Defining the relevant combinatorial space of the PKC/CARD-CC signal transduction nodes

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Abstract — **Biological signal transduction typically display a so-called bow-tie or hour glass topology: Multiple receptors lead to multiple cellular responses but the signals all pass through a narrow waist of central signaling nodes. One such critical signaling node for several inflammatory and oncogenic signaling pathways in humans are the CARD-CC/Bcl10/MALT1 (CBM) complexes, which get activated by upstream protein kinase C (PKC). In humans, there are four phylogenetically distinct CARD-CC family (CARD9, -10, -11 and -14) proteins and 9 true PKC isozymes (α to ι). At this moment, less than a handful of PKC/CARD-CC relationships are known from experimental evidence. In order to explore the biologically relevant combinatorial space out of all 36 potential permutations in this two-component signaling event, we made use of CRISPR/Cas9 genome-edited HEK293T cells to knock out CARD10 for subsequent pairwise co-transfections of all CARD-CC family members and activated mutants of all true PKCs. By quantitative reporter gene expression readout, we could define specific strong and weak PKC/CARD-CC relationships. Surprisingly as many as 21 PKC/CARD-CC combinations were found to have synergistic effects. This information will be valuable for future studies of novel signaling pathways dependent on the CBM complex signaling nodes. The expanded scope of signaling pathways can also have practical applications, where the information can be exploited for pharmacological manipulation of the signaling pathways via downstream components like MALT1 protease activity for more specific effects and potentially less of the side effects associated with PKC inhibition.**

Index Terms — **Biochemistry, Biological information theory, Biological systems, Genetic engineering, Protein engineering, Signal transduction**

I. Introduction

SIGNAL transduction aims to transmit a signal from an external or internal stimulus resulting in a cellular response. The signals and responses to stimuli need to be tightly regulated in order to be sensitive enough while still filtering out environmental noise [1]–[3]. A common network topology for intracellular molecular signaling pathways is the so-called bow-tie (or hourglass) architecture: multiple receptors and environmental signals pass their information through a few common signaling nodes but end up with a wide diversity of cellular (*i.e.* transcriptional) responses [4], [5]. This narrow waist line network topology is thought to contribute to filtering out informative signals from noise and to ensure appropriate cellular responses [6], [7]. One class of such narrow-waist signaling nodes is represented by the CARD-CC/Bcl10/MALT1 (CBM) signaling complexes. Several different CBM signaling complexes, which are composed of Bcl10, MALT1 and specific CARD-CC family proteins (CARD9 [8], CARD11 (also known as CARMA1) [9], CARD14 (also known as CARMA2) [10]–[12] and CARD10 (also known as CARMA3) [13]) and which are formed upon stimulation of distinct receptors in several immune and non-immune cells. Part of the CBM complex specificity comes from cell type specific expression of the different CARD-CC proteins: in general, CARD9 is expressed in myeloid cells, CARD11 in lymphocytes, CARD14 in skin and CARD10 in most of the other cells (https://www.proteinatlas.org/) [14]. Due to their cell-type specific expression, the different CARD-CC proteins play different roles. For example: Loss of *CARD9* function causes susceptibility to fungal infections due to defective innate immune responses [8]. Loss of *CARD11*, on the other hand, cause severe deficiencies in adaptive immunity [15], whereas over-active mutants cause B cell lymphomas [16]. The role of *CARD14* is currently unknown, but activating mutations in this CARD-CC has been shown to cause psoriasis [17], and elevated *CARD14* expression has been found in an androgen-insensitive and highly tumorogenic prostate cancer sub-clone [18]. There are no known human loss- or gain-of-function mutants known in *CARD10*, and the mouse knock-out mutant is often embryo lethal [19]. On the other hand, some cancers show elevated *CARD10* expression [20]. Receptor signaling via the ITAM (immunoreceptor tyrosine-based activation motif) binding SH2 domain tyrosine kinases Zap70 or Syk activates PKC, leading to CARD-CC phosphorylation and the recruitment of a pre-existing Bcl10/MALT1 complex [9], [21], [22] (Fig. 1). Studies on CARD11 have shown that phosphorylation leads to release of intramolecular auto-inhibition and aggregation of the CARD-CC protein together with the other CBM complex components in huge macromolecular filamentous structures that acts as a platform for downstream signaling [23]. CARD9 has been shown to be activated by PKCδ [24], CARD10 is activated by PKCα [25] and CARD11 is activated by PKCβ and PKCθ in B and T cells [26]–[28]. It is very likely that there are more CBM-activating PKC isozymes, and that there are more PKC::CARD-CC kinase/substrate relationships in different cells and pathways. The PKC family consists of nine true members in the "conventional" (α, β, γ), "novel" (δ, ε, η, θ) and "atypical" (ζ,

