Protein Kinase Cε Binds Peripherin and Induces Its Aggregation, Which Is Accompanied by Apoptosis of Neuroblastoma Cells*

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A hallmark of the afflicted nervous tissue in amyotrophic lateral sclerosis is the presence of protein aggregates, which to a large extent contain the intermediate filament protein peripherin. Here we show that activation of protein kinase C (PKC) or overexpression of PKCε induces the aggregation of peripherin in cultured neuroblastoma cells with elevated amounts of peripherin. The formation of aggregates was coupled to an increased apoptosis, suggesting a functional link between these events. Both induction of aggregates and apoptosis were suppressed in cells that had been transfected with small interfering RNAs targeting PKCε. PKCε and peripherin associate as shown by co-immunoprecipitation, and the interaction is dependent on and mediated by the C1b domain of PKCε. The interaction was specific for PKCε since corresponding structures from other isoforms did not co-precipitate peripherin, with the exception for PKCζ and -θ, which pulled down minute amounts. PKCε interacts with vimentin through the same structures but does not induce its aggregation. When the PKCε C1b domain is expressed in neuroblastoma cells together with peripherin, both phorbol ester-induced peripherin aggregation and apoptosis are abolished, supporting a model in which PKCε through its interaction with peripherin facilitates its aggregation and subsequent cell death. These events may be prevented by expressing molecules that bind peripherin at the same site as PKCε.

Extensive neuronal cell death is an underlying problem of many neurodegenerative diseases. This is frequently accompanied with, and may in several instances be caused by, the formation of large aggregates of different proteins in the afflicted neuronal tissue. The perhaps most notable examples are the amyloid plaques and neurofibrillar tangles seen in Alzheimer neuronal tissue. The perhaps most notable examples are the amyloid plaques and neurofibrillar tangles seen in Alzheimer disease (1, 2). These accumulations are supposedly generated as a consequence of misfolded proteins that are not properly degraded.

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Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by a progressive death of upper and lower motor neurons present in the cerebral cortex, brainstem, and spinal cord. This leads to skeletal muscle atrophy, paralysis, and finally death (3–6). As seen in many neurodegenerative diseases, different protein aggregates can be found in the afflicted neurons (5, 7). These frequently contain the intermediate filament proteins neurofilament and peripherin (8, 9). Peripherin is a class III intermediate filament that is specifically expressed in neuronal tissue and at particularly high levels in cells of the peripheral nervous system (10, 11).

Besides the findings in patients, there are a number of studies linking peripherin aggregates to ALS and/or motor neuron degeneration. Transgenic mice that overexpress peripherin exhibit motor neuron loss that is preceded by the appearance of peripherin inclusions in the cells (12, 13) and by defective axonal transport (14). Furthermore, the aggregation of peripherin makes neurons more susceptible to cell death stimuli (12), suggesting a causal link between the formation of aggregates and cellular degeneration. The putative pathogenic role of the aggregates raises the possibility that their dissolution or a block of their further expansion may suppress the advancement of the disease. However, there is not much known about the molecular mechanisms underlying the formation of peripherin aggregates.

In this study, we demonstrate that protein kinase C (PKC) promotes the aggregation of peripherin. PKC constitutes a serine/threonine kinase family that is subdivided in classical (PKCa, -βI, -βII, and γ), novel (PKCd, -ε, -η, and -θ), and atypical (PKCe and -ζ) PKC isoforms based on structural similarities (15). The PKC isoforms are central regulators of a wide range of cellular processes. We have seen that PKCe induces outgrowth of neurites in many cell types of neural origin (16–18). The effect is independent of the catalytic activity and mediates this effect, we immunoprecipitated the neuritogenic PKCe structure and analyzed which proteins were co-precipitated. One of these was peripherin, and the interaction was

* The abbreviations used are: ALS, amyotrophic lateral sclerosis; ECFP, enhanced cyan fluorescent protein; GFP, green fluorescent protein; EGFP, enhanced GFP; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; z-VAD, benzylcarbonyl-Val-Ala-Asp-(O-methyl)fluoromethylketone; DMSO, dimethyl sulfoxide; siRNA, small interfering RNA; PBS, phosphate-buffered saline; ANOVA, analysis of variance.

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confirmed for endogenous proteins. When analyzing a putative function of the interaction in the regulation of cellular morphology, we discovered that either activation of PKC or over-expression of PKC markedly increase the number of cells with peripherin aggregates. Furthermore, the aggregate formation was accompanied by an increased apoptosis. Thus, the results in this study provide important clues to our understanding of the molecular basis for the formation of peripherin aggregates and its consequences for neuronal cell survival, a feature that is characteristic for ALS.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Expression vectors encoding full-length human PKC isoforms, isolated domains of PKC isoforms, or kinase-dead full-length PKCe (K438R mutation) fused to enhanced green fluorescent protein (EGFP) have been described previously (17–19). To generate an expression vector encoding FLAG-tagged peripherin, cDNA was amplified with PCR using primers containing EcoRI and SalI sites and a peripherin full-length clone (RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH, clone ID: RIT p97D00254D6) as template. The cDNA was inserted into the p3XFLAG-CMV™-7.1 vector (Sigma) and sequenced to verify that no mutations had been introduced in the PCR.

pCMV-script vectors (Stratagene) encoding either wild-type vimentin or mutants with N-terminal serine residues (Ser-4, -6, -7, -8, -9) mutated to alanine (vimentin Ala) or aspartate (vimentin Asp) were kindly provided by Dr. J. Ivaska (20). To produce ECFP-tagged peripherin or peripherin and EGFP as (vimentin Asp) were kindly provided by Dr. J. Ivaska (20). To produce ECFP-tagged peripherin or peripherin and EGFP as separate proteins, the FLAG-tagged peripherin cDNA was digested with EcoRI/SalI, and the fragment was inserted in either the pECFP-C1 expression vector (Clontech) or the pCMS-EGFP expression vector (Clontech).

