BIOCHEMICAL LETTERS

Specificity of cysteine-rich domains in diacylglycerol kinases and protein kinases C

Protein kinase C (PKC) and diacylglycerol kinase (DGK) are two distinct enzymes that interact with the second messenger diacylglycerol (DG). PKC is activated by DG, whereas DGK is believed to act as an attenuator of PKC activity by phosphorylating DG to phosphatidic acid. Most PKC and DGK isotypes contain two cysteine-rich domains (CRD1 and CRD2) through which DG interaction is thought to occur (Figure 1) [1–3]. This concept is mainly based on studies with phorbol esters which closely mimic DG as an activator of PKC through binding to its CRDs (also referred to as C1 domains) in a highly specific manner (see below). No clear distinction has so far been made between CRD1 and CRD2 in PKC or DGK isotypes [3]. On the basis of our knowledge of the PKC–CRD interaction with phorbol esters and DG, it has been proposed that DGK binds DG in a similar fashion, at their CRDs [4], which then somehow should co-operate with the catalytic domain to phosphorylate DG. Indeed, we found that a mutant of the DGK α isotype lacking the CRDs is enzymically inactive (D. Schaap and W. J. van Blitterswijk, unpublished work).

We recently cloned a new DGK isotype, DGK θ , that contains three instead of two CRDs [5]. In fact, no other protein has been reported to contain three such domains. In search of a possible function for this third domain, we compared the structure of the three CRDs in DGK θ with those in other DGK and PKC isotypes, using an improved sequence alignment of amino acids (see the legend to Figure 2). It appears that the first and second CRD of DGK θ are very similar to the CRD1 in other DGKs and to both CRDs in PKC. Interestingly, however, the third CRD (CRD3) in DGK θ is very similar to CRD2 in all other DGK isotypes, but quite distinct from that of PKC. Apart from the typical core structure:

HXWX_{8–10}CX₂CX_{11–19}CX₂CX₄HX_{2–4}CX_{5–9}C

that binds two zinc ions in a tetrahedral geometry [6], the sequence contains an additional 30 conserved amino acids, 25 of which (blue-coloured in Figure 2) are exclusively present in the CRD2s of all DGK isotypes known. Interestingly, the sequence similarity extends 15 amino acids immediately adjoining the Cys₆/His₂ core structure in the C-terminal direction. Such a con-

served C-terminal elongation is not found for DGK–CRD1 or other CRDs in any protein kinase (in PKC, the N-terminus of CRD1 is adjacent to the pseudosubstrate). This ‘extended CRD’ (extCRD, Figure 1) can thus be considered as unique for DGK and is likely to be essential, in conjunction with the conserved catalytic domain, for the unique enzymic function of DGK. It is tempting to speculate that it binds DG, albeit probably in a manner different from that by which PKC binds DG (see below), and that the CRD elongation is somehow involved in presenting DG to the catalytic domain for eventual phosphorylation. Of course, this hypothesis needs experimental verification, for instance by deletion and/or swapping of CRD2/3 and CRD1 to see how this would affect enzymic activity.

From the three-dimensional structure of the CRD2 of PKC, resolved by NMR [6] and crystallographic studies [7], and from site-directed mutagenesis [8], we know in much detail which amino acid residues determine phorbol ester and, by inference, DG binding (marked by asterisks in Figure 2). Unexpectedly, phorbol ester does not significantly alter the conformation of the domain [7]. Instead, it only covers the hydrophilic cleft between residues 9 and 12 and between 20 and 24 (Figure 2) and, as such, makes the CRD surface more hydrophobic, thus facilitating and stabilizing insertion into the membrane [7]. When one compares the amino acid sequences of CRD1 and CRD2 of phorbol-ester-binding PKC isotypes, n-chimaerin and Unc-13, some small but clear differences can be noted (grey-coloured in Figure 2). Interestingly, these differences are mainly located at positions 5–10 and 15–20, immediately preceding the aforementioned peptide segments most crucially involved in phorbol-ester binding. This distinction between CRD1 and CRD2 of PKC may relate to the recently found non-equivalency of the two CRDs in the context of the intact enzyme. Whereas both CRD1 and CRD2 can bind phorbol ester, CRD2 plays the predominant role in phorbol-ester-induced translocation of PKC [9].

