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Comparison of the PKCα and the PKCε C1b Domains: Identification of Residues Critical for PKCε-mediated Neurite Induction

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We showed earlier that over-expression of protein kinase C (PKC) ε induces neurite outgrowth. The effect is mediated by a region (PKCεPSC1V3) encompassing the pseudosubstrate, the two C1 domains and part of the V3 region, and is independent of the catalytic activity of the enzyme. In this region, residues immediately N-terminal of the C1b domain are crucial for neurite outgrowth. However, in this study we show that the PKCε C1b domain itself is necessary for neurite induction, since a mutant in which the PKCε C1b domain has been replaced with the C1b domain from PKCα, PKCεPSC1a(αC1b)V3 lacks neurite-inducing capacity. The molecular basis for the importance of the PKCε C1b domain was investigated by mutation studies of the PKCα C1b domain. Point mutations were done in the PKCα C1b domain of the PKCεPSC1a(αC1b)V3 construct, in which the PKCα residues were mutated into the corresponding residues in PKCε. This highlighted residues in the C-terminal part of the primary sequence of the C1b domain, located in the base of the C1b domain, as important for neurite outgrowth. The mutations S48P, D32K and L49N all influenced neurite induction positively. Furthermore, the mutation of L49N alone was sufficient to make PKCεPSC1a(αC1b)V3 neuritogenic in phorbol ester-stimulated cells, and mutation of this residue in full-length PKCε into the corresponding residue in PKCα, N291L reduced the neurite-inducing effect of PKCε. In conclusion, we have identified residues in the PKCε C1b domain, in particular Asn49, that are essential for neurite outgrowth.

Keywords: protein kinase C; neurite outgrowth; neuroblastoma cells; C1 domains

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Introduction

The protein kinase C (PKC) isoforms constitute a family of serine-threonine kinases involved in the regulation of a multitude of processes in the cell. There are at least ten PKC isoforms, which can be divided into three subclasses according to structure and requirements for the activation of the enzyme. The classical PKC isoforms (PKCα, βI, βII and γ) need diacylglycerol (DAG) and Ca^{2+} for their activation, novel isoforms (PKCδ, ε, η and θ) require DAG but are independent of Ca^{2+}, and the atypical PKC isoforms (PKCζ and ι/λ) are insensitive to both DAG and Ca^{2+}.[1] The PKC molecule consists of a C-terminal catalytic and an N-terminal regulatory domain. The regulatory domain contains two C1 domains, which bind DAG/phorbol esters, and one C2 domain, which in the classical PKC isoforms binds Ca^{2+} and phosphatidylserine, and a pseudosubstrate motif that keeps PKC in a closed conformation in the absence of activators.

It is clear that PKC isoforms have unique effects that conceivably depend on structural differences. The outgrowth of neurites, long branches from the cell body that later become axons or dendrites, is one process where PKC isoforms have different effects. Especially the novel isoforms PKCδ and PKCε have been suggested to be positive regulators of neurite outgrowth,[2–6] although there have

Abbreviations used: PKC, protein kinase C; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

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been contradictory reports.[7] Our group showed earlier that PKCε induces neurite outgrowth in neuroblastoma cells.[8] This is mediated *via* the regulatory domain of PKCε, and is independent of the kinase activity. A region encompassing the C1 domains and flanking amino acid residues is sufficient for the effect. Recently, we identified a motif immediately N-terminal of the PKCε C1b domain crucial for neurite outgrowth.[9] However, this sequence was not sufficient to confer neurite-inducing capacity to the PKCα C1 region, indicating that other structures within the PKCε C1 domains are required.