**Cell Culture and Transfections**—Human neuroblastoma cells SK-N-BE(2)C were grown in minimum essential medium (Sigma) supplemented with 10% fetal bovine serum (EuroClone), 100 IU/ml penicillin (Invitrogen), and 100 μg/μl streptomycin (Invitrogen). MDA-MB-231 breast carcinoma cells were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 100 IU/ml penicillin, and 100 μg/μl streptomycin. Cells were kept at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air.

For immunoprecipitation experiments, SK-N-BE(2)C or MDA-MB-231 cells were seeded at a density of 1.5–2 × 10⁶ cells/100-mm cell culture dish. For immunofluorescence experiments, 200,000 SK-N-BE(2)C cells were seeded on glass coverslips in 35-mm cell culture dishes. For differentiation of SK-N-BE(2)C cells, 200,000 cells were seeded on glass coverslips in 35-mm cell culture dishes and incubated in 10 μM all-trans-retinoic acid (Sigma). Transfections were performed with 2 μg of DNA and 2 μl of Lipofectamine 2000 (Invitrogen) per ml OptiMEM 1 medium (Invitrogen) according to the supplier’s protocol.

For siRNA transfections 70,000 SK-N-BE(2)C cells were transfected on three consecutive days with 50 nM Stealth™ RNA interference oligonucleotides (Invitrogen) and 1.5 μl of Lipofectamine 2000 per ml OptiMEM 1 medium. Two off-target oligonucleotides with 44 and 48% GC-content, respectively, were used as controls. The PKCe oligonucleotides were 5’-CACAAGUUGAUAACCACACCAUCU-3’ (siPKCe1), 5’-GCAAGGUGAUGUGGCAGAACUA-3’ (siPKCe2), and 5’-CCACAGUGUACUGCCACCACAU- CUU-3’ (siPKCe3). When indicated, cells were incubated with 16 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma), 2 μM GF109203X (bisindolylmaleimide I; Calbiochem), or 20 μM benzylocarbonyl-Val-Ala-Asp-(O-methyl)fluoromethylketone (z-VAD, Sigma).

**Immunoprecipitation**—Cells were treated as indicated in the protocol supplied with the μMACS epitope-tagged protein isolation kit (Miltenyi Biotec). Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer, supplied with the kit, and a complete protease inhibitor mixture (Roche Applied Science) for 30 min on ice. Lysates were cleared by centrifugation at 14,000 × g for 10 min at 4 °C and incubated either with anti-GFP-conjugated microbeads for 30 min or with 2 μg of anti-peripherin antibodies (Sigma) for 1 h prior to addition of protein G-coupled microbeads and an additional incubation for 30 min on rotation at 4 °C. Mouse IgG1 antibodies (ImmunonKontact) were used as controls. The immune complexes were recovered by applying the cell lysates on μ columns placed in the magnetic field of a μMACS separator and then washed and eluted with an elution buffer included in the kit.

**Identification of Co-precipitated Proteins**—Immunoprecipitates were obtained as described under the “Immunoprecipitation” section. Thereafter iodoacetamide was added to give a final concentration of 100 mM, and the precipitated proteins were incubated for 20 min in darkness. The samples were electrophoretically separated by SDS-PAGE using a 7.5% polyacrylamide gel. The gel was silver-stained as described previously (21), first washed in 50% methanol with 5% acetic acid for 20 min and placed in 50% methanol for 10 min and then sensitized with 0.02% sodium thiosulfate before staining with 0.1% silver nitrate for 20 min at 4 °C and developed in a solution of 0.04% formalin and 2% sodium carbonate for less than 10 min followed by 5% acetic acid stopping the reaction. Bands in the lane with precipitate from SK-N-BE(2)C cells transfected with PKCe PSC1V3 and absent in the lane with precipitate from EGFP-transfected cells were excised and treated for subsequent in-gel digestion as described (22). Briefly, after destaining and rehydration using neat acetonitrile, the samples were proteolyzed overnight using porcine modified trypsin (Promega). The generated peptides were analyzed by peptide mass fingerprinting using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Ultraflex tandem time-of-flight, Bruker Daltonics). The instrument settings were optimized for analytes from 600 to 4500 Da and α-cyano-4-hydroxycinnamic acid was used as matrix. The peptide mass lists were used to search sequence databases for protein identification using ProFound at the PROWL web site as search engine.

**Immunoblotting**—Proteins were electrophoretically separated by SDS-PAGE and transferred to a polyvinylidine difluoride membrane (Millipore). The membranes were incubated with primary monoclonal antibodies toward GFP (Zymed Laboratories Inc.), peripherin (Sigma), or vimentin (DakoCytomation) or with polyclonal antibodies toward PKCe, -pIL, -β, or -ε (Santa Cruz Biotechnology). The membranes were thereafter
incubated with horseradish peroxidase-labeled secondary antibody (Amersham Biosciences), which was detected using the SuperSignal system (Biological Industries) as substrate. The chemoluminescence was captured with a charge-coupled device camera (Fujifilm).

