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PKC-θ is a negative regulator of TRAIL-induced and FADD-mediated apoptotic spectrin aggregation

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Abstract
Introduction. During studies on chemotherapy-induced apoptosis in lymphoid cells, we noted that aggregation of spectrin occurred early in apoptosis, *i.e.* before activation of initiator caspase(s) and prior to exposure of phosphatidylserine (PS). We also found that protein kinase C theta (PKC-θ) co-localized with spectrin in these aggregates. Our previously published studies indicated that in formation of early apoptotic spectrin aggregates, either PKC-θ or other apoptosis-related proteins are involved. Taking into consideration above data, we decided to test the effect of PKC-θ and Fas-associated death domain protein (FADD) on spectrin aggregation in these cells during tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis.
Material and methods. For PKC-θ gene (*PRKCQ*) or *FADD* gene expression silencing in Jurkat T cells we used lentiviral particles containing shRNA and scrambled shRNA, respectively. Spectrin aggregates were detected by Western blotting after Triton-X 100 extraction in pellet and soluble fractions or by confocal imaging.
Results. TRAIL-induced apoptosis results in spectrin aggregation and leads to translocation and aggregation of PKC-θ. We found that phorbol-myristate acetate, a PKC activator and translocation inducer, has only a small effect on spectrin aggregation. To further confirm this, we have also shown that knock down of *PRKCQ* in Jurkat T cells accelerates the formation of TRAIL-induced spectrin aggregates. Transient overexpression of the β-spectrin C-terminal fragment, containing multiple S/T phosphorylation sites, potential substrate sites for PKC-θ, accelerated the formation of spectrin aggregates. Silencing of downstream TRAIL receptor effector gene, *FADD*, delayed aggregation of spectrin, but did not reduce PKC-θ localization to the plasma membrane.
Conclusions. In summary, our results show for the first time involvement of spectrin aggregation in TRAIL receptor-FADD apoptotic pathway and indicate that TRAIL-induced spectrin aggregate formation is mediated by FADD and negatively regulated by PKC-θ. *(Folia Histochem Cytobiol. 2016, Vol. 54, No. 1, 1–13)*.

Key words: apoptosis; FADD; TRAIL; PKC theta; spectrin aggregation; Jurkat T cell; shRNA

Introduction

During studies on chemotherapy-induced apoptosis (fludarabine, mitoxantrone, and dexamethasone, regimen; FND) in non-Hodgkin Lymphoma (NHL) lymphoid cells, we noted that rearrangement of spectrin, a central structural protein of the membrane skeleton occurred. Observed aggregation of spectrin occurred early in apoptosis, before activation of initiator caspase(s) and prior to exposure of phosphatidylserine (PS). We also found that protein kinase C theta (PKC-θ) co-localized with spectrin in these aggregates in FND-treated Jurkat T cells [1, 2].

Spectrin is a structural protein that regulates cell mechanics [3, 4], but it has also been shown to be involved in signal transduction [5–9]. Non-erythroid

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Table 1. Antibodies used in this study

Antibody	Working dilutions	Source
Monoclonal rabbit anti-PKC-θ	1:1000 (WB), 1:100 (IF)	AbCam, Cambridge, UK
Monoclonal mouse anti-spectrin	1:1000 (WB), 1:100 (IF)	Santa Cruz Biotechnology Inc.
Monoclonal mouse anti-FADD	1:1000 (WB)	Santa Cruz Biotechnology Inc.
Monoclonal rabbit anti-phosphoserine/tyrosine	1:500 (WB)	Santa Cruz Biotechnology Inc.
Polyclonal rabbit anti-actin	1:5000 (WB)	GeneTex Inc.
Donkey anti-rabbit-HRP	1:10000 (WB)	Santa Cruz Biotechnology Inc.
Goat anti-mouse-HRP	1:10000 (WB)	Santa Cruz Biotechnology Inc.
Donkey anti-rabbit-Cy5	1:100 (IF)	Jackson ImmunoResearch Laboratories
Goat anti-rabbit-Alexa488	1:400 (IF)	AbCam, Cambridge, UK
Donkey anti-mouse-Cy5	1:400 (IF)	Jackson ImmunoResearch Laboratories