Manuscript received MM DD YYYY. Work in the Beyaert lab has been financed by the Fund for Scientific Research Flanders (FWO), the Belgian Foundation Against Cancer, Interuniversity Attraction Poles, Concerted Research Actions (GOA) and the Group-ID Multidisciplinary Research Partnership of Ghent University.

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ι/λ) sub-classes [29]. The extended definition of the PKC superfamily also include the related three PKN and three PRKD kinase family members. PRKD1 was sometimes called PKCμ, and PRKD3 was called PKCν. Aberrant activation of PKC has been associated to several types of cancer [25], [30], [31], indicating that at least some of those might represent an oncogenic process dependent on over-active CBM signaling complexes.

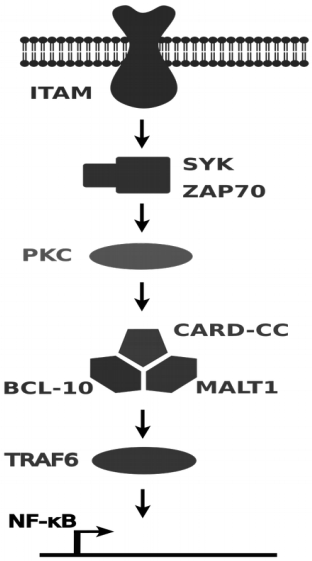

Fig. 1. CBM complex signaling model. Upstream ITAM-containing receptors activate different types of PKC via the activity of the SH2 domain tyrosine kinases Syk or Zap70. Upon phosphorylation, the CBM complex is assembled which recruits TRAF6 and activates downstream NF-κB transcriptional activity.

MALT1 in the activated CBM complex recruits critical downstream proteins, such as TRAF6, for activation of NF-κB -dependent gene expression [32]. Apart from this NF-κB -activating "scaffold" function, MALT1 (PCASP1 [33]) also is a protease (paracaspase), which is a drugable catalytic activity. The anti-inflammatory role of many of the known MALT1 protease substrates coupled with the critical role for MALT1 in pro-inflammatory signaling has sparked an interest in targeting MALT1 protease activity as a therapeutic strategy for autoimmune diseases [34]. The proteolytic activity of MALT1 was also found to be critical for certain cancers [35], which has stimulated an interest in MALT1 protease activity as a cancer therapy target as well. The aim of this study is to discover the full scope of CBM complex dependent signaling pathways downstream of PKC activation. Defining the full scope of the functions of MALT1 could ultimately be important in the context of future MALT1 inhibitor-based therapies [36].

II. Materials and Methods

A. *CARD-CC and activated PKC expression plasmids*

Plasmids of the cloned genes were deposited in the BCCM/LMBP plasmid collection along with detailed descriptions of cloning strategy and plasmid sequence (http://bccm.belspo.be/about-us/bccm-lmbp). Expression plasmids with wild-type Flag-tagged CARD9 (LMBP: 9609), CARD10 (LMBP: 10187), CARD11 and CARD14 (LMBP: 9863) were used together with expression plasmids with E-tagged activated mutant PKCα-A25E (LMBP: 8931), PKCβII-A25E (LMBP: 8918), PKCγ-A24E (LMBP: 8919), PKCδ-RR144/145AA (LMBP: 8924), PKCε-A159E (LMBP: 8920). PKCθ-A148E (LMBP: 8925), PKCη-A160E (LMBP: 8922), PKCζ-A119E (LMBP: 8921) and PKCι-A129E (LMBP: 8923). For set-ups without PKC transfection, an equal amount of pCAGGS (LMBP: 2453) empty vector (EV) was used.