**Immunofluorescence and Confocal Microscopy**—16 h after transfection, cells were washed once in PBS, fixed with 4% paraformaldehyde in PBS for 4 min, washed twice in PBS, and thereafter permeabilized and blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 30 min. Cells were incubated with primary antibodies toward peripherin (Sigma), vimentin (DAKO), or FLAG (Sigma) in PBS for 1 h. Following washes in PBS, cells were incubated with secondary Alexa Fluor 546-conjugated goat anti-mouse IgG antibody (Molecular Probes) diluted 1:800 in PBS for 1 h followed by extensive washes in PBS and mounted on object slides using 20 μl of PVA-DABCO (9.6% polyvinyl alcohol, 24% glycerol and 2.5% 1,4-diazabicyclo[2.2.2]octane) in 67 mM Tris-HCl, pH 8.0). For phalloidin staining, cells were incubated with Alexa Fluor 546-conjugated Phalloidin (Molecular Probes) diluted 1:200 in PBS for 20 min after the secondary antibody incubation. The coverslips were studied by immunofluorescence microscopy or by confocal microscopy using a Bio-Rad Radiance 2000 confocal system fitted on a Nikon microscope with a ×60/NA 1.40 oil lens. Excitation wavelengths were 457 nm (ECFP), 488 nm (EGFP), and 543 nm (Alexa Fluor 546), and the emission filters used were HQ485/30 (ECFP), HQ515/30 (EGFP), and 600LP (Alexa Fluor 546). In aggregation experiments, 200 transfected cells, identified by the fluorescence of EGFP, were scored for aggregate contents.

**Analysis of Apoptosis**—SK-N-BE(2)C cells were washed in PBS and fixed with 4% paraformaldehyde in PBS for 4 min and thereafter washed twice in PBS followed by incubation for 20 min in a DNA staining solution containing 3.5 μM Tris-HCl, pH 7.6, 10 mM NaCl, 5 μg/ml propidium iodide, 20 μg/ml RNase, and 0.1% v/v Nonidet P-40. The coverslips were examined by immunofluorescence microscopy where 200 transfected cells, identified by EGFP positivity, were counted and then scored for fragmented or condensed nuclei.

**RESULTS**

**Peripherin Associates with PKCe in Neuroblastoma Cells**—Our previous work has shown that PKCe, via its regulatory domain, induces neurites in a wide range of neural cell types. More specifically, the effect is mediated by a structure encompassing the two C1 domains and flanking residues in the pseudosubstrate and the V3 region (PKCe PSC1V3) (17, 23).

PKCe Induction of Peripherin Aggregates

To identify proteins that interact with the PKCe PSC1V3 region, SK-N-BE(2)C neuroblastoma cells were transfected with a vector encoding this PKCe structure fused to EGFP (Fig. 1A). Cell lysates were thereafter immunoprecipitated with an anti-GFP antibody coupled to magnetic beads. The precipitate was separated with SDS-PAGE, and the gel was subjected to silver staining (Fig. 1B). As control, precipitates of lysates from cells transfected with empty EGFP vector were used to visualize proteins unspecifically precipitated. Silver staining revealed several bands that were only present in the lane with the PKCe PSC1V3 precipitate. One of these, representing a protein of ~50 kDa was, following trypsin digestion and mass spectrometry analysis, identified as peripherin, a class III intermediate filament, with an estimated molecular mass of 54 kDa.
To confirm the interaction, the association of endogenous PKCε with peripherin was investigated. Lysates from SK-N-BE(2)C neuroblastoma cells were immunoprecipitated using anti-peripherin antibodies, and PKCε was detected in the precipitate (Fig. 1C). Of the other classical and novel isoforms expressed in neuroblastoma cells, neither PKCβII nor PKCδ co-precipitated with peripherin. However, there was a faint band corresponding to PKCα. Isotype-matched control antibodies did not precipitate the PKC isoforms.

The interaction of PKC with its binding partners has in several cases been shown to depend on the conformation of the PKC molecule. To investigate whether this is the case for the interaction of PKCε with peripherin, SK-N-BE(2)C cells were treated for 30 min with TPA prior to immunoprecipitation with antibodies against peripherin. When compared with untreated cells, no effect of TPA on the amount of co-precipitated PKCε could be discerned (Fig. 1C). Thus, the interaction of peripherin with PKCε is largely isoform-specific and not influenced by activation of PKC with phorbol esters.

**The Interaction Is Mediated via the C1b Domain in PKCε—** Peripherin was found as a PKCε-binding protein by using a fragment of the regulatory domain, PSC1V3, suggesting that this is the site responsible for the association. However, it cannot be excluded that other parts of the PKCε molecule also participate in the association with peripherin. To compare the interaction capacity of different PKCε domains, SK-N-BE(2)C cells were transfected with vectors encoding either the regulatory or the catalytic domain together with a vector encoding full-length PKCε fused to EGFP (Fig. 2A). Immunoprecipitation of the GFP-tagged proteins revealed that peripherin binds to the regulatory domain and not to the catalytic domain of PKCε. The interaction with the isolated regulatory domain seemed to be stronger than the interaction with the holo-enzyme, perhaps implying that relevant structures are conformationally masked in the full-length protein.

To further delineate the specific structures in the PKCε regulatory domain that mediate the interaction, the C2 domain and the PSC1V3 domain of PKCε fused to EGFP were expressed in SK-N-BE(2)C cells and immunoprecipitated (Fig. 2B). This demonstrated that the C2 domain does not contribute to the interaction with peripherin. In addition, a construct encoding PKCε PSC1V3 with the C1b domain exchanged for the PKCα C1b domain (PKCε PSC1V3, C1b) was evaluated for peripherin interaction. The amount of peripherin that was co-precipitated with this chimera (Fig. 2B, lane 4) was substantially lower when compared with the amount that was precipitated with the pure PKCε construct. Furthermore, the isolated PKCε C1b domain co-precipitated with peripherin, whereas this was not observed for either the isolated PKCε C1a or the isolated PKCα C1b domain (Fig. 2C). Thus, the peripherin interaction seems to be mediated primarily by the PKCε C1b domain. However, the tandem PKCε C1aC1b domain displayed a stronger interaction than the isolated C1b domain (Fig. 2C), indicating that the C1a domain or structures flanking the C1b domain contribute to or promote the interaction.