In DGK, CRD1 is very similar to the CRDs in PKC, yet contains some unique conservations, green-coloured in Figure 2, and shows differences or deletions in the stretches of amino acids located at positions 7–23 and 28–29 that are crucially involved in phorbol-ester binding [8]. For instance, DGK does not contain a W/Y at position 22. These differences may explain why DGK does not bind phorbol ester [10,11].

In conclusion, the sequence alignments shown here reveal a

Figure 1 Domain architecture of two representative DGK isotypes compared with classical PKCs

Abbreviations: extCRD, extended CRD; Catalyt, catalytical domain (grey for DGKs, black for PKC); EF-hands and C2, Ca²⁺-binding domains in DGK and PKC respectively; PR, proline-rich domain; PH, pleckstrin homology domain; RA, Ras-associating domain.

Figure 2 Sequence comparison of CRDs in PKC (also named C1 domains) and in DGK isotypes

Consensus profiles are shown at the bottom. Asterisks (in PKC consensus) denote essential phorbol-ester/DG-binding sites [7,8]. For PKC, only a representative selection of isotypes from a variety of species is listed, supplemented by phorbol-ester-binding n-chimaerin and Unc-13. First, second and third CRDs are indicated in parentheses in the second column, followed by the number of the first residue (His) of the respective CRD in the protein (third column). Optimal alignment was achieved manually by proper insertion of amino acid gaps (dashes). Note that there are a few more gaps (the peptide is slightly more 'stretched') than in previous alignments (e.g. in [3]), thus revealing a higher number of conserved residues without violation of the tertiary structure. The numbering of residues indicated on top ignores these gaps, to conform to the numbering given by others [3]. Numerals $-n-$ within sequences indicate insertion of n amino acid residues. Fully conserved residues are in **bold**, of which Cys/His core residues are in white over black; other conserved residues shared between PKC and DGK CRDs are in yellow; conserved residues exclusively present in DGK-CRD2 (and DGK θ -CRD3), including the unique conserved 15 amino acids extension beyond Cys51) are in blue; differences in conserved residues between CRD1 and CRD2 of PKCs are in black letters on grey; conserved residues in DGK-CRD1 that differ from PKC-CRDs are in green.

previously unrecognized specificity in CRDs of DGK and PKC isotypes. Most significantly, in DGK, CRD2 is quite distinct from CRD1 and has a unique conservation, which extends C-terminally beyond the His/Cys core structure. Furthermore, subtle differences exist between CRD1 (or C1A domain) and CRD2 (or C1B domain) of PKC, and between CRD1 of DGK and the CRDs of PKC, that may relate to their different affinity towards phorbol ester.

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Hypothesis: ceramide conditionally activates atypical protein kinases C, Raf-1 and KSR through binding to their cysteine-rich domains

Classical protein kinase C (PKC) has long been known to be activated by phorbol esters or, physiologically, by the lipid second messenger diacylglycerol (DG). These activators bind to the two cysteine-rich domains (CRD) of the protein [1,2]. More recently, crystallographic [3], NMR [4] and site-directed-mutagenesis [5] studies have revealed precisely how phorbol ester, and by inference DG, bind to the second CRD (or C1B domain) of PKC δ (Figure 1). The hydrophilic portion of these molecules inserts into a hydrophilic cleft of the CRD in a very specific, tightly fitting, manner. Importantly, and for an 'activator' unexpectedly, the inserted phorbol moiety does not significantly affect the conformation of the domain. Phorbol ester only covers a hydrophilic cleft between residues 9–12 and 20–24 (Figure 2) in the otherwise hydrophobic globular structure, so that the top third of the domain displays a contiguous hydrophobic surface which facilitates and stabilizes membrane insertion of the CRD [3]. In the PKC protein as a whole, a conformational change and subsequent kinase activation then follows in a concerted mechanism by (co)factors other than DG. These factors include the increased hydrophobicity imposed upon the whole protein, the binding of negatively charged phospholipids and Ca²⁺ (in case of conventional PKCs), specific anchoring or scaffold proteins and a substrate [1].