IF — immunofluorescence; WB — western blotting

spectrin αIIβII forms a membrane skeletal network *via* association with adducin [10, 11], actin [12, 13], and protein 4.1 [14, 15], among numerous other proteins (for a review see 3). This 'network' arrangement is characteristic of unstimulated cells. Various stimuli may lead to changes in the cellular distribution of spectrin, including its aggregation. Formation of aggregates was observed upon phosphorylation of adducin by PKC activated by phorbol-myristate acetate (PMA) [16, 17] which resulted in the dissociation of spectrin from the spectrin-actin complex [18]. Aggregation of spectrin was also shown during induced hyperthermia [19] or lymphocyte activation [20], occurring concomitantly with PKC-β and PKC-θ activity. In these aggregates β spectrin was found phosphorylated, and the whole process was inhibited by bisindolylmaleimide-1, the PKC inhibitor [19].

PKC-θ, is a member of the novel of PKC family. It is present in a wide variety of cells, from hematopoietic cells through skeletal muscle [22, 23] to neurons [24, 25]. PKC-θ plays a role in the regulation of cell migration, lymphoid cell motility [26], and signaling pathways (for review see 21), including T cell activation [27] or survival [28] and activation-induced cell death (AICD) by FasL (CD95L) [29, 30]. Its role in mitosis and cell cycle regulation was also implicated [31, 32]. Interestingly, most of these processes involve cellular relocation of PKC-θ and cytoskeletal rearrangements; however, the mechanistic details of the regulatory role of PKC-θ on cytoskeleton and membrane skeleton are not fully understood.

The previously published studies [1, 2] indicated that in spectrin aggregate formation during early apoptosis, either PKC-θ or other apoptosis-related proteins are involved. Taking into consideration that PKC-θ is a PKC isoform expressed at a higher level than its close homolog, PKC-δ, in Jurkat T cells, we decided to test the effect of PKC-θ and Fas-associated death domain protein (FADD) on spectrin aggregation in these cells. Here, we found that PMA that is known PKC-θ activator and inducer of its translocation [33, 34], has only a small effect on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptotic spectrin aggregation. In Jurkat T cells with PKC-θ gene (*PRKCQ*) knock-down (KnD) spectrin aggregate formation was accelerated. Transient overexpression of the beta-spectrin C-terminal fragment, as a potential PKC-θ substrate, accelerated formation of spectrin aggregates but delayed translocation of PKC-θ to these aggregates. Silencing of *FADD* gene expression delayed apoptotic aggregation of spectrin reported in the present study shed more light on the molecular mechanism of early apoptotic spectrin aggregate formation, indicating that either PKC-θ or FADD are involved in the process.

Material and methods

Antibodies. The antibodies used in this study for Western blotting and immunofluorescent staining are listed in Table 1.

Primers. The following primers were used for C-terminal fragment of spectrin conjugated to Green Fluorescent Protein (Spc-GFP) generation: FP 5' GAG CGA GAA AAA CGC TTC AGC TCC TAG G, RP 5' GAG CGA GAA AAA CGC TTC AGC TTC.

Cell cultures, apoptosis induction, and PKC activation. Jurkat T cells obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures), were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum,

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29.2 mg/mL glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin (all from Lonza Group, Basel, Switzerland) at 37°C, 5% CO2 and 95% humidity. Cells were passaged every 2–3 days. For experiments, cells in the middle of the logarithmic growth phase (48 h after passage) were used. Apoptosis was induced via activation of the TRAIL-receptor pathway by adding TRAIL (recombinant protein that bind to TNFRSF10A/ /TRAILR1, TNFRSF10B/TRAILR2 to induce apoptosis, at 100 ng/mL final concentration; AbCam, Cambridge, UK) to the culture medium and with of hydrogen peroxide (100 μM final concentration, POCH, Poland). In experiments with PKC activator, we used phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 100 nM.

PRKCQ /FADD gene expression silencing in Jurkat T cells. PRKCQ (PKC-θ) or FADD shRNA containing lentiviral particles (Santa Cruz Biotechnology Inc., Dallas, TX, USA) were used for gene silencing. The transduction procedure was done according to the manufacturer's protocols. Lentiviral particles containing scrambled shRNA were used as a negative control for silencing of gene expression. Briefly: The suspension of lentivirus particles designed to silence *PRKCQ* (PKC-θ) or *FADD* gene expression contained 1 × 10^6 infectious units of virus in 200 μL. Jurkat T cells (cells should be approximately 50% confluent) were incubated with the lentiviral transduction particles (20 μL) in the presence of 5 μg/mL of polibrene (Santa Cruz Biotechnology Inc.) for 16 h, then the medium was replaced with RPMI medium containing puromycin (Santa Cruz Biotechnology Inc.) at a final concentration of 2 μg/mL and cultured for additional 48 h.

