Identification of Conserved Amino Acids N-terminal of the PKCεC1b Domain Crucial for Protein Kinase Cε-mediated Induction of Neurite Outgrowth

We have shown previously that protein kinase C (PKC) ε can induce neurite outgrowth independently of its catalytic activity via a region encompassing its C1 domains. In this study we aimed at identifying specific amino acids in this region crucial for induction of neurite outgrowth. Deletion studies demonstrated that only 4 amino acids N-terminal and 20 residues C-terminal of the C1a domain are important for neurite induction. The corresponding regions from all other novel isoforms but not from PKCε were also neurogenic. Further mutation studies indicated that amino acids immediately N-terminal of the C1a domain are important for plasma membrane localization and thereby for neurite induction. Addition of phorbol ester made this construct neurite-inducing. However, mutation of amino acids flanking the C1b domain reduced the neurite-inducing capacity even in the presence of phorbol esters. Sequence alignment highlighted an 8-amino acid-long sequence N-terminal of the C1b domain that is conserved in all novel PKC isoforms. Specifically, we found that mutations of either Phe-237, Val-239, or Met-241 in PKCε completely abolished the neurite-inducing capacity of PKCε C1 domains. Phorbol ester treatment could not restore neurite induction but led to a plasma membrane translocation. Furthermore, if 12 amino acids were included N-terminal of the C1b domain, the C1a domain was dispensable for neurite induction. In conclusion, we have identified a highly conserved sequence N-terminal of the C1b domain that is crucial for neurite induction by PKCε, indicating that this motif may be critical for some morphological effects of PKC.

The induction and elongation of neurites are cellular processes driven by cytoskeletal changes. These are under the control of different intracellular transduction pathways mediating signals from other cells or the extracellular matrix. The members of the PKC1 family constitute one important family controlling the outgrowth of neurites. PKC isoforms have been suggested to both positively and negatively influence the outgrowth of neurites.

There are 10 different PKC isoforms that are divided into three subclasses according to their structure and requirements for activation, classical PKCs (α, βI, βII, and γ), novel PKCs (δ, ε, η, and θ), and atypical PKCs (ζ, η, and ζ). Of these isoforms, particularly PKCδ (1–3) and PKCε (3–8) have been suggested to positively influence neurite outgrowth in several different cell types. However, there are also indications that PKC isoforms, for instance PKCε, can counteract outgrowth (9). Our group has shown previously that overexpression of PKCε in neuroblastoma (7, 8) and in immortalized neural precursor (3) cells leads to neurite outgrowth. A similar morphological effect of PKCε has also been observed in fibroblasts (10, 11). The neurite-inducing effect of PKCε is independent of its catalytic activity, and a region from the regulatory domain of the enzyme encompassing the two C1 domains with flanking structures is necessary and sufficient for the effect (7).

Many proteins, besides PKC isoforms, contain C1 domains (12). These domains can roughly be subgrouped as typical C1 domains that bind phorbol esters and atypical C1 domains that do not bind phorbol esters (13). Classical and novel PKC isoforms contain two typical C1 domains. The structure of PKCα C1b (14), PKCδ C1b (15), and PKCγ C1b (16) domains has been determined, revealing that the C1 domain is a compact globular structure. The integrity of the domain depends on the coordinated binding of two Zn2⁺ ions by conserved cysteine and histidine residues. On the tip of the domain is a diacylglycerol/phorbol ester binding pocket located between two β strands (15). The sides of the pocket are covered by hydrophobic residues, and binding of lipid generates a continuous hydrophobic surface that facilitates the insertion of the domain into the membrane. Besides the binding of lipids, C1 domains have been shown to mediate protein-protein interactions (17–21). Amino acids flanking the C1 domains have also been found to mediate interactions with other proteins. For instance, in PKCε an F-actin-binding site is located C-terminal of the C1a domain (22).

One important function of C1 domains is likely the targeting of the protein to specific intracellular locations. Several C1 domain-containing proteins are localized to the Golgi apparatus, and this is mediated via a C1 domain (23–28), and the C1a domain of PKCβII has been shown to bind the centrosomal protein pericentrin (19). The fact that C1 domains bind membranes and can mediate protein interaction implies that they also may serve as anchoring proteins at different membranes. We hypothesize that the PKCε C1 domains induce neurites by interacting with one or several proteins and either anchor them at the membrane, perhaps as a signaling complex, or, if they are membrane-residing proteins, induce a conformational change in them.