**Peripherin Preferentially Binds to PKCε—** To further investigate the isoform specificity of the interaction, SK-N-BE(2)C cells were transfected with vectors encoding full-length ver-

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**FIGURE 2.** The interaction with peripherin is mediated by the C1b domain of PKCε. SK-N-BE(2)C cells were transfected with vectors encoding EGFP fusions of either full-length PKCε (PKCεFL), the PKCε regulatory domain (PKCεRD), or the PKCε catalytic domain (PKCεCD) (A); the PKCε C2 domain (PKCεC2), the PKCε PSC1V3 domain (PKCεPSC1V3), or a PKCε PSC1V3 variant where the PKCε C1b domain was exchanged for the PKCα C1b domain (PKCε PSC1V3, c1bV3) (B); or the PKCε regulatory domain (PKCεRD), the PKCε C1a domain (PKCεC1a), the PKCε C1b domain (PKCεC1b), the tandem PKCε C1a and C1b domains together (PKCεC1ab), or the PKCε C1b domain (PKCεC1b) (C). An empty EGFP vector was used as control. Lysates were immunoprecipitated using anti-GFP-conjugated magnetic beads and thereafter subjected to Western blotting using a peripherin antibody. The positions of molecular mass markers are shown to the left of the blots. Please note that the two rightmost constructs in the total cell lysate blot of B are weak and located immediately above an unspecific band and therefore hard to detect. The presence of the constructs are clearly seen in the blot with the precipitates.
the precipitates indicate that these isoforms may also contain the structures necessary for association with peripherin.

Next, the isolated C1b domains from PKCa and all novel PKC isoforms were compared in terms of association with peripherin (Fig. 3C). A substantial interaction with peripherin was observed for the PKCe C1b domain, although minor amounts of peripherin were detected in PKCη C1b and PKCθ C1b precipitates, reflecting the results with the corresponding regulatory domains (Fig. 3, A and B).

**Overexpressed Peripherin Aggregates in a PKC-dependent Manner—**

To analyze a putative functional importance of the interaction of PKCe with peripherin, we first overexpressed peripherin tagged to the FLAG epitope in SK-N-BE(2)C neuroblastoma cells and thereafter incubated the cells for 16 h with the PKC activator TPA and/or the PKC inhibitor GF109203X (Fig. 4, A and B). Transfected cells were then visualized by the green fluorescent protein, and exogenous peripherin was visualized with immunofluorescence toward the FLAG tag. Overexpression of peripherin resulted in the formation of large peripherin-positive aggregates in some cells. Exposure to TPA markedly increased the number of cells with aggregates (Fig. 4A, panel d) from 31 to 54% (Fig. 4B), and this effect was suppressed by GF109203X (Fig. 4A, panel f, and B). The peripherin aggregates are reminiscent of what is observed in nervous tissue of patients with ALS, and the PKC-mediated aggregation of peripherin may therefore shed mechanistic light on this disease (3, 6, 12).

To analyze whether PKCe directly influences the peripherin aggregation, cells were co-transfected with vectors encoding FLAG-tagged peripherin and PKCe fused to EGFP (Fig. 4A, panels g and h, and B). Co-expression with PKCe markedly potentiated the aggregation (Fig. 4A, panel h, and B). More than 80% of the cells transfected with both PKCe-EGFP and peripherin contained aggregates when compared with 31% for cells overexpressing peripherin together with EGFP alone. The effect of PKCe overexpression could not be further potentiated by TPA, but it was markedly suppressed by GF109203X. In the presence of the inhibitor, the number of PKCe-transfected cells