B. *Genome-editing of human model cell lines*

As positive controls, we generated large-deletion *MALT1*$^{\Delta Ex1\text{-}9}$ (clone #M24) and *Bcl10*$^{\Delta Ex1\text{-}3}$ (clone #B51) knock-out HEK293T cell lines. *MALT1* was knocked-out by co-transfecting two Cas9::guide constructs targeting exon 1 (LMBP: 10533) and exon 9 (LMBP: 10401). *Bcl10* was knocked-out by co-transfecting two Cas9::guide constructs targeting exon 1 (LMBP: 10402) and exon 3 (LMBP: 10403). *CARD10* (clone #C8) was knocked out by using a single Cas9::guide construct targeting exon 12 (LMBP: 10406). Two days after transfection, single-cell sorting for GFP-positive (=successfully transfected) cells was performed on an ARIA II (BD Biosciences) fluorescence activated cell sorter (FACS) machine. Individual clones were evaluated for knock-out by PCR and functional assays. Full *CARD10* knock-out was verified by PCR of the genomic region with the primers GT-hCARD10-Ex12-F : TCCCTGCCCAATCAGACTT and GT-hCARD10-Ex12-R : GCTTGTGACTTGAGATTTGAAGG. Sequencing of the amplified region was done with the nested primers Seq-hCARD10-Ex12-F : AATTAAGCCTGGCTGAGCA and Seq-hCARD10-Ex12-R : ATGATCAGCATTTCCCAGGT. Alignment of the complex indel chromatogram with a wild-type reference chromatogram was done using the TIDE calculator (https://tide-calculator.nki.nl/) [37]. Lack of mRNA expression of *CARD10* due to nonsense-mediated decay was furthermore verified by RT-PCR using the primers RT-hCARD10-F : GTGGATCAGCTCAAGCTCAAA and RT-hCARD10-R : TGGTCTCGCTCCTTCTCAAT.

C. *Cell culture, transfection and inhibitors*

HEK293T cells were grown under standard conditions (DMEM, 10% FCS, 5% CO_2, 37 °C) and transfected with the calcium phosphate method [38]. PKC inhibition was done with two broad range PKC inhibitors : 2μM Sotrastaurin (Selleckchem) or 10μM Gö6983 (Tocris Bioscience). Inhibitors were added before transfection and when media was refreshed 6h after calcium phosphate transfection.

D. *Luciferase reporter gene assay and analysis*

For evalutation of NF-κB induction, candidate inducing constructs were co-transfected with a NF-κB-dependent luciferase reporter expression plasmid (LMBP: 3249) and an actin promoter-driven β-galactosidase (LacZ) expression plasmid (LMBP: 4341) as transfection control. The CARD-CC/PKC screening transfecion set-ups were based on a 1 µg DNA master mix containing 100 ng luciferase reporter plasmid and 100 ng β-galactosidase expression plasmid. The amount of the different CARD-CC expression plasmids was determined individually based on basal spontaneous activation from overexpression : CARD9 (200 ng), CARD10 (5 ng), CARD11 (100 ng) and CARD14 (5 ng). The remaining DNA (600-795 ng) consisted of expression plasmid DNA for the activated PKC constructs or empty vector (EV). The 1 µg master mix was transfected over 4 24-well plate wells ($4 \cdot 10^4$ cells per well). The cells used for luciferase analysis were washed with PBS and lysed in luciferase lysis buffer (25mM Tris pH7.8, 2mM DTT, 2mM CDTA, 10% glycerol, 1% Triton X-100). For the colorimetric determination (at 595nm) of β-galactosidase activity, chlorophenol red-β-D-galactopyranoside (CPRG) (Roche diagnostics) was used as a substrate. Luciferase activity was measured by using beetle luciferin (Promega) as a substrate and luminescence was measured with the GloMax® 96 Microplate Luminometer (Promega). Luciferase data processing and calculation of 95% confidence intervals (Student's t-distribution [39]) was done in Libreoffice (www.libreoffice.org) Calc. To specifically look at the synergistic effects, the data was formatted according to formula (1), which results in a unitless fold-induction value.

A(x) = LacZ normalized luciferase mean value for CARD-CC + x
B(x) = LacZ normalized luciferase mean value for x
x = {EV,PKCα-PKCι}

$$\frac{A(x)-B(x)}{A(EV)-B(EV)} \quad (1)$$

E. *Protein expression analysis*

Cells transfected for luciferase activity evaluations were lysed directly in Laemmli buffer (0.1% 2-mercaptoethanol, 5ppm bromophenol blue, 10% Glycerol, 2% SDS, 63 mM Tris-HCl (pH 6.8)). Protein lysates were run on a 10% PAGE gel and blotted on nitrocellulose. Expression of the CARD-CC proteins was determined with anti-Flag antibody (F-3165, Sigma). Expression of the E-tagged PKC clones with anti-E-tag antibody (ab66152, Abcam).