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To understand further such an interaction, this study was designed to identify a specific motif in the C1 region of PKCe which is crucial for induction of neurite outgrowth. We show that a stretch of eight amino acids N-terminal of the C1b domain, which is evolutionarily conserved in all novel PKC isoforms, is critical for neurite induction. Furthermore, we identify in this 

**TABLE I**

| Construct | Primer |
|-----------|--------|
| PKCe 4+C1V3 | F1/R1 |
| PKCe 4+C1+39 | F1/R2 |
| PKCe 4+C1+20 | F1/R3 |
| PKCe 4+C1+12 | F1/R4 |
| PKCe 4+C1+6 | F1/R5 |
| PKCe 12+C1b+20 | F2/R3 |
| PKCe 22+C1b+20 | F3/R3 |
| PKCe PSC1V3-scr | F4/R6 |
| PKCe PSC1V3(R236A) | F5 |
| PKCe PSC1V3(F237A) | F6 |
| PKCe PSC1V3(V239G) | F7 |
| PKCe PSC1V3(N240G) | F8 |
| PKCe PSC1V3(M241G) | F9 |
| PKCe PSC1V3(V186L/N167E, G221T/L222R) | F10/R7, F11/R8, F12/R9 |
| PKCe PSC1V3(N240L/M241E, V295T/D296R) | F10/R10, F13/R11, F14/R9 |

Forward primers

| F1 | CGCGACGCGTGCCAGAGGAATCGCCAAAGTA |
| F2 | GCAGATCTCGACCATGGGTGAAGCCCCTAAAGACAAT |
| F3 | CAGGTCGACATTTTGTCTGGGGTAACGCC |
| F4 | CGCGTCGACTGCGGGGACTCGGCACCAGC |
| F5 | GACCTTCACAGCATATAC |
| F6 | CGCAGATCTCGACCATGCAGGTCAACGGCCACAAGTTCGGTATCCACAAC |
| F7 | GCGACGCGTAGCACACTTTGTGATTATGAG |
| F8 | CGCCTCGAGCTGATGGACCCTGCGCCTGAC |
| F9 | GGGCTGCATGCTGGAGCCCACCTGGTCGGGGGT |
| F10 | CAGGTGGGCTCCCAGGCCTTCAGCGTCAACATGCC |
| F11 | CGCGTCGACTGCGGGGACTCGGCACCAGC |
| F12 | CAGCGGTTCAGCGTCAACGGGCCCCACAAGTTCGGTATC |
| F13 | TTCAACCGGGTCCACAAGTTCGGTATCCACAAC |
| F14 | CGCGTCGACTGCGGGGACTCGGCACCAGC |

Reverse primers

| R1 | GACCTTCACAGCATATAC |
| R2 | CAGGTCGACATTTTGTCTGGGGTAACGCC |
| R3 | CGCGTCGACTGCGGGGACTCGGCACCAGC |
| R4 | CAGGTCGACATTTTGTCTGGGGTAACGCC |
| R5 | CGCGTCGACTGCGGGGACTCGGCACCAGC |
| R6 | CGCGTCGACTGCGGGGACTCGGCACCAGC |
| R7 | CGCGTCGACTGCGGGGACTCGGCACCAGC |
| R8 | CGCGTCGACTGCGGGGACTCGGCACCAGC |
| R9 | CGCGTCGACTGCGGGGACTCGGCACCAGC |
| R10 | CGCGTCGACTGCGGGGACTCGGCACCAGC |
| R11 | CGCGTCGACTGCGGGGACTCGGCACCAGC |

The ExSite PCR-based site-directed mutagenesis kit (Stratagene) was used to replace the sequence QRFSVNMP in PKCePSC1V3-EGFP with the scrambled sequence SMQFPNNRV. The DpnI-treated PCR products were purified in 1% agarose gel and were thereafter ligated. All PCR products were sequenced to ensure that no mutations were introduced in the PCRs.

The scrambled sequence SMQFPNNRV was also introduced in full-length PKCe by using the ScaI and SacI restriction enzyme sites to cleave out a PKCe fragment containing the scrambled sequence. The fragment was thereafter ligated into the full-length PKCe-EGFP vector.

The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce the R236A, F237A, V295T, D296R, and R236A mutations in the PKCePSC1V3 sequence using the expression vector encoding PKCePSC1V3-EGFP as template. The mutations were verified by sequencing. All primers are listed in Table I.