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**FIGURE 3. PKCe is the isoform with the strongest interaction with peripherin.** SK-N-BE(2)C cells were transfected with vectors encoding EGFP only or EGFP fused to the full-length versions (A); the regulatory domains (B); or the C1b domains (C) of the different PKC isoforms α, β, ε, η, or θ. Cell lysates were immunoprecipitated (IP) using anti-GFP-conjugated magnetic beads and thereafter subjected to Western blotting using anti-peripherin antibody. The positions of the molecular mass markers for 66, 45, and 30 kDa are shown to the left of the blots. RD, regulatory domain.
FIGURE 4. Activation of PKC or overexpression of PKCe potentiates peripherin aggregation. A, SK-N-BE(2)C cells were co-transfected with an empty EGFP vector (panels a–f), full-length PKCe (PKCeFL) fused to EGFP (panels g and h), or kinase-dead PKCe (PKCeKD) fused to EGFP together with a vector encoding peripherin tagged to the FLAG epitope. The cells were thereafter treated with either 16 nM TPA alone (panels c and d) or TPA combined with 2 μM GF109203X (GFX, panels e and f) for 16 h. Exogenous peripherin was visualized by immunofluorescence with an anti-FLAG antibody. Cells were examined with a confocal microscope, and images show EGFP (panels a, c, and e), PKCe-EGFP (panel g), and FLAG-tagged peripherin (panels b, d, f, and h). Cells treated with DMSO were used as controls (panels a and b and panels g and h). B, SK-N-BE(2)C cells transfected and treated as in A were examined with fluorescence microscopy, and 200 transfected cells, visualized by the EGFP fluorescence, were scored for the presence of peripherin aggregates. A Western blot demonstrating relative expression of PKCe-EGFP fusions is shown above the graph. Data (mean ± S.E., n = 3) shows the percentage of transfected cells with peripherin aggregates. C, SK-N-BE(2)C cells transfected with a vector encoding peripherin fused to ECFP were treated with 16 nM TPA, and the peripherin aggregates were examined by confocal microscopy using the fluorescence of ECFP. D, SK-N-BE(2)C cells were transfected with a vector encoding peripherin and EGFP as separate proteins, treated with or without 16 nM TPA for 16 h, and subjected to immunofluorescence toward peripherin. Cells were examined with a confocal microscope and images show EGFP (top photos) and peripherin (bottom photos). E, shows a quantification of the amount of cells with peripherin aggregates treated with 16 nM TPA for 16 h when compared with untreated cells (mean ± S.E., n = 3). 200 cells were counted. F, SK-N-BE(2)C cells were treated with 10 μM all-trans-retinoic acid for 72 h and then co-transfected with EGFP vector and CMS-PRPH vector. The cells were thereafter treated with 16 nM TPA or with DMSO as control for 16 h. The peripherin aggregates were visualized by immunofluorescence using a primary anti-peripherin antibody and a secondary antibody conjugated to Alexa Fluor 546. The cells were analyzed by fluorescence microscopy. G, quantification of the amount of differentiated cells with peripherin aggregates after treatment with DMSO or with 16 nM TPA alone or in combination with 2 μM GF109203X for 16 h (mean ± S.E., n = 3). H, SK-N-BE(2)C cells were transfected with three different siRNAs targeting PKCe (si-1–3) or two control oligonucleotides (con 1–2) followed by transfection with a vector encoding FLAG-peripherin. After immunofluorescence toward the FLAG tag, the percentage of cells containing peripherin aggregates was quantified (mean ± S.E., n = 3). Western blots demonstrating expression of PKCe following siRNA treatment and actin as indicator of total protein loaded are included. * indicates statistically significant (p < 0.05) differences after analysis with ANOVA followed by Duncan’s multiple range test (B, G, and H) or Student’s t test (E). In G, the difference is significant when compared with the absence of TPA or the presence of TPA and GF109203X. ATRA, all-trans-retinoic acid. In H, the differences are significant when compared with mock- or control oligonucleotide-transfected cells. ∆ indicates statistically significant difference when compared with the absence of TPA, the presence of GF109203X alone, and the combination of the two agents.
PKCɛ Induction of Peripherin Aggregates

FIGURE 5. TPA disrupts the endogenous peripherin network and induces punctate peripherin structures. SK-N-BE(2)C cells were treated with 16 nM TPA alone or in combination with 2 μM GF109203X (GFX) for 16 h. As control, cells treated with DMSO were used. The endogenous peripherin network was visualized by immunofluorescence using a primary anti-peripherin antibody and a secondary antibody conjugated to Alexa Fluor 488 (A). The arrows point to punctuate structures found after TPA treatment. B, SK-N-BE(2)C cells were treated with 16 nM TPA alone or in combination with 2 μM GF109203X or with DMSO as control for 96 h, and peripherin was visualized by immunofluorescence using the same antibodies as in A. The number of punctuate peripherin structures was quantified. The data (mean ± S.E., n = 2) show the number of punctuate peripherin structures per cell (×100). C, SK-N-BE(2)C cells were treated with 16 nM TPA alone or together with 2 μM GF109203X or 2 μM GF109203X alone for 16 h. DMSO was used as control. The endogenous peripherin network was visualized by immunofluorescence by a primary anti-peripherin antibody and a secondary antibody conjugated to Alexa Fluor 488 together with phalloidin conjugated to Alexa Fluor 546 to visualize F-actin. D, the peripherin and vimentin networks were stained simultaneously in TPA-treated cells using Alexa Fluor-conjugated antibodies to visualize peripherin (Alexa Fluor 488) and vimentin (Alexa Fluor 546). The arrow points to a peripherin structure that is slightly positive when stained for vimentin. All images were captured by confocal microscopy. * indicates statistically significant difference (p < 0.05) when compared with other groups using ANOVA followed by Duncan’s multiple range test.

with aggregates was reduced to 51%, indicating that PKCɛ catalytic activity is needed to obtain maximal potentiation of aggregate formation. This is further supported by the finding that co-expression with kinase-dead PKCɛ augments the peripherin aggregation to a much lesser extent than wild-type PKCɛ does (Fig. 4B).

Our confocal analysis of the aggregates, visualized by immunofluorescence toward the FLAG tag, suggested that peripherin mainly localizes to the rims of the aggregates (Fig. 4A, panels d and h). This result may be due to an inability of the antibody to penetrate the aggregates. To circumvent this problem, we generated a vector encoding peripherin fused to the fluorescent protein ECFP. SK-N-BE(2)C cells were transfected with this vector, and the aggregates were analyzed with confocal microscopy (Fig. 4C). This demonstrated that peripherin is present throughout the aggregates.

To eliminate the possibility that the observed aggregation is dependent on the FLAG or ECFP tag, a vector was generated from which peripherin and EGFP are expressed as two separate proteins. SK-N-BE(2)C cells were transfected with the vector and treated with TPA. Peripherin was thereafter visualized with immunofluorescence, and transfected cells were identified by the expression of free EGFP (Fig. 4D). As for tagged peripherin, exposure to TPA led to a marked increase in the number of cells containing peripherin aggregates (from 17 to 64%, Fig. 4E), demonstrating that the aggregation is not dependent on the tags.

To investigate whether the aggregation also takes place in cells with a more differentiated phenotype, the SK-N-BE(2)C cells were treated with all-trans-retinoic acid for 3 days to induce neuronal differentiation. The cells were thereafter transfected with vectors encoding untagged peripherin and EGFP and exposed to TPA overnight (Fig. 4F and G). This demonstrated that TPA also induces peripherin aggregation in differentiated cells. As in undifferentiated neuroblastoma cells, the effect was blocked by GF109203X, indicating that it is due to PKC activity.