III. Results

A. *Verification of the screening tools*

A general activator of conventional and novel PKCs is the diacylglycerol (DAG) analog phorbol 12-myristate 13-acetate (PMA) [31]. To functionally verify that we have generated a cell line suitable for screening of PKC/CARD-CC interactions, we treated wild-type and mutant HEK293T cells with PMA for 6 hours to activate a broad range of PKCs present endogenously in HEK293T cells and measured NF-κB - driven luciferase expression via the luciferase enzymatic activity. HEK293T cells are chosen as model cell because they easily can be transiently transfected at a very high efficiency. Bcl10 and MALT1 are common for all CBM complexes and the knock-out cell lines are thus completely un-responsive to PMA stimulation (Fig. 2). CARD10 is the dominant CARD-CC isoform (transcript per million (TPM): 11 vs 0.4 for *CARD9*, 1.0 for *CARD11* and 0.8 for *CARD14*) in HEK293T cells [14], but the lack of complete loss of PMA responsiveness in the *CARD10* knock-out HEK293T clone indicate weak expression of (an)other partially redundant CARD-CC family member(s) or alternative activation mechanisms.

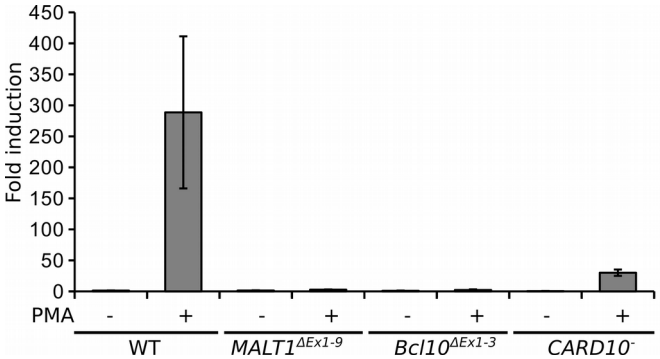

Fig. 2. PMA-induced NF-κB driven luciferase expression in wild-type (WT) and *MALT1*, *Bcl10* and *CARD10* knock-out HEK293T cells after 6 hours stimulation. Values represent LacZ-normalized luciferase enzymatic activity expressed as fold induction (unitless) of the PMA-stimulated (+) cells compared to the average background level of the non-stimulated (-) cells. Error bars represent 95% confidence intervals (student's t distribution).

The dramatically reduced PMA responsiveness of the *CARD10* knock-out HEK293T cell does however enable systematic analysis of specific PKC/CARD-CC interactions through reconstituton by transient transfection. In order to verify that NF-κB activation from overexpression of the activated PKC mutants was due to their kinase activities, we evaluated their NF-κB activating potential either non-treated (NT) or in presence of the two broad-range PKC inhibitors Gö6983 or Sotrastaurin in wild-type HEK293T cells. The broad-range inhibitor Sotrastaurin could completely inhibit luciferase induction downstream of most of the activated PKC isoforms.

	Fold	% (NT)	% (Gö6983)	% (Sotrastaurin)
PKCα	8.2 ± 1.4	100 ± 20	45.7 ± 12	-5.4 ± 1.4
PKCβ	52.3 ± 1.8	100 ± 3.4	3.9 ± 0.4	-0.4 ± 0.2
PKCγ	4.0 ± 0.2	100 ± 7.2	128.9 ± 38	27.1 ± 7.0
PKCδ	106.1 ± 5.4	100 ± 5	7.2 ± 5.9	0.7 ± 0.2
PKCε	4.8 ± 0.4	100 ± 9.6	191.2 ± 14	-6.8 ± 2.1
PKCθ	7.5 ± 0.5	100 ± 7.8	33 ± 12	-6.5 ± 0.0
PKCη	3.1 ± 0.2	100 ± 9.2	367.5 ± 35	18.7 ± 3.0
PKCζ	4.6 ± 0.2	100 ± 6.9	58.4 ± 4.8	20.6 ± 6.0

Table 1. Verification of kinase-dependent NF-kB from overexpression of activated PKC mutants in wild-type HEK293T cells. Fold indicates the NF-κB activation compared to empty vector for each non-treated activated PKC isoform ± 95% confidence interval. NF-κB signals and confidence intervals after PKC inhibition are normalized against the mean value of the corresponding PKC without inhibitor treatment. (NT) and expressed as %.