**Cell Culture and Transfections—**Human neuroblastoma SK-N-BE (2C) cells and SH-SY5Y cells stably transfected with cDNA encoding TrkA (30) were grown in minimum essential medium with Earle’s salts and L-glutamine and supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Rat pheochromocytoma PC12 cells were grown in RPMI 1640 medium with L-glutamine supplemented with 10% horse calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. All cell culture solutions were from Invitrogen. For transfections, cells were trypsinized and seeded on glass coverslips at a density of 200,000 (SK-N-BE/2C) cells or 300,000 cells (SH-SY5Y/TrkA and PC12 cells) per 35-mm cell culture dish. The cells were transfected 24 h later using 2 μl of Lipofectamine2000 (Invitrogen) and 1 μg of DNA in 1 ml of serum-free medium according to the supplier’s protocol. Six hours later, the transfection mixture was...
replaced with medium containing serum and antibiotics. Where indicated, cells were treated with 16 nM TPA (Sigma) or 50 μM C2-ceramide (Sigma) for 17 h or 100 ng/ml NGF (Promega) for 2 days.

**Morphology Studies**—Seventeen hours, or 2 days for experiments with NGF, after transfection, cells were fixed and mounted as described previously (7). Transfected cells, identified by the fluorescence of EGFP, were considered to have neurites if the process was longer than two cell bodies. For PC12 cells, processes longer than the length of one-half cell body were considered as neurites. 200 transfected cells per experiment were counted.

**Western Blotting**—Cells were seeded at a density of 2.5 × 10^5 cells/100-mm culture dish or 1.2 × 10^5 (SK-N-BE(2)C) or 2.2 × 10^5 (SH-SY5Y/TraK and PC12) cells/60-mm culture dish and transfected with 6 μl of Lipofectamine2000 and 3 μg of DNA in 3 ml of serum-free medium. The day after transfection cells were washed in phosphate-buffered saline and lysed in buffer (10 mM Tris, pH 7.2, 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA) followed by centrifugation. Equal amounts of proteins were electrophoretically separated on a SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). Proteins were detected with primary antibodies toward GFP (1:250; Zymed Laboratories Inc.) and visualized with horseradish peroxidase-labeled secondary antibody (Amersham Biosciences) using the SuperSignal system (Pierce) as a substrate. The chemiluminescence was detected with a CCD camera.

**Immunofluorescence**—Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 2 min, permeabilized, and blocked with 5% normal goat serum and 0.3% Triton X-100 in Tris-buffered saline for 30 min. Syntaxin 6 was detected with a primary monoclonal antibody (Pharminen) diluted 1:25 followed by a secondary Alexa Fluor 546-conjugate antibody (Molecular Probes) diluted 1:1000 in Tris-buffered saline. The coverslips were thereafter mounted as for morphology studies.

**Confocal Microscopy**—Live cells were examined the day after transfection. The coverslips were washed twice with buffer H (20 mM Hepes, 137 mM NaCl, 3.7 mM KCl, 1.2 mM MgSO4, 2.2 mM KH2PO4, 1.6 mM CaCl2, 10 mM glucose, pH 7.4) and mounted on the heated stage of a Nikon microscope. The localization of PKCe-EGFP was examined using a × 100 objective (NA 1.4) and a Bio-Rad Radiance 5000 confocal system with excitation wavelengths at 488 nm and emission filter 515HPQ30 prior to and 1 min after addition 1 mM carbaxol. Fixed cells were examined with the same settings with the modification that Alexa Fluor 546 was detected using 543 nm excitation and 600LP emission filter.

**RESULTS**

**The Importance of the Pseudosubstrate and the V3 Region for Neurite Outgrowth Induced by the Regulatory Domain of PKCe**—Our group has shown previously that overexpression of PKCe induces neurite outgrowth in SK-N-BE(2) neuroblastoma cells via the regulatory domain and that a region of PKCe encompassing the pseudosubstrate, the two C1 domains, and the V3 region (PKCeC1V3) is sufficient for this effect (7). To elucidate further which structures in PKCe are necessary for neurite outgrowth, the importance of amino acids N- and C-terminal of the C1 domains was investigated. For N-terminal amino acids we studied PKCe variants in which either all amino acids N-terminal of the C1a domain had been removed (PKCeC1V3) or in which four amino acids prior to the C1a domain were included (PKCeC4+C1V3). SK-N-BE(2)/C neuroblastoma cells, transfected with vectors encoding PKCePSC1V3 or the truncated variants fused to EGFP, were cultured in the absence or presence of 16 nM TPA for 17 h. Thereafter the number of transfected cells with neurites was quantified (Fig. 1A). Removal of the pseudosubstrate resulted in a construct with markedly reduced capacity to stimulate neurite outgrowth, and inclusion of four amino acids N-terminal of the C1a domain generated a construct with an intermediate effect. However, TPA treatment of cells overexpressing the truncated mutants led to neurite outgrowth to a similar extent as for cells overexpressing PKCePSC1V3. Thus, the necessity of the pseudosubstrate for optimal neurite induction can be compensated for by TPA treatment. Western blot on lysates from transfected cells confirmed the expression of proteins of proper size (Fig. 1B).