To further establish that PKCɛ mediates the TPA effect on peripherin aggregation, SK-N-BE(2)C cells were transfected with three siRNAs targeting different parts of the PKCɛ mRNA (Fig. 4H). Each PKCɛ siRNA led to suppression, but not complete down-regulation, of PKCɛ protein levels. Cells treated with siRNAs were transfected with peripherin vector, and after an overnight incubation, transfected cells were analyzed for peripherin aggregation. All PKCɛ siRNAs, but none of the control oligonucleotides, markedly reduced the number of cells with aggregates.

Treatment with TPA Leads to the Formation of Punctuate Structures of Endogenous Peripherin—Since the PKC activator TPA potentiates the formation of peripherin aggregates, we analyzed whether TPA also has an effect on the endogenous peripherin network. SK-N-BE(2)C cells were subjected to TPA treatment alone or in combination with the PKC inhibitor GF109203X for 16 h, and the peripherin network was thereafter visualized using anti-peripherin antibody (Fig. 5A). After incubation with TPA, punctuate peripherin structures appeared in the cytoplasm of some cells. Such structures could not be seen in untreated cells. Co-incubation with GF109203X seemed to suppress the formation of the peripherin punctuate structures.

Although structures were visible after 16 h, the structures became more prominent after longer TPA incubation. After treatment with TPA for 96 h, a clear increase in punctate peripherin formations could be discerned (Fig. 5B). The punctuate structures were counted, and the number of structures per cell was more than three times higher in TPA-treated compared with control cells. This increase was largely prevented by co-exposure to GF109203X.

The TPA treatment also seems to alter the morphology of the peripherin network (Fig. 5C). In cells exposed for TPA for 16 h, peripherin was more concentrated to the perinuclear area.
Peripherin is closely related to vimentin, which is also expressed in neuroblastoma cells (24). Furthermore, PKCe has been suggested to influence the formation of punctuate vimentin structures (20). We therefore investigated whether we could detect vimentin in the observed peripherin ring structures. After TPA treatment, SK-N-BE(2)C cells were co-stained with anti-vimentin antibodies and anti-peripherin antibodies (Fig. 6A). Only small amounts of vimentin were seen in the circular peripherin structures, indicating that vimentin constitutes at most a minor part of the aggregates.

**Vimentin Associates with PKCe but Does Not Aggregate upon PKC Activation**—Since we could detect some vimentin in the punctuate peripherin structures, we asked whether PKCe and TPA can influence vimentin to the same extent as peripherin. It has previously been shown that vimentin complexes with PKCe in mouse embryo fibroblasts (20). We therefore aimed at elucidating whether vimentin also interacts with PKCe in a similar manner as peripherin and whether it aggregates in response to PKC activation. EGFP-tagged PKCe PSC1V3 construct and full-length PKCe were expressed in MDA-MB-231 breast carcinoma cells that express high levels of vimentin. EGFP precipitates were subjected to immunoblotting using anti-vimentin antibody (Fig. 6A). As predicted, full-length PKCe interacts with vimentin, and so does the isolated PSC1V3 domain. Thus, there may be a common structural basis for the interaction of PKCe with class III intermediate filaments.

Overexpressed vimentin has been shown to accumulate in punctuate patterns upon PKC inhibition or when the phosphorylation of N-terminal serine residues is abolished (20). To investigate whether this aggregation is related to the observed phenomenon with peripherin aggregation, SK-N-BE(2)C cells were transfected with vectors encoding wild-type vimentin or vimentin with N-terminal serines mutated to alanines or aspartates (20). As seen in rat embryonic fibroblasts, vimentin with N-terminal serines mutated to alanines accumulated in a punctuate pattern, which was also observed for the aspartate mutant (Fig. 6B). However, overexpressed wild-type vimentin assembled into the endogenous vimentin network and was not influenced by exposure to TPA. Exposure to TPA did not influence the localization patterns of the mutants either. Furthermore, the punctuate vimentin structures do not contain peripherin (Fig. 6C), indicating that these vimentin structures are unrelated to the peripherin aggregates seen after PKC activation. Taken together,
Simultaneous Expression of the Interacting PKCe Domain Abrogates the Peripherin Aggregation—Since PKCe both interacts with peripherin and induces its aggregation, we speculated that the interaction may promote the formation of aggregates. The interaction is mediated via the PKCe C1b domain, and we therefore hypothesized that this domain may antagonize the TPA-mediated peripherin aggregation. We therefore transfected SK-N-BE(2)C cells with vectors expressing the interacting PKCe domains PSC1V3 or C1b together with peripherin tagged to the FLAG epitope and analyzed aggregate formation following TPA exposure (Fig. 8A). The expression of both PSC1V3 and C1b prevents peripherin from forming aggregates in the presence of TPA when compared with control (EGFP, 42%; ePSC1V3, 16%; eC1b, 16%). These marked effects were not observed by co-expression of the PKCe C1a or the PKCe PSC1V3 region with the C1b domain exchanged for the PKCa C1b domain.

We next investigated whether the same dominant negative effect can be obtained for the apoptotic effect of TPA on peripherin-overexpressing cells. SK-N-BE(2)C cells overexpressing peripherin together with PKCe PSC1V3 or C1b domains fused to EGFP were treated with TPA or left untreated and then stained with propidium iodide. The number of transfected cells, identified by EGFP fluorescence, with fragmented or condensed nuclei was calculated (Fig. 8B). Both the PKCe PSC1V3 and the C1b constructs suppressed the apoptosis-inducing effect of TPA from 11% apoptotic cells expressing EGFP to 4% for PKCe PSC1V3- and PKCe C1b-expressing cells. No protective effect was obtained with PKCe C1a or the PKCe PSC1V3 region with the C1b domain exchanged for the PKCa C1b domain.