C1 domains are important components of many different proteins, including PKCs, diacylglycerol kinases, chimaerins, raf and many others.[10,11] All C1 domains have in common conserved cysteine and histidine residues that are crucial for its tertiary structure.[12] The residues coordinate two zinc ions in the interior of the domain, and this maintains the domain in a globular structure. The crystal structure of the PKCδ C1b domain in complex with phorbol ester has been determined, showing the DAG/phorbol ester binding pocket at the top of the C1b domain.[13] The pocket is surrounded by hydrophobic residues that penetrate the membrane upon DAG/phorbol ester binding and has a hydrophilic groove that is capped when DAG/phorbol ester binds. In addition, cationic residues in the middle part of the C1 domain are involved in interactions with anionic phospholipids, thereby further contributing to membrane binding.[14]

The functions of the C1 domains primarily seem to be to target the protein to membranes and/or mediate interactions with other proteins.[15–21] The targeting to membranes can be mediated by intracellular messengers, such as DAG, or exogenous agents, such as phorbol esters. The responsiveness to phorbol esters varies considerably between C1 domains, which has led to the subdivision of the domains into typical C1 domains, which respond to phorbol esters, and atypical C1 domains, which do not bind phorbol esters.[22] The C1 domains in classical and novel PKC isoforms are all considered as typical.

This study shows that, despite the fact that the C1b domains of PKCα and PKCε are typical C1 domains and have a high degree of sequence homology, they are not interchangeable in terms of functional effects. This further indicates that a relatively limited number of amino acid residues are determinants for unique effects of C1 domains. The identification of these may provide important information about what determines the molecular basis for the isoform-specific effects of different PKC isoforms. This study was designed to define the individual residues in the C1b domain that are critical for the neurite-inducing effect of PKCε. We identified several residues in the PKCε C1b domain, in particular Asn49, which could account for the isoform specificity of the neurite-inducing capacity of PKCε.

Results

Residues flanking the PKCε C1b domain are not sufficient to make the tandem C1 domains of PKCα neuritogenic

Our group showed earlier that over-expression of PKCε induces outgrowth of neurites from neuroblastoma cells.[8] The effect is mediated by the regulatory domain of PKCε and a region encompassing the C1 domains is sufficient for neurite induction. The tandem C1 domains of all novel PKC isoforms, but not the classical isoforms α and β, have been shown to have neurite-inducing capacity.[9] Furthermore, we have identified a motif N-terminal of the PKCε C1b domain that is evolutionarily conserved among the novel PKC isoforms and necessary for neurite outgrowth. To test if the motif by itself possesses the capacity of PKCε to induce neurites, we inserted it into a PKCα region encompassing the C1 domains. The eight residue sequence QRFSVNMP was inserted immediately N-terminal of the C1b domain in PKCαC1ab, a part of PKCα comprising the C1 region including four residues N-terminal of the C1a domain and 21 residues C-terminal of the C1b domain, analogous to the neurite-inducing region of PKCε (Figure 1(a)). SK-N-BE(2)C neuroblastoma cells were transfected with vectors encoding PKCεC1ab, PKCαC1ab and PKCαC1ab(8 insert) fused to EGFP and empty EGFP vector. After transfection, cells were grown for 17 h in the absence or in the presence of the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Figure 1(d)). All chimeras were expressed with proper size (Figure 1(e)). As shown earlier,[9] the wild-type variant of PKCα did not induce neurites. Insertion of the eight residue sequence from PKCε did not confer significant neurite-inducing capacity to the PKCα construct. In the presence of TPA, only 9% of the cells expressing this construct had neurites, which is comparable to 7% of EGFP-expressing and 9% of PKCαC1ab-expressing cells but substantially less than the 34% neurite-expressing cells expressing PKCεC1ab. This indicates that the motif is not sufficient, although it is crucial, for neurite induction. Thus, there are other parts in PKCε of importance for neurite outgrowth.

One region of conceivable importance is the sequence immediately C-terminal of the C1b domain in PKCε, since the neurite-inducing capacity of PKCε is diminished substantially when 12 instead of 20 residues are included C-terminal of the C1b domain. Furthermore, a region of PKCε consisting of only the C1b domain, including 22 residues N-terminal of the C1b domain (i.e. the complete region between the C1a and C1b domains) and 20 residues C-terminal of the C1b domain, PKCε22+C1b+20, has neurite-inducing capacity in the presence of TPA.[9] We hypothesized that the C-terminal sequence together with the motif N-terminal of PKCε C1b may be mediating the neurite-inducing effect, and that insertion of amino acid residues