Next, the importance of the C-terminal part of the PKCe regulatory domain for neurite outgrowth was investigated by gradually deleting parts of the V3 region. The PKCePSC1V3 region contains 80 amino acids C-terminal of the C1b domain. Constructs encoding a PKCe regulatory domain encompassing the pseudosubstrate of the C1a domain and encompassing 39 (PKCeC4+C1+39), 20 (PKCeC4+C1+20), 12 (PKCeC4+C1+12), or 6 (PKCeC4+C1+6) residues C-terminal of the C1b domain were generated, and SK-N-BE(2)/C cells were transfected with the vectors. Neurite outgrowth was prominent and similar in cells overexpressing PKCeC4+C1V3, PKCeC4+C1+39, or PKCeC4+C1+20, whereas no neurite induction could be detected in cells overexpressing PKCeC4+C1+12 or PKCeC4+C1+6 (Fig. 2A). Western blot on lysates of transfected cells demonstrated that non-neurite-inducing variants were not expressed at lower levels than neurite-inducing proteins (Fig. 2B), showing that the lack of neurite induction is not due to lower expression levels.

To investigate whether the PKCeC4+C1+12 region is capable of inducing neurite outgrowth when stimulated with TPA, cells overexpressing PKCeC4+C1+12, PKCeC4+C1V3, or PKCePSC1V3 were treated with TPA for 17 h after transfection (Fig. 2C). Still, the PKCeC4+C1+12 construct did not induce neurite outgrowth, suggesting that more than 12 residues C-terminal of the C1b domain are required and that 20 residues C-terminal of the C1b domain are sufficient for neurite induction.

To confirm that the PKCeC4+C1V3 and PKCeC4+C1+12 are responsive to phorbol esters, cells expressing these EGFP fusion proteins were treated with TPA and subjected to analysis with confocal microscopy (Fig. 2D). TPA treatment led to a plasma membrane translocation of both proteins demonstrat-
Motif in PKCe Crucial for Neurite Induction

The number of transfected cells with neurites was thereafter quantified. The number of transfected cells with neurites was thereafter quantified. The number of transfected cells with neurites was thereafter quantified.

The C1 Domains of All Novel PKC Isoforms Are Capable of Inducing Neurites in Neuroblastoma Cells—We have seen previously that the regulatory domains of all novel PKC isoforms to various extents have neurite-inducing capacity (3, 7, 8). This led us to investigate if the region corresponding to PKCe4+C1+20 in other PKC isoforms also is capable of inducing neurite outgrowth. SK-N-BE(2)C cells were therefore transfected with vectors encoding EGFP fused to the C1 domains of PKCa, -β, -γ, and -δ flanked by 4 N-terminal and 20 C-terminal amino acids. Cells were grown for 17 h in the absence or presence of TPA and thereafter examined for neurite outgrowth (Fig. 3A). Overexpression of PKCe4+C1+20, but not the corresponding regions in PKCa, -γ, and -δ, led to neurite outgrowth. After TPA stimulation, overexpression of C1 domains from all novel isoforms induced neurites, whereas overexpression of the corresponding region from the classical isoform PKCa had no effect. The expression levels of the EGFP-fused proteins were analyzed by Western blotting demonstrating that all proteins were expressed (Fig. 3C). However, the η and θ variants were expressed at lower levels, and there is therefore a possibility that the C1 domains of these isoforms may induce neurites also in the absence of TPA, if expressed at higher levels. Thus, the C1 domains of all novel PKC isoforms, but not of PKCa, have a capacity to induce neurite outgrowth.

Furthermore, we investigated if only one of the two PKCε C1 domains is sufficient to induce neurites. For this purpose, we generated constructs encoding the isolated C1a or C1b domain containing 4 residues N-terminal and 20 residues C-terminal of the C1 domain. Neuroblastoma SK-N-BE(2)C cells were transfected with the expression vectors and cultured in the absence or presence of TPA and examined for neurite outgrowth (Fig. 3B). Neither the isolated C1a nor the C1b domain could induce neurites by itself or after stimulation with TPA, indicating that overexpression of only one PKCe C1 domain is not sufficient to induce neurites. This is not due to lower expression levels of the isolated C1 domains because they are expressed at higher levels than PKCe4+C1+20 (Fig. 3C).