Grey cells indicate complete inhibition. Negative values indicates that the PKC inhibitor-treated sample shows lower NF-κB signal than empty vector tranfected cells, since background values from empty vector transfected cells are subtracted before normalization. PKCι did not induce NF-κB in this set-up.

Since we could verify that the PKC-induced NF-κB activation was kinase dependent, and that we had managed to make a model cell line where the dominant CARD-CC was deleted, we are confident to use these tools for screenings of synergistic PKC/CARD-CC interactions. CARD9 and CARD11 could easily be investigated using a default transfection set-up in the CARD10 knock-out HEK293T cells since basal activation by overexpression was very low (Fig. 3).

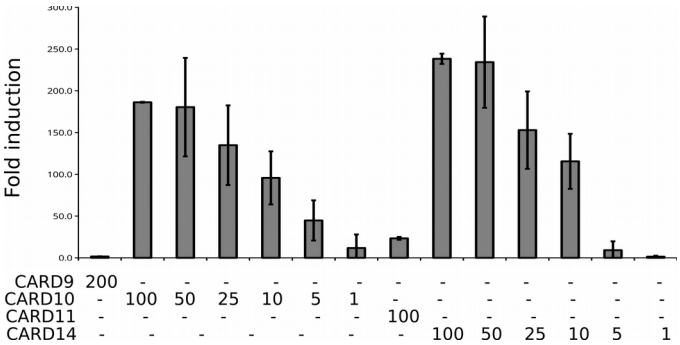

Fig. 3. Basal NF-κB activation from CARD-CC overexpression vs empty vector transfected control, based on luciferase activity in wild-type HEK293T cells. Numbers indicate ng/µg in transfection master mix.

CARD10 and CARD14, on the other hand, seem to have a much poorer auto-inhibition and in order to observe synergistic effects with activated PKCs, we had to titrate down the amount of expression plasmid DNA to the level where the basal activation was closer to that of CARD9 and CARD11 (Fig. 3).

B. Identification PKC/CARD-CC functional interactions

Analysis of CARD9 activation by overexpression of activated PKCs revealed a surprisingly strong novel activation by PKCη , PKCβ and PKCε, followed by the published PKCδ [24] and PKCθ.

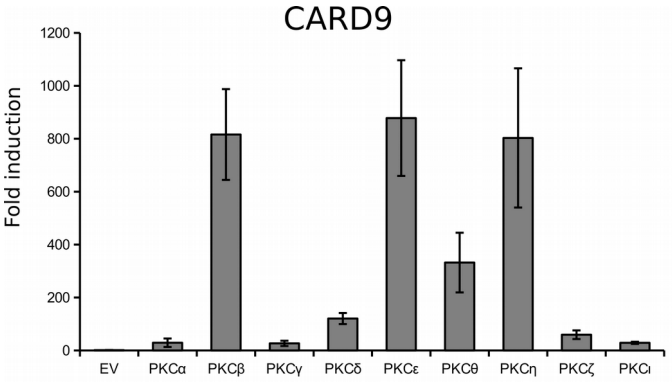

Fig. 4. CARD9 activation by activated PKC mutants expressed as fold-induction of NF-κB-induced luciferase expression, compared to CARD9 expressed without activated PKC (EV). Background induction from PKC alone is subtracted before normalization according to formula (1).

CARD10, on the other hand, gets activated by a wide range of PKCs, including the atypical sub-class, which was inactive against CARD9. Also, the strongly CARD9-activating PKCη only show weak or intermediate CARD10 activation, demonstrating that different CARD-CC proteins get differentially activated by different PKCs.