**DISCUSSION**

PKCe is a versatile enzyme regulating cellular processes of importance both for the normal function of the organism as well as for pathological processes. One effect induced by PKCe is the profound change in cellular morphology exemplified by the outgrowth of neurites in a wide range of neuronal cell types (17, 25–27). The effect is independent of the catalytic activity of PKCe and mediated via a structure encompassing the C1 domains (17). We have identified single residues of crucial importance for the effect immediately N-terminal of the C1b domain (23) and in the base of the C1b domain (28). To understand the mechanisms of this PKCe effect, we utilized the most potent neurite-inducing construct, the PKCe PSC1V3 region, to identify interacting proteins that may mediate the PKCe effect. Through this approach, peripherin was identified as a novel PKCe-interacting protein. Judging from the silver-stained gel and the immunoprecipitation experiments, it is conceivable that only a small percentage of cellular PKCe and peripherin associate with each other and that PKCe interacts with several other proteins in the neuroblastoma cells as well.

The association with peripherin is dependent on primarily the PKCe C1b domain, which is in line with the molecular basis for neurite induction. C1 domains were originally identified as binding sites for the PKC activators phorbol esters and diacylglycerol and responsible for the membrane targeting of PKC (29). It has later become evident that they also mediate the

down-regulated by transfection with siRNAs targeting PKCe (Fig. 7C). In cells that had been transfected with any of the three PKCe siRNAs, the induction of apoptosis was markedly reduced when compared with mock-transfected cells or cells that had been transfected with control oligonucleotides.

**FIGURE 7. Overexpression of peripherin makes SK-N-BE(2)C cells go into apoptosis upon activation of PKC.** SK-N-BE(2)C cells were transfected with either an empty FLAG vector or a vector encoding FLAG-tagged peripherin together with a vector encoding EGFP and subsequently incubated in the absence or presence of 16 nM TPA for 16 h (A). Cells were stained with propidium iodide to visualize the nuclei. Transfected cells, identified by EGFP fluorescence, were scored for apoptosis, identified by fragmented or condensed nuclei. In B, SK-N-BE(2)C cells were transfected with an EGFP vector together with a FLAG vector or a vector encoding FLAG-tagged peripherin and were subjected to 16 nM TPA together with 2 μM GF109203X (GFX) or 20 μM z-VAD for 16 h. DMSO-treated cells were used as control. Cells were stained with propidium iodide to visualize the nuclei and scored for apoptosis as in A. C, SK-N-BE(2)C cells were transfected with siRNAs targeting PKCe (si-ε 1–3) or control oligonucleotides (con 1–2) followed by transfection with a FLAG-peripherin-encoding vector and treatment with TPA. Apoptosis was thereafter analyzed as in A and B. Data (mean ± S.E., n = 3, 200 cells were scored in each experiment) are expressed as the percentage of transfected cells that were apoptotic. * indicates statistically significant differences (p < 0.05) using ANOVA followed by Duncan’s multiple range test. The difference is significant when compared with all other groups (B) and mock-transfected (MOCK) or control oligonucleotide-transfected cells (C).
PKC\textsuperscript{e} Induction of Peripherin Aggregates

**A** SK-N-BE(2)C cells were co-transfected with vectors encoding EGFP only or EGFP fusions of PKC\textsuperscript{e} PSC1V3 (PKC\textsuperscript{e}PSC1V3), C1b (PKC\textsuperscript{e}C1b), or C1a domain (PKC\textsuperscript{e}C1a) or a PKC\textsuperscript{e} PSC1V3 construct where the C1b domain has been exchanged with the C1b domain (PKC\textsuperscript{e}PSC1a(c1b)V3) together with a vector encoding FLAG-tagged peripherin. Cells were incubated with or without 10 \textmuM TPA for 16 h and thereafter stained with an antibody directed toward the FLAG tag. Cells were scored for peripherin aggregates (A). B, SK-N-BE(2)C cells treated as in A were stained with propidium iodide to visualize the nuclei. 200 transfected cells, identified by EGFP fluorescence, were counted and scored for apoptosis, identified by fragmented or condensed nuclei. Data are mean ± S.E. from three independent experiments. * indicates statistically significant differences (p < 0.05) when compared with the absence of TPA using ANOVA followed by Duncan’s multiple range test.

**Figure 8.** PKC\textsuperscript{e} C1b obstructs TPA-induced peripherin aggregation and apoptosis in peripherin overexpressing cells. A. SK-N-BE(2)C cells were co-transfected with vectors encoding EGFP only or EGFP fusions of PKC\textsuperscript{e} PSC1V3 (PKC\textsuperscript{e}PSC1V3), C1b (PKC\textsuperscript{e}C1b), or C1a domain (PKC\textsuperscript{e}C1a) or a PKC\textsuperscript{e} PSC1V3 construct where the C1b domain has been exchanged with the C1b domain (PKC\textsuperscript{e}PSC1a(c1b)V3) together with a vector encoding FLAG-tagged peripherin. Cells were incubated with or without 16 \textmuM TPA for 16 h and thereafter stained with an antibody directed toward the FLAG tag. Cells were scored for peripherin aggregates (A). B, SK-N-BE(2)C cells treated as in A were stained with propidium iodide to visualize the nuclei. 200 transfected cells, identified by EGFP fluorescence, were counted and scored for apoptosis, identified by fragmented or condensed nuclei. Data are mean ± S.E. from three independent experiments. * indicates statistically significant differences (p < 0.05) when compared with the absence of TPA using ANOVA followed by Duncan’s multiple range test.