Amino Acids Flanking the C1b Domain Are Critical for Neurite Outgrowth—Our next approach was to investigate whether the C1 domains can be exchanged for each other or whether each C1 domain has unique properties of importance for the neurite-inducing effect. To test this we aimed at generating constructs encoding PKCePSC1V3 with tandem C1a (PKCePSC1aC1aV3) or C1b (PKCePSC1bC1bV3) domains. For this purpose PCR primers were designed to introduce restriction enzyme sites on both sides of each C1 domain-encoding sequence (Fig. 4A). The cDNA encoding the C1 domain was thereafter inserted in the PKCePSC1V3 sequence, from which the other C1 domain had been excised. To obtain proper controls, we also created PKCePSC1aC1bV3 in which the restriction enzyme sites flanking the C1 domains were inserted. Neither the PKCePSC1aC1aV3 nor the PKCePSC1bC1bV3 construct had the capacity to induce neurites (not shown), but neither did control constructs containing the PKCePSC1V3 region with the mutations resulting from insertion of the restriction enzyme sites flanking the C1 domains, PKCePSC1V3(V166L/N167E, G221T/L222R) and PKCePSC1V3-(N240L/M241E, V295T/D296R) (Fig. 4B). Thus, amino acids flanking the C1 domains seem to be critical for neurite induction.

To further pin-point the importance of amino acids flanking the C1 domains in PKCe, PKCePSC1V3 variants containing mutations on only one side of each C1 domain were also generated: PKCePSC1V3(V166L/N167E), PKCePSC1V3(G221T/L222R), PKCePSC1V3(N240L/M241E), and PKCePSC1V3-(V295T/D296R). SK-N-BE(2)C cells were transfected with the expression vectors and grown in the absence or presence of TPA, and EGFP-positive cells were scored for neurites (Fig.
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that of the wild-type that mediates neurite induction also in the

C-terminal of the C1a domain (G221T/L222R) was similar to

PKC

FIG. 3. The two C1 domains of all

novel isoforms are capable of induc-

neurites. A and B, SK-N-BE/2C

cells were transfected with vectors encod-

ing a region encompassing the C1a and

C1b domains of PKCa (αC1ab), PKCb

(βC1ab), PKCe (εC1ab), PKCη (γC1ab), or

PKCθ (δC1ab) (A) or the isolated C1a

(αC1a) or C1b (εC1b) or both C1 (εC1ab)
domains of PKCe (B) fused to EGFP. In all

constructs four amino acids N-terminal

and 20 amino acids C-terminal of the C1a domain were included. A vector encoding only EGFP was used as control (con).

Cells were grown in medium in the absence

or presence of 16 nM TPA for 17 h

after transfection. Cells were thereafter

fixed and mounted, and EGFP-positive

cells with neurites were counted. Data are

means ± S.E. (n = 3) and expressed as

percentage of transfected cells with neu-

rites. C, cell lysates were subjected to

Western blotting, using anti-GFP anti-

body. The positions of the molecular mass

markers for 30 and 46 kDa are indicated to

the right of the blot.

4B). As shown previously, PKCePSC1V3 with mutations both

N-terminal and C-terminal of the C1a domain had no neurite-

inducing effect, but treatment with TPA made it neurotogenic.

The same pattern was observed in cells overexpressing

PKCePSC1V3 only mutated N-terminal of the C1a domain

(V166L/N167E), whereas the effect of PKCePSC1V3 mutated

C-terminal of the C1a domain (G221T/L222R) was similar to

that of the wild-type that mediates neurite induction also in the

absence of TPA.

We have seen previously that the neurite induction of PKCe

C1 domains seems to depend on its plasma membrane localiza-

tion (7, 8), and we therefore examined the subcellular localiza-

tion of the mutated PKCePSC1V3 variants with confocal mi-

croscope (Fig. 4C). PKCePSC1V3 with mutations C-terminal of

the C1a domain (G221T/L222R) had a similar plasma mem-

brane localization as the wild-type. On the other hand, PKCe

PSC1V3 mutated N-terminal of the C1a domain (V166L/

N167E) is to a large extent localized to the cytoplasm but a clear

plasma membrane translocation can be induced by TPA.

Thus, for these mutants localization to the plasma membrane

seems to correlate to the neurite-inducing capacity of the mu-

tant. It is therefore conceivable that the mutations suppress

the neurite-inducing effect by altering the subcellular localiza-

tion of the C1 domains.

In contrast to the mutations flanking the C1a domain, mu-

tations either N-terminal or C-terminal of the C1b domain both

led to constructs that completely lacked neurite-inducing ac-

tivity (Fig. 4B) and for these mutants, neurite induction could

not be restored by treatment with TPA. Both mutants had a

perinuclear localization pattern (Fig. 4C). Treatment with TPA

led to a distinct plasma membrane localization, but as men-

tioned above, this was not accompanied by neurite induction.

