PKCi effect on NF-κB. The lentiviral-delivered shRNA kd was performed in 3 experiments as described in C. * P<0.025. (E) Similar cultures expressing LacZ or A120E-PKCi were transduced with lentiviral particles expressing a luciferase (luc) NF-κB transcriptional reporter and puromycin selected. The cells were analyzed by immunoblot with the antibodies indicated on the left side. (F) Quantification of luciferase luminescence in 3 experiments as described in E. * P<0.001.

Discussion

Our results indicate that both aPKC and Par3 are antagonistic of pro-inflammatory signaling. The inhibitory effect of PKCi on NF-κB activation described here is likely not present in malignant cells where aPKC expression acts as an oncogene.
because in those cases NF-κB transactivation is necessary for the malignant phenotype (Lu et al., 2001).

Because HEK293 and Caco-2 cells express the same Par-complex proteins (Par3, Par6 and aPKC) and PB1 domain binding partners (p62 and Par6) (Fig. 3B), the difference between both cell lines must be downstream of the Par-complex. This conclusion is highlighted by the fact that the ca-PKCi with a deleted PB1 domain, which clearly did not incorporate into the Par-complex in Caco-2 cells, had a similar effect on NF-κB as the normally localized kinase. Both ca-PKCi expression or Par3 knock-down induce sustained changes in IκB steady-state levels and relA(p65) nuclear translocation, suggesting that the effect of Par-complex proteins takes place via the canonical activation mechanism for NF-κB. Hence, the data is consistent with the interpretation that a “switch” mechanism determining NF-κB inhibition in the epithelium (as opposed to activation in other cells) is upstream of relA(p65) cytosolic retention. In addition, we also conclude that Par3 effect on the TNFR pathway is likely independent of aPKC activation. It is of note that Par3 is known to interact with Gab1, which is also involved in epithelial polarity (Yang et al., 2012). In turn, Gab1 participates in the activation of NF-κB by TNFα in endothelial cells (Che et al., 2002).

The molecular switch or switches that control the activating or inhibitory effect of aPKC on NF-κB in different cell types and the molecular relationship between Par3 and the TNFR pathway will require additional investigations and may have profound implications not only for epithelial response to pro-inflammatory stimuli, but also for diseases involving the aPKC – NF-κB axis such as diabetes and obesity (Sajan et al., 2009a; Sajan et al., 2009b). The functional implication of these mechanisms is that the Par-complex may modulate responses that include secretion of cytokines in addition to the well-known tight junction opening (Pasparakis, 2012), an as yet unsuspected possible mechanism in chronic inflammation.

Materials and Methods

Lentivirus constructs and expression vectors

Lentiviral constructs expressing shRNA and puromycin resistance were obtained from Sigma (anti-Par3, TRCN 0000118134, scrambled non-mammalian shRNA SHC002V). Human PKCε ORF was obtained from Origene (SC118455 NM_002740) The mutagenesis of A120E-PKCε was achieved by PCR using modified primers (Kadowaki et al., 1989). Deletion of the PB1 domain was performed by primer extension PCR (Ho et al., 1989) using the A120E mutant as template. The mutants were verified by PCR sequencing. For protein expression, these constructs were cloned in a pLenti6.3/V5-DEST™ Gateway® Vector (K5330-Life Technologies), and packaged using ViralPower™ HiPerform™ Lentiviral Expression Systems (blastcidin resistance) (K5310-00, Life Technologies). For tet-inducible expression of PKCε-A120E, the cells, transduced and selected as described above, were passed 1:4, immediately transduced with lentiviral particles expressing TR, and selected in geneticin in addition to blasticidin. This order of transduction was found to be essential. Tet-transduced and selected as described above, were passed 1:4, immediately transduced with lentiviral particles expressing TR, and selected in geneticin in addition to blasticidin. This order of transduction was found to be essential. Tet-transduced cells were transduced with lentivirus expressing anti-Par3 shRNA (kd) and visualized by immunofluorescence. Bars, 10 μm. (B) Similar cultures were analyzed by immunoblot, in cells with or without stable ca-PKCε expression (A120E). (C) Quantification of the IκB bands in cells expressing or not Par3 shRNA, relative to tubulin signal (n=4; * P<0.025), and A120E-PKCε (n=5). All the values were normalized for tubulin signal. (D) IL-8 mRNA was measured by RT-qPCR in scrambled shRNA-transduced and Par3 kd cultures previously incubated for 24 hours in TNFα (+) or control (−). Results are expressed as fold changes respect to the mock control levels. * P<0.025 (n=3). (E) Immunofluorescence determination of subcellular distribution of relA(p65) in Caco-2 cells grown on filters and stimulated or not with basolateral TNFα. Bars, 10 μm. (F) Quantification of average intensities of nuclear/cyttoplasmic pixels from experiments as described in E, graphed as “whisker” boxes (scrambled, 39 cells; scrambled TNFα, 41 cells; Par3 kd, 33 cells; Par3 kd TNFα, 27 cells; * paired comparison P<0.001). Suspected outlier points (beyond the third quartile) are shown individually.
of scrambled-expressing control cells for each transcript. The fold differences were normalized to the values measured by RT-qPCR. Grey bars represent values from cells maintained during this period as well. For detection, cells were extracted in PBS and RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed using the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA). IL-8 and GRO-1 -3 gene expression was quantified by Quantitative PCR analysis using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) on an iCycler instrument (Bio-Rad, Hercules, CA, USA). RNA expression levels were normalized to internal control GAPDH. To compare the transcript levels between different samples the ΔΔCT comparative cycle threshold calculation was used (Livak and Schmittgen, 2001).

Immunofluorescence and image analysis

Immunofluorescence images were acquired with a Leica SP5 confocal microscope, under 63× oil immersion (NA 1.4) objective, using Leica LAS software. Images are shown as single confocal sections, except for DAPI nuclear signal which was often at a different focal plane and is presented as stack maximum projections. Pixel intensity measurements were performed as described before (Wald et al., 2011).

Statistics

Average and standard deviation were used. Differences between averages were analyzed by Student’s t test. For nuclear/cytoplasm signal ratios, descriptive statistics were used showing data in the form of box-and-whisker plots (McGill et al., 1978). In this case, significance of the difference between groups was analyzed by Kruskal-Wallis test (Kruskal and Wallis, 1952).

Acknowledgements

We are indebted to Ms Yolanda Figueroa-Menendez for superb technical help, and to Drs Richard Rotundo and Robert Warren for critically reading the manuscript. TROMA I monoclonal antibody developed by R. Kemler was obtained from the Developmental Studies Hybridoma Bank (University of Iowa).

Funding

This work was supported by NIDDK [grant numbers R01-087359, R01-076652 to P.J.S.]; a F31 post-doctoral fellowship to R.F.; and a CCFA fellowship to F.A.W.

Author Contributions

R.F. performed experiments, analyzed data and conducted statistical analyses. F.A.W. performed experiments, analyzed data and prepared constructs. A.M. performed experiments, analyzed data, and prepared and tested lentiviral particles. Z.K. performed experiments, analyzed data and conducted qPCR studies. P.J.S. conceived the original hypotheses, analyzed data, coordinated efforts, and wrote the manuscript.

Competing Interests

The authors have no competing interests to declare.

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Quantitative real-time polymerase chain reaction (qPCR)

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed using the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA). IL-8 and GRO-1 -3 gene expression was quantified by Quantitative PCR analysis using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) on an iCycler instrument (Bio-Rad, Hercules, CA, USA). RNA expression levels were normalized to internal control GAPDH.
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