Detection of apoptosis by flow cytometry. Apoptotic cells were quantified as the percentage of cells exposing phosphatidylserine (PS). Staining of Annexin V-FITC was performed in accordance with the manufacturer's instructions (Annexin V: FITC Apoptosis Detection Kit II, BD BioScience, Franklin Lakes, NJ, USA). Stained cells were analyzed using a flow cytometer (BD FACSCalibur, with CellQuestPro software, Becton Dickinson).

Triton X-100 extraction, electrophoresis, and Western Blotting. Cells were incubated in lysis buffer 1% Triton X-100, 100 mg/mL PMSF (phenylmethylsulfonyl fluoride) and 1 mM EDTA, in an ice bath for 10 min, with occasional rapid stirring. The resulting cell lysates were centrifuged (30,000 g, 4°C, 20 min), and the supernatants or pellets were solubilized in a reducing reagent (4× concentrated, 277.8 mM Tris-HCl, pH 6.8 sample buffer, 44.4% (v/v) glycerol, 4.4% SDS, 0.02% bromophenol blue) and subjected to standard SDS-PAGE (12% polyacrylamide electrophoresis gel, for 2–3 h, 30 mA) and immunoblotting (16 h, 400 mA). Band density was determined by Image J software (NIH, Bethesda, MD, USA). Presented Western blots are representative from group of biological replicates (at least three) and the data in the graphs are average of biological replicates with standard deviation (SD) bars.

Immunofluorescence. Cells were pelleted onto slides by centrifugation (800 g, 5 min) and dried for 24 h at room temperature. Slides were fixed in 4% formaldehyde for 5 min at room temperature (RT) and permeabilized by incubation in 0.1% Triton X-100 for 30 min. To reduce non-specific interactions, slides were incubated for 1 h with 1% fetal calf serum. Then slides were incubated overnight with the appropriate dilution of each primary antibody. Afterwards slides were incubated with appropriate secondary antibodies (2 h, RT). Slides were rinsed 3 times in phosphate-buffered saline (PBS) between incubations. Appropriate negative controls were performed by omitting primary antibody. Observations were performed by using a Zeiss microscope LSM510-Meta with an Apochromat 63× objective with a numerical aperture of 1.4 (Zeiss, Jena, Germany).

Generation of C-terminal β spectrin fragment-GFP vector. mRNA was isolated from Jurkat T cell line using a Titanium One Step RT-PCR Kit (Clontech, Mountain View, CA, USA). RT-PCR was performed using the appropriate primers to produce the desired 730 bp cDNA encoding a spectrin fragment consisting of aminoacid residues from 2142 to 2384. The primers were designed to incorporate the restriction sites for HindIII and BamHI at the 5'-ends of the forward and reverse primers, respectively (all from Thermo Fisher Scientific, Inc., Waltham, MA, USA). The fragment was inserted into P-max C-Green (Lonza Group) at the HindIII and BamHI sites using T4 ligase (Thermo Fisher Scientific, Inc.). The ligation mixture was transformed into *E. coli* DH5α bacteria by the heat-shock method: 30 min on ice, 90 sec at 42°C, 2 min on ice. Eight milliliters of SOC (super optimal catabolite repression, mixture containing yeast extract, tryptone, NaCl, $MgCl_2$, KCl) was added and bacteria were incubated at 37°C for 4 h with shaking. Bacteria were plated on LB (Luria Bertani) agar medium containing 50 μg/mL kanamycin (all from Sigma-Aldrich). Positive colonies bearing reaction plasmid detected by PCR were inoculated into liquid LB medium (30 mg/mL kanamycin) to purify plasmid. Isolation of plasmid DNA was performed using a QiaPrep Spin Miniprep kit (Qiagen, Venlo, Netherlands). Transfection of Jurkat T cells with plasmid DNA was performed using a commercially available electroporator, CLB-Transfection Device with CLB-Transfection Pulse optimal for Jurkat T cells (CELL 9, Lonza Group) according to the manufacturer's protocol (Cell Line Nucleofector® Kit, Lonza). Transfection efficiency was measured by GFP signal intensity using a BD FACSCalibur flow cytometer.