Thus, it is likely that the amino acids mutated in these con-

structs are of importance for neurite outgrowth beyond affect-

ing the localization of the protein.

All mutated constructs were expressed at similar levels ex-

cept for PKCePSC1V3 with mutations on both sides of the C1b

domain (N240L/M241E and V295T/D296R) and PKCePSC1V3

with mutation C-terminal of the C1b domain (V295T/D296R)

that had lower expression levels (Fig. 4B). Therefore, it cannot

be excluded that the lack of neurite induction by the latter

construct is due to the fact that it is expressed at lower levels.

Identification of a Motif N-terminal of the C1b Domain

Crucial for Neurite Outgrowth—The previous results indicate an

important role for amino acids N-terminal of the PKCeC1b

domain for PKCe-mediated neurite induction. A sequence

alignment of amino acids N-terminal of the C1b domain of

novel isoforms revealed that a stretch of residues N-terminal

of the C1b domain is highly conserved among the human

novel PKCe isoforms and their Caenorhabditis elegans

and Drosophila melanogaster counterparts (Fig. 5A). The analo-

gous amino acids of the non-neurite-inducing PKC isoforms

have no similarity with this sequence (not shown). To investi-

gate whether this amino acid sequence, which is conserved in

all neurite-inducing isoforms, is crucial for neurite induction, we

replaced it with a scrambled version in full-length PKCe

(PKCe-scr) and in PKCePSC1V3 (PKCePSC1V3-scr). This

modification completely abolished the neurite-inducing capac-

ity of both constructs (Fig. 5B), further highlighting the

importance of amino acids N-terminal of the C1b domain for

neurite outgrowth.

We next analyzed the role of PKCe and the amino acids

N-terminal of the C1b in neuronal differentiation driven by

NGF. For this purpose we studied PC12 cells, in which PKCe

has been shown to potentiate NGF-stimulated neurite out-

growth (4) and SH-SY5Y neuroblastoma cells stably expressing

TrkA (30) in which PKCe has been indicated to play a role in

NGF-stimulated neurite outgrowth (5, 7, 8). The cell lines were

transfected with vectors encoding EGFP fusions of full-length

PKCe or PKCePSC1V3 or their scrambled counterparts prior to

treatment with NGF for 2 days (Fig. 5, C and D).

In PC12 cells (Fig. 5C), overexpression of either full-length

PKCe or PSC1V3 potentiated both basal and NGF-stimulated

neurite outgrowth. In contrast, the scrambled variants did not

cause such a potentiation. In SH-SY5Y/TrkA cells (Fig. 5D),

only PKCePSC1V3 potentiated basal and NGF-stimulated neu-
rite outgrowth. In cells expressing scrambled full-length PKCe, the NGF-induced neurite outgrowth was actually significantly lower than in NGF-stimulated cells that expressed EGFP alone, indicating a dominant negative effect of the construct. A tendency to such suppression was also observed in cells expressing the scrambled PKCe/H9280PSC1V3 variant.

The scrambled PKCe/H9280PSC1V3 was consistently expressed at lower levels in the neuroblastoma cells (Fig. 5, B and D), which could explain its lower neurite-inducing capacity. However, its expression in PC12 cells and the expression of the scrambled full-length variant were not markedly lower than wild type variants. Furthermore, when point-mutated PKCe/H9280PSC1V3 variants were investigated (Fig. 7), these were expressed at the same level as wild type PKCe/H9280PSC1V3, and some of them lacked neurite-inducing capacity. Thus, the reduced neurite-inducing capacity of the mutants is most conceivably explained by an altered function of the constructs and not by reduced expression levels.

The findings raised the question if other properties of PKCe are altered by the mutation. To examine whether this is the case, we chose to study the localization of PKCe/H9280-scr. Wild type PKCe responds to carbachol stimulation of SK-N-BE(2)C cells with a sustained translocation to the plasma membrane (29). As shown in Fig. 6, A and B, PKCe/H9280PSC1V3(V166L/N167E, G221T/L222R), PKCe/H9280PSC1V3(N240L/M241E, V295T/D296R) in the absence or presence of TPA was examined with confocal microscopy.

![Image](image_url)
by the C1b domain (28), and we therefore studied whether the PKC<P sub C1V3-scr construct would be enriched in the Golgi complex (Fig. 6, E–G). The wild type PKCEC1V3 causes profound morphological changes of neuroblastoma cells and is primarily present in the plasma membrane (7), but the PKC<P sub C1V3-scr shows a high degree of colocalization with syntaxin 6, a marker for the trans-Golgi network (37). Thus, scrambling of the eight amino acids N-terminal of the C1b domain does not negatively influence the localization of PKCe to the Golgi complex. Taken together, these data demonstrate that several properties of PKCe, receptor-stimulated membrane translocation and enrichment in the Golgi complex, are not abrogated by scrambling of the motif. Thus, the motif is of specific importance for neurite outgrowth and does not lead to a general annihilation of PKCe properties.