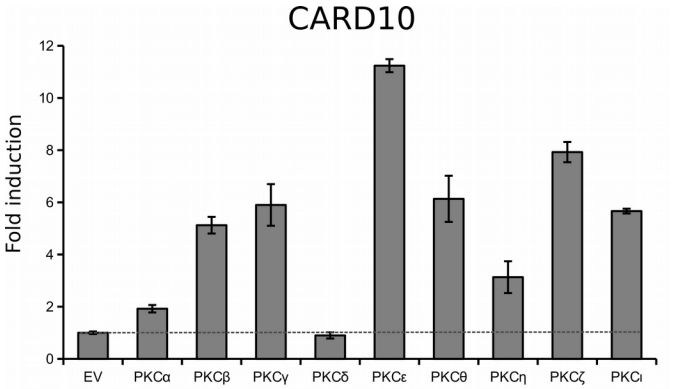

Fig. 5. CARD10 activation by activated PKC mutants expressed as fold-induction of NF-κB-induced luciferase expression, compared to CARD10 expressed without activated PKC (EV). Background induction from PKC alone is subtracted before normalization according to formula (1). Dashed line represents the average induction by CARD10 without PKC.

As expected, CARD11 gets activated by PKCβ and PKCθ but CARD11 is also activated by PKCε and PKCη, making the CARD11 activation profile more CARD9-like than CARD10-like.

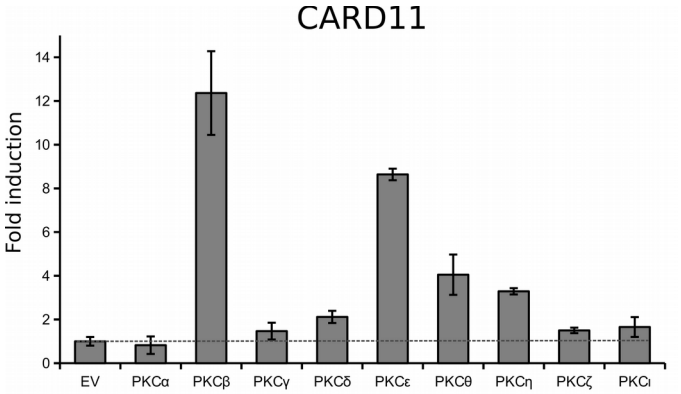

Fig. 6. CARD11 activation by activated PKC mutants expressed as fold-induction of NF-κB-induced luciferase expression, compared to CARD11 expressed without activated PKC (EV). Background induction from PKC alone is subtracted before normalization according to formula (1). Dashed line represents the average induction by CARD11 without PKC.

In contrast to the other CARD-CC proteins, no upstream receptor pathway is known for CARD14, where activated mutations are implicated in psoriasis [10]. The autoinhibition is less pronounced than for CARD9 and CARD11 (Fig. 3). By careful adjustment of the CARD14 expression level, we could however verify that also CARD14 gets activated by PKCβ, ε and θ, and the atypical PKCζ and ι. Despite having approximately the same basal activation level as CARD10, CARD14 responded much less to activated PKCs.

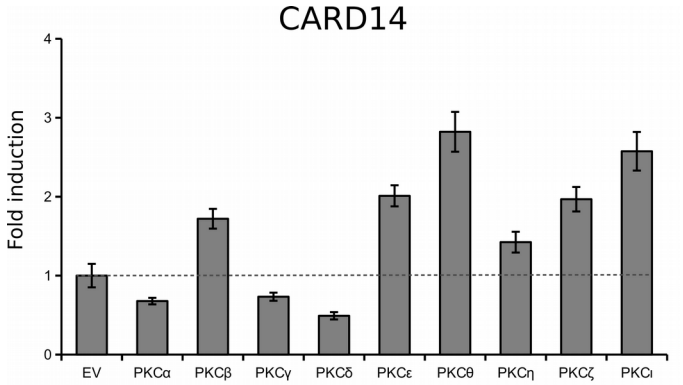

Fig. 7. CARD14 activation by activated PKC mutants expressed as fold-induction of NF-κB-induced luciferase expression, compared to CARD14 expressed without activated PKC (EV). Background induction from PKC alone is subtracted before normalization according to formula (1). Dashed line represents the average induction by CARD14 without PKC.

C. Verification of protein expression levels

The activated PKCα was very poorly expressed and in order to see any clear effects, it had to be transfected at very high levels. Because of this, the effects seen by this PKC could be under-estimated simply be due to a limitation of the screening system using activated mutants. It is known that some PKCs get destabilized and degraded upon activation [40]. Due to the massively different protein expression levels between the different activated PKC isoforms, protein expression from luciferase experiments from figure 4-7 were evaluated per PKC.