Atypical PKCs (PKC ζ and λ/ι), Raf and KSR kinases contain only single CRD copies that have been classified as 'atypical' because they do not bind phorbol ester and are not activated by DG, at least *in vitro* [2] (see below). Instead, and intriguingly enough, in some systems these Ser/Thr protein kinases have recently been shown to bind to, and to be activated by, ceramide, both *in vitro* and *in vivo* [6–8]. Ceramide is another (putative) lipid second messenger, implicated in stress and induction of apoptosis [9,10] and having a molecular structure/shape and physico-chemical properties that are very similar to those of DG

(Figure 2). Could it be, therefore, that ceramide binds to these enzymes in a manner similar to that by which DG binds to PKC, i.e. at their CRD? Indeed, like PKC, the Raf-1-CRD can translocate and bind to membrane lipids [11]. The three-dimensional structure of the CRD of Raf-1, revealed by NMR [12], is very similar to that of PKC, with a similar cavity for possibly accommodating a DG-like molecule (ceramide?). Only small differences are apparent, particularly at the loops (shaded in Figure 2) near/at already mentioned amino acid residues 9–12 and 20–24, known to be important segments for phorbol ester binding. In PKC, these loops are relatively flexible and are slightly pushed apart by insertion of the rigid phorbol moiety [12]. In Raf-1, PKC ζ and KSR, several of these loop residues are absent or altered (shaded in Figure 1). Consequently, the loop near residue 20 is shortened and immobilized, as demonstrated for Raf-1 [12] (Figure 2A). These are likely reasons why the CRD in these kinases cannot bind phorbol ester. However, this does not necessarily exclude the possibility of ceramide or even DG binding *in vivo*: The binding of the more flexible DG molecule to the CRD of PKC, for example, is much less stringent (its affinity is two orders of magnitude less) than the perfectly fitting rigid phorbol ester ('super' DG) [13]. Moreover, it is important to realize that, in the cell, CRDs also interact with other molecules in signalling complexes, as demonstrated most extensively for Raf-1 (see below) [14]. One can imagine that, depending on the cell system, these interactions may slightly affect dimensions within the CRD cleft (comparable with the phorbol ester-imposed peptide loop distortions [12] mentioned), sufficient to allow or to prevent the binding of ceramide or DG. This might explain why, in Rat-1 fibroblasts *in vivo*, we unexpectedly found activation of PKC- ζ by DG [15,16], whereas others found ceramide activation of PKC- ζ [6], Raf-1 [7] or KSR [8] in different cell systems.

The mechanism of Raf-1 activation in cells is only partially understood and appears to be very complex [14]. Its CRD hydrophobic surface binds to phosphatidylserine in the plasma membrane [11,12]. Reconstitution experiments have revealed another (unidentified) lipid cofactor that significantly contributes to Raf-1 activation [17]. In inactive Raf-1, the CRD binds to a 14-3-3 protein [18,19], which may mask a (second) Ras-binding site that is unveiled during Raf-1 activation [14,20,21]. This binding site for Ras encompasses amino acid residues 5–12 [22], precisely the loop that hypothetically binds to ceramide (analogous to phorbol ester/DG binding to PKC). Hence, it is conceivable that in many systems where 14-3-3 or activated Ras bind to Raf-1, the CRD cleft may be shielded from possible ceramide binding. On the other hand, in signalling systems where

Figure 1 Sequence comparison of the single CRDs of PKC ζ , Raf-1 and KSR with the second CRD (or C1B domain) of PKC δ