Point Mutation of PKCe Phe-237, Val-239, or Met-241 Abolishes the Neurite-inducing Effect—In the conserved sequence N-terminal of C1b there are three completely conserved residues, Arg-236, Phe-237, and Pro-242 (numbering from human PKCe). Furthermore, for Val-239 and Met-241, there are analogous hydrophobic residues in all novel isoforms, and at the corresponding site of Asn-240 there is either an asparagine or an aspartate. Ser-238, on the other hand, is not conserved. We decided to explore the importance of each individual conserved or semi-conserved residue, but we excluded Pro-242 due to its immediate vicinity to the first cysteine of the C1b domain. By using site-directed mutagenesis, the coding sequence of PKC<P sub C1V3 was altered to encode either alanine (for Arg-236 and Phe-237) or glycine (for Val-239, Asn-240, and Met-241). SK-N-BE(2)C cells were transfected with the plasmids and thereafter grown in the absence or presence of TPA (Fig. 7A). Overexpression of PKC<P sub C1V3(N240G) led to neurite outgrowth from the same number of cells as did overexpression of wild type PKC<P sub C1V3. On the other hand, mutation of the Phe-237, Val-239, or Met-241 residues led to PKC<P sub C1V3 variants that almost completely lacked neurite-inducing capacity, even after TPA treatment, suggesting that these residues are critically involved in neurite outgrowth induced by PKCe. All the mutated proteins were expressed at levels similar to that of the corresponding wild-type region (Fig. 7A).

All three mutants (PKC<P sub C1V3 P237A, V239G, or M241G) were clearly enriched in the plasma membrane after TPA exposure (Fig. 7B), showing that the lack of neurite induction is...
the non-neurite-inducing PKC body for analysis of expression levels. transfection and subjected to Western blotting, using anti-GFP anti-transfected cells with neurites. Cell lysates were also prepared after variants PKC PSC1V3(V239G), PKC/H9280 PKC PSC1V3(N240G), or PKC PSC1V3(M241G) after carbachol addition. B, SK-N-BE(2/C) cells expressing PKC PSC1V3-scr fused to EGFP were treated with 1 mM carbachol, and the localization of PKC variants was examined with confocal microscopy. Images demonstrate the localization of PKC PSC1V3-scr EGFP (E), syntaxin 6 (F), and a merged image (G).

FIG. 6. Scrambling of amino acids N-terminal of the C1b domain does not influence the localization of PKC. A and B, SK-N-BE(2/C) cells expressing PKCe-scr fused to EGFP were treated with 1 mM carbachol, and the localization of PKCe-scr-EGFP was followed with confocal microscopy. Images were taken prior to (A) and 60 s (B) after carbachol addition. C and D, SK-N-BE(2/C) cells expressing wild type PKCe (C) or PKCe-scr fused to EGFP (D) were grown in the presence of 50 μM C2-ceramide for 17 h after transfection. Cells were fixed and mounted, and the localization of the PKCe variants was examined with confocal microscopy. E–G, SK-N-BE(2/C) cells expressing PKCe/PSC1V3-scr fused to EGFP were subjected to immunofluorescence using primary monoclonal mouse antibody against the trans-Golgi marker syntaxin 6 and secondary antibody Alexa Fluor 546-conjugated goat anti-mouse and analyzed with confocal microscopy. Images demonstrate the localization of PKCe/PSC1V3-scr-EGFP (E), syntaxin 6 (F), and a merged image (G).

not due to these mutated variants being resistant to TPA-mediated plasma membrane localization.

The C1a Domain Is Dispensable for Neurite Outgrowth—We then speculated that the C1b domain alone is capable of inducing neurites if more than four amino acids N-terminal of the domain are included. To test this hypothesis, expression vectors were generated encoding the C1b domain including 22 (PKCe22+C1b+20) or 12 (PKCe12+C1b+20) amino acids N-terminal of the domain. Twenty amino acids were included C-terminal of the C1b domain, SK-N-BE(2/C) cells were transfected with the expression vectors and grown in the absence or presence of TPA. The effects of these proteins on neurite outgrowth were compared with the effect of a construct containing both the C1a and the C1b domain (PKCe4+C1+20) or the single C1b domain with only four amino acids N-terminal of the C1 domain (PKCe4+C1b+20) (Fig. 8). The neurite-inducing capacity of both PKCe22+C1b+20 and PKCe12+C1b+20 was reduced compared with the construct with both C1 domains, but following stimulation with TPA they induced neurites. The expression vectors encoding PKCe22+C1b+20, PKCe12+C1b+20, and PKCe4+C1b+20 were all expressed at higher levels than PKCe12b, indicating that the reduced capacity of the constructs to induce neurites is not due to lower expression levels (Fig. 8). Thus, the C1a domain is needed for optimal induction of neurite outgrowth, but the C1b domain alone has a significant neurite-inducing effect if 12 amino acids N-terminal of the domain are included in the construct.