Statistical analysis. Statistical analysis of the results was performed using Student's t-Test considering $P \leq 0.05$ or $P \leq 0.01$ as significant.

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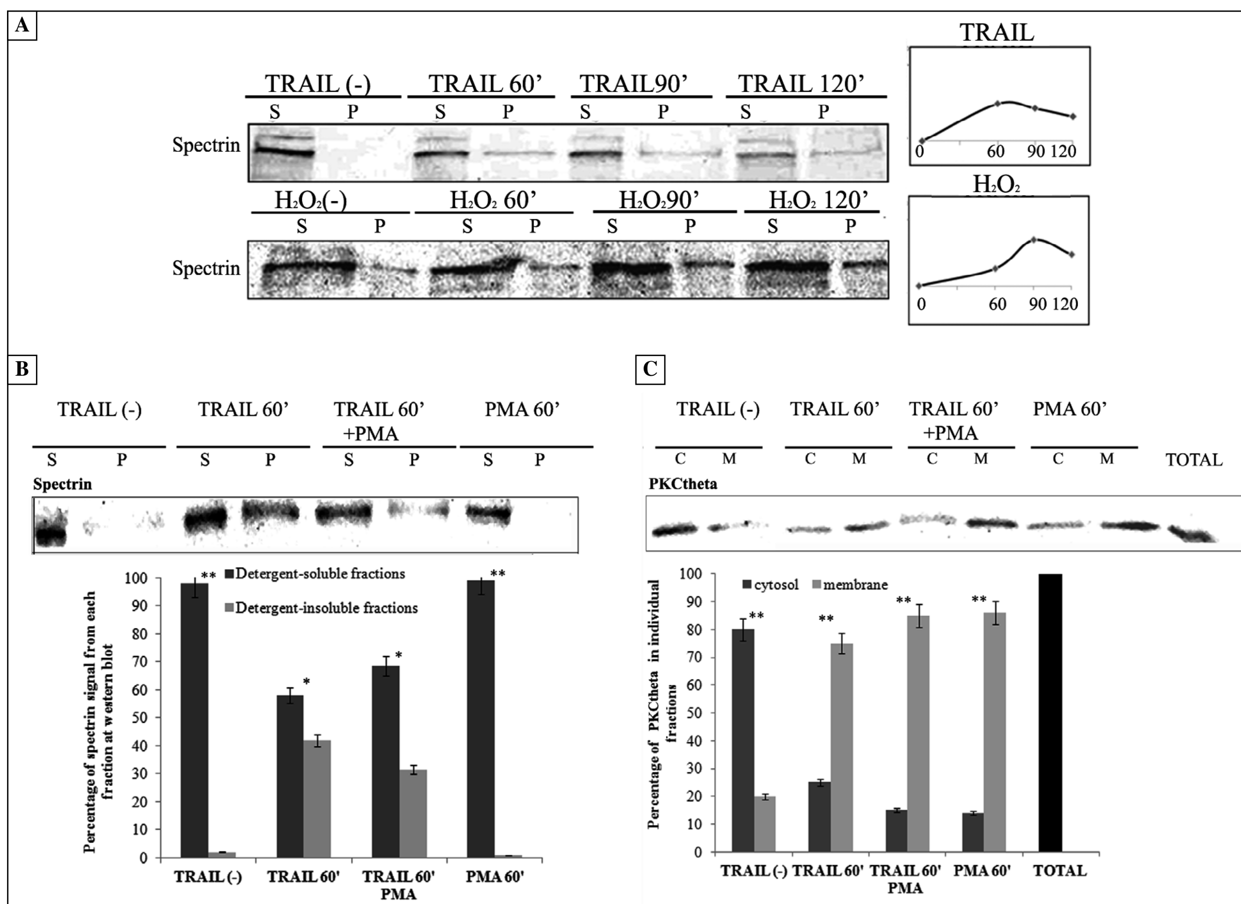