Important differences (alterations and deletions) are shaded. Sequence alignment was done as described in the accompanying BJ Letter [26]. Numbering of residues indicated on top conforms to that used in a related [3] and the companion paper [26]. The conserved Cys/His core residues are in white over black. Peptide folding (β -strands and α -helix) [3,4,12] is indicated at the bottom. Residues essential for phorbol ester/DG binding to PKC δ [3,5] are marked by asterisks. Pro¹¹, Gly²³ and Gln²⁸ play critical roles in maintaining the precise unzipped structure of the upper β -sheets which form the phorbol-ester/DG-binding site (Figure 2B). Important phorbol-binding segments are located near Pro¹¹ and the LLWGL sequence at position 20–24, in/near β 2 and β 3 strands respectively, and are shaded in the ribbon structure of Figure 2(B). Precisely at these sites the CRDs of PKC ζ , Raf-1 and KSR differ markedly (shaded) from the sequence of PKC δ . Accession codes are from the Swissprot or GenBank[®] databases. Sources: m, murine; h, human.

Figure 2 Hypothetical model for binding of ceramide and DG to the CRD of Raf-1 (A) and the second CRD of PKC δ (B) respectively, visualized in ribbon structures [12]

Loops that determine DG or ceramide binding are shaded, and the most relevant amino acids in these loops are indicated (with sequence number) at their approximate positions. The small difference in structure between the polar regions of DG and ceramide is highlighted by the shadow. Boxes indicate insertion of DG or ceramide moieties into the pocket (cleft) between the shaded loops. For DG, the carbonyl at the *sn*-1 position is presumed to form a hydrogen bond with Gly²³, while the hydroxy group does so with Thr¹² and Leu²¹ (B), analogous to the perfect fit of the phorbol moiety in this cleft in the PKC-CRD [3]. How precisely the proposed insertion of ceramide in the Raf-CRD cleft would occur, i.e. which amino acids bind to which sites in the ceramide head, remains to be investigated.

Ras or 14-3-3 do not play a prominent role, such as in interleukin-1 β -stimulated mesangial cells, direct ceramide binding to and activation of Raf-1 was demonstrated [7]. In tumour-necrosis-factor signalling, ceramide was recently found to activate KSR, which, in turn, activated Raf-1 [8]. In this system, KSR, 14-3-3 and Raf-1 may form an oligomeric complex [23], possibly in a way that precludes direct ceramide-Raf-1 interaction, but which allows ceramide to bind and activate KSR [8].

Similar to Raf-1, the CRD in atypical PKCs may bind to other signalling proteins with a potential influence on lipid binding. Lambda-interacting protein activates the atypical PKC λ/ι *in vitro* and *in vivo* through binding to its CRD [24]. Par-4 protein, expressed during apoptosis, binds to the CRDs of both PKC ζ and PKC λ/ι and inhibits their enzymic activity [25]. Ceramide, implicated in apoptotic signalling, can promote this association [25], consistent with the concept that these interactions all occur at the surface of the CRD.

In conclusion, whereas the CRDs of Raf-1, KSR and PKC ζ show structural similarity to those in classical PKC, determining their similar, zinc-mediated peptide folding, their detailed amino acid sequence differs enough to make them incapable of binding

phorbol ester. This structural distinction likely also relates to their specific interaction with other molecules in signal transduction, including DG and ceramide. These lipid second messengers may not be exclusively directed to one particular protein kinase. This promiscuity particularly applies to ceramide, and may possibly relate to the stringency of fitting of the lipid in the CRD cleft, which might be influenced by other CRD-interacting molecules in the signalling complex. Thus the specificity of these lipids as signalling molecules (messengers) may be limited. They rather seem to act as a trigger that induces and stabilizes CRD insertion in the membrane, necessary for further activation of the enzyme. The specificity in kinase activation is then likely to be determined by protein-protein interactions.

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