FIG. 7. Point mutation of PKCe Phe-237, Val-239, or Met-241 abolishes the neurite-inducing effect. A, SK-N-BE(2/C) cells were transfected with vectors encoding the PKCe/PSC1V3 region or mutated variants PKCe/PSC1V3(R236A), PKCe/PSC1V3(F237A), PKCe/PSC1V3(V239G), PKCe/PSC1V3(N240G), or PKCe/PSC1V3(M241G) fused to EGFP. A vector encoding only EGFP was used as control. Cells were grown in medium in the absence or presence of 16 nM TPA for 17 h after transfection. Cells were thereafter fixed and mounted and scored for neurites. Data are mean ± S.E. (n = 4), expressed as percentage transfected cells with neurites. Cell lysates were also prepared after transfection and subjected to Western blotting, using anti-GFP antibody for analysis of expression levels. B, the intracellular localization of the non-neurite-inducing PKCe/PSC1V3 mutants, in cells grown in the absence or presence of TPA, were analyzed by confocal microscopy.

DISCUSSION
This study identifies an evolutionarily conserved motif in novel PKC isoforms that is crucial for the ability of PKCe to induce neurite outgrowth. The PKC isoforms are serine/threonine kinases that presumably mediate their effects by phosphorylation of target proteins and thereby alter their functions. However, there are also several studies indicating that PKC isoforms have biochemical or cellular effects that are independent of their catalytic activity. The induction of neurites is one such kinase-independent effect of PKCe (7), but PKC effects independent of catalytic activity also include suppression of sulfation of glucosaminoglycans in the Golgi apparatus by PKCe (25), activation of phospholipase D by PKCo (32), inhi-
proteins. The position of the molecular mass marker for 46 kDa is indicated to the right of the blot.

The lack of TPA effect was not due to a resistance to TPA. This was observed for constructs in which the PKC isoforms from both C. elegans and D. melanogaster indicate that the PKC expression, induction of neurites, has developed early in the evolution of multicellular organisms. The importance of the motif was also indicated by the fact that full-length PKCe with the motif scrambled failed to potentiate NGF-stimulated neurite outgrowth in PC12 cells and suppressed the same effect in SH-SY5Y/TrkA cells.

The question remains as to the function of the motif. Our data clearly indicate that it does not abrogate PKCe properties in general. The mutated PKCe responded to receptor-stimulated phospholipase C activation and also displayed a similar Golgi localization as wild type PKCe. TPA treatment induces a clear plasma membrane localization of the constructs in which the motif had been mutated, but this still does not lead to neurite outgrowth. Given the fact that C1 domains also mediate interactions with other proteins (17–21), we hypothesize that interaction with other proteins at the plasma membrane is an important mechanism mediating the induction of neurites by the PKCe C1 domains. Point mutations revealed that the hydrophobic residues Phe-237, Val-239, and Met-241 were critical. Conservation of phenylalanine or methionine on the protein surface has been suggested to imply a protein interaction site (36) supporting the hypothesis that the motif is important for protein-protein interaction.

However, the mere presence of the motif N-terminal of the C1 domain is not by itself sufficient to make a PKC isoform neurite-inducing. We made a construct encoding both C1 domains of PKCα in which the motif had been introduced immediately N-terminal of the PKCα C1 domain, but this construct lacked neurite-inducing effects (data not shown). We also hypothesized that expression of a peptide, either EGFP-tagged or Myc-tagged, containing the motif would break a putative protein-protein interaction and consequently block neurite outgrowth. However, this was not the case (data not shown). This may be due to aberrant folding of a short peptide, but it may also, perhaps more conceivably, be explained by a need for other components of the PSC1V3 construct in order to obtain proper binding.

In conclusion, in this study we have identified an evolutionarily conserved motif N-terminal of the C1 domain in novel PKC isoforms. The motif is necessary, but not sufficient, for neurite outgrowth by PKCe and does not generally alter other properties of the enzyme.

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Identification of Conserved Amino Acids N-terminal of the PKC\(\gamma\)C1b Domain Crucial for Protein Kinase C\(\gamma\)-mediated Induction of Neurite Outgrowth
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