The interaction of PKC, in many cases in an isoform-specific manner, with other proteins (29–33). The interaction with peripherin clearly displays isoform specificity. Very limited association was observed for classical PKCs or for PKC\textsuperscript{\delta}. However, full-length PKC\textsuperscript{\gamma} and the isolated C1b domains of both PKC\textsuperscript{\gamma} and PKC\textsuperscript{\theta} seemed to have the capacity to interact with peripherin. Thus, the C1b domains of PKC\textsuperscript{\epsilon}, -\gamma, and -\theta conceivably share a common structure critical for the association with peripherin, but for PKC\textsuperscript{\delta}, this may not be sufficient for binding of the full-length protein.

Although the C1b domain mediates the interaction, the association of full-length PKC\textsuperscript{\epsilon} with peripherin was not potentiated by TPA. This may appear contradictory since TPA supposedly induces an open conformation of the enzyme with consequent exposure of previously concealed domains. The finding can have several explanations. One reason could be that TPA and peripherin have a common binding site in the C1b domain. However, TPA may then be expected to suppress the interaction, which it does not do. Alternatively, the peripherin-binding site may be already exposed in the inactive and closed PKC\textsuperscript{\epsilon} and PKC\textsuperscript{\delta}o raPKC\textsuperscript{\epsilon}C1b together with a vector encoding FLAG-tagged peripherin. Cells were incubated with or without 16 \textmuM TPA for 16 h and thereafter stained with an antibody directed toward the FLAG tag. Cells were scored for peripherin aggregates (A). B, SK-N-BE(2)C cells treated as in A were stained with propidium iodide to visualize the nuclei. 200 transfected cells, identified by EGFP fluorescence, were counted and scored for apoptosis, identified by fragmented or condensed nuclei. Data are mean ± S.E. from three independent experiments. * indicates statistically significant differences (p < 0.05) when compared with the absence of TPA using ANOVA followed by Duncan’s multiple range test.

In this context, peripherin is an attractive candidate as a pathogenic mediator. The fact that deletion of the peripherin gene does not abrogate the neuronal death observed in SOD1 mutant mice (46) may speak against a crucial role for peripherin in the development of the disease. However, as mentioned above, only a minor fraction of ALS cases is linked to mutated SOD1, whereas peripherin aggregation is a common feature for all ALS cases. Besides the presence of peripherin aggregates in afflicted tissues, there have emerged a number of studies suggesting a pathogenic role of peripherin. The peripherin (PRPH) gene is mutated in a few cases of familial ALS (47, 48), and peripherin levels are elevated in disease tissue (49). Overexpression of peripherin in motor neurons leads to their degeneration in transgenic mice (13), which is also seen in peripherin aggregation (13) and a suppression of axonal transport (14). Overexpression of peripherin also leads to the death of motor neurons cultured in vitro, further suggesting a causative link between peripherin aggregation and neuronal death (12).

In this study, the induction of apoptosis clearly paralleled the formation of peripherin aggregates. All manipulations that resulted in an attenuation of peripherin aggregation also suppressed apoptosis, and overexpression of peripherin per se had only minor effects on cellular survival unless aggregation was induced. Thus, this study provides substantial evidence for a cell death-inducing effect of peripherin aggregates, thereby supporting a causative role for these aggregates in the observed motor neuron death in ALS.
The observations highlight the importance of understanding what causes the aggregation of peripherin molecules. We, in this study, and others (50) have shown that increased levels of peripherin enhance its tendency to aggregate. Here we identify PKCe as another important factor facilitating peripherin aggregation. The aggregation could be induced either by activation of endogenous PKC or by elevating the levels of PKCe and was suppressed either by down-regulation of PKCe with siRNA or by inhibition of PKC activity. Although the number of aggregates was markedly elevated in cells overexpressing peripherin, punctuate peripherin structures could also be observed in non-transfected cells after PKC activation. This further underscores the putative importance of PKCe in promoting the aggregation of peripherin.

Based on the effects by TPA and GF109203X, it is conceivable that the kinase activity of PKCe is important, suggesting that increased phosphorylation of peripherin may be one step that leads to its aggregation. Altered phosphorylation of N-terminal residues in the related protein vimentin has been shown to influence its localization in cells (20), but we could show that these are different from peripherin aggregates (Fig. 5). We have so far been unable to see any changes in overall peripherin structures that associate with this study, and others (50) have shown that increased levels of what causes the aggregation of peripherin molecules. We, in this study, and others (50) have shown that increased levels of peripherin enhance its tendency to aggregate. Here we identify PKCe as another important factor facilitating peripherin aggregation. The aggregation could be induced either by activation of endogenous PKC or by elevating the levels of PKCe and was suppressed either by down-regulation of PKCe with siRNA or by inhibition of PKC activity. Although the number of aggregates was markedly elevated in cells overexpressing peripherin, punctuate peripherin structures could also be observed in non-transfected cells after PKC activation. This further underscores the putative importance of PKCe in promoting the aggregation of peripherin. 

A role for PKCe in aggregation is further supported by our findings that the minimal PKCe structures that associate with peripherin (C1b and PSC1V3) also suppress phosphol e.stimulated aggregation and apoptosis. Thus, we have identified molecules that can prevent the potentially hazardous aggregation of intracellular peripherin. This opens up options for novel molecular approaches to block this putative pathogenic event and thereby slow down or halt the ALS disease process.

In conclusion, in this study, we identify peripherin as a novel PKCe interaction partner. Through this interaction, PKCe can markedly enhance the aggregation of peripherin, an event that subsequently leads to neuronal cell death. Both the PKC-induced aggregation and apoptosis can be prevented by the isolated C1b domain of PKCe, thereby providing a promising approach for interference with the peripherin aggregation process.

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