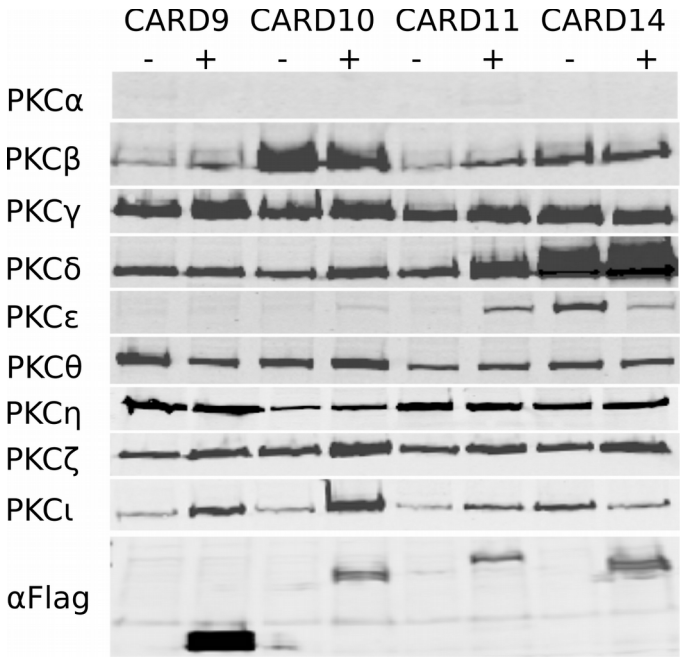

Fig. 8. Verification of protein expression levels of activated PKCs and CARD-CC proteins in the experiments for figure 4-7. Anti-flag is a representative example of CARD-CC expression in the different experiments, since CARD-CC protein expression did not change in presence of different PKCs. PKC expression was determined by anti-E-tag development.

IV. Conclusions

Systematic evaluation of all pairwise PKC and CARD-CC functional interactions revealed a surprising number of highly potent synergistic activations. It is important to note that the very high fold inductions seen for CARD9 more has to do with a very low basal activation of CARD9 compared to the other CARD-CC proteins. Similarly, some of the PKC contributions could very well be underestimated. The very poor protein expression of activated PKCα could mean that the CARD-CC activating potential of this particular isoform is underestimated. In contrast, two PKCs (PKCβ and PKCδ) still show relatively high basal activity also in the CARD10 knock-out HEK293T cells without complementation by expression of a CARD-CC protein, which also could lead to an underestimation of these two PKCs. Nevertheless, our novel screening system for functional interactions uncovered several previously unknown potential pathways which can be further explored in the future. Some of the novel functional interactions (like for example PKCη and CARD9) that we found using our screening system are much stronger than the already published (like for example PKCδ-CARD9) activation pathways. It has been shown that PKCη is activated by PMA and other stimuli in myeloid cells leading to foam cell formation [41], which thus could be a CARD9 CBM complex-dependent process. Other generally CBM -activating PKC isoforms like PKCε is known to be involved in a wide range of inflammation- and cancer-related processes [42]. Also the surprising dependency of CBM complexes downstream of the atypical PKCs further expands the potential use of CBM-interfering interventions as a therapeutic strategy. For example, the PKCζ → NF-κB pathway has been found to be important in cardiac hypertrophy [43] and PKCι/λ has been shown to act as an oncogene in many different types of cancer [44]. This demonstrates that our findings can lead to new hypotheses to be tested in different biological systems which ultimately might lead to novel pharmacological treatment opportunities, for example by using MALT1 protease inhibitors [36]. Since we surprisingly could find synergistic activations between all PKCs (including the atypical) and CARD-CC proteins, we might also have to consider the broader context of the entire PKC superfamily in the future (including the PRKD and PKN proteins) as potential CBM activators. There may also be other CARD-CC activating mechanisms from other classes of kinases or other protein-modifying enzymes that are currently unknown, as suggested for DNA damage-induced CBM activation [45].

V. Acknowledgments

We would like to thank Dr. Corinne Rösselt and Prof. Dr. Wim Declercq, Ghent University, for gateway entry vectors with activated mutant PKCs. pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138) [46]. Flag-tagged CARD11 expression construct was a gift from Dr. Mathijs Baens, KU Leuven [47]. Cell sorting was performed by Gert Van Isterdael, IRC, VIB-UGhent.

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