Figure 1. Effects of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and phorbol-myristate acetate (PMA) on spectrin aggregation. **A.** Time course reveals that spectrin aggregation is more sensitive to TRAIL-induced (100 ng/mL) than H_2O_2-induced (100 μM) apoptosis. Western blot analysis was performed using mouse anti-spectrin and developed by using appropriate secondary antibodies as detailed in Table 1. Densitometric analyses of the data (signals from control insoluble fractions are subtracted from signals from apoptotic insoluble fractions). S — supernatant, Triton X-100 soluble fraction; P — pellet, Triton X-100 insoluble fraction; **B.** PMA treatment does not lead to spectrin aggregation. Cells treated with TRAIL, PMA, or TRAIL and PMA (concentrations as above) for 60 min were extracted with cold 1% Triton X-100 and centrifuged at 30,000 *g*. Supernatant and pellet at the same protein concentrations were subjected to SDS-PAGE (12%) electrophoresis, and transferred onto nitrocellulose. S — supernatant, Triton X-100 soluble fraction; P — pellet, Triton X-100 insoluble fraction; *,** $P \leq 0.05$ and $P \leq 0.01$, respectively; **C.** Protein kinase C theta (PKC-θ) translocates to the "membrane fractions" during apoptosis induced by TRAIL (100 ng/mL) and PMA (100 nM) treatment. A simplified subcellular fractionation experiment and Western blot were performed using mouse anti-spectrin and rabbit anti-PKC-θ and developed by using appropriate secondary antibodies as detailed in Table 1 in Material and methods. Densitometric analyses show the percentage of PKC-θ in each membrane and cytosol fraction. Graph represents means of biological triplicates, with error bars being standard deviations. C — cytosol fraction; M — membrane fraction

Results

TRAIL-treatment induces spectrin aggregation

We tested whether spectrin aggregation observed during early apoptosis was induced by the extrinsic, intrinsic or both apoptotic pathways. Cells were treated with TRAIL (an activator of the extrinsic receptor-mediated apoptotic pathway) or hydrogen peroxide (an inducer of the intrinsic mitochondrial apoptotic pathway). We found that spectrin aggregates were formed during apoptosis induced by both pathways, but this process occurred faster *via* the TRAIL-induced extrinsic pathway (Figure 1A).

To detect spectrin and PKC-θ aggregation upon TRAIL-induced apoptosis, we analyzed presence of both proteins in Triton X-100-insoluble and soluble fractions (pellets and supernatants, respectively). As a control treatment for PKC-θ translocation we used PMA. Spectrin aggregation, observed within 60 min of TRAIL treatment, was significantly reduced in both TRAIL and TRAIL with PMA treatments and was not detectable upon PMA treatment only. However, the exposition of cells to PMA and TRAIL decreased the amount of spectrin present in the aggregates from 42% (TRAIL treatment only) to 31% (TRAIL and PMA treatment) (Figure 1B).

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PKC-θ translocation from cytosol to the membrane fraction was observed within 60 min of TRAIL, TRAIL and PMA or PMA treatment (Figure 1C). The data show that the effects of these two factors (TRAIL and/or PMA) on PKC-θ were not additive.

The obtained results indicate that PMA treatment alone induces PKC translocation but has no effect on spectrin aggregation status. In contrast to PMA, TRAIL leads to spectrin aggregation and PKC-θ translocation.

FADD plays a role as an inducer of spectrin-aggregation

Because spectrin aggregates within activation of TRAIL receptor and before caspase 8 activation [2], therefore we reasoned that in the interval between of these two signals, there might be a trigger to the formation of spectrin-based apoptotic complexes. FADD is a candidate for this trigger as it acts following TRAIL-R activation, but before spectrin aggregation and caspase 8 activation [36]. In order to test this, we silenced *FADD* gene and evaluated effects of its KnD on TRAIL-induced spectrin aggregation. Lentiviral-based silencing resulted in a ~70% reduction in the expression of *FADD* in Jurkat T *FADD* KnD cells (Figure 2A). Analysis of PS exposure indicated that after 320 min of TRAIL-treatment *FADD* KnD Jurkat T cells were less prone to apoptosis (P ≤ 0.01) than the control Jurkat T cells (Figure 2B). Early apoptotic spectrin aggregation in *FADD* KnD cells, measured as the amount of spectrin in Triton X-100-insoluble fraction, was delayed (120 min) compared to control Jurkat T cells (60 min, Figure 2C, D). The data indicate that *FADD* silencing had positive effect on PKC-θ translocation, as PKC-θ was mostly present in the membrane zone or membrane fraction in *FADD* KnD cells (Figure 2D, E), and it also indicates that TRAIL-FADD signaling is involved in induction of spectrin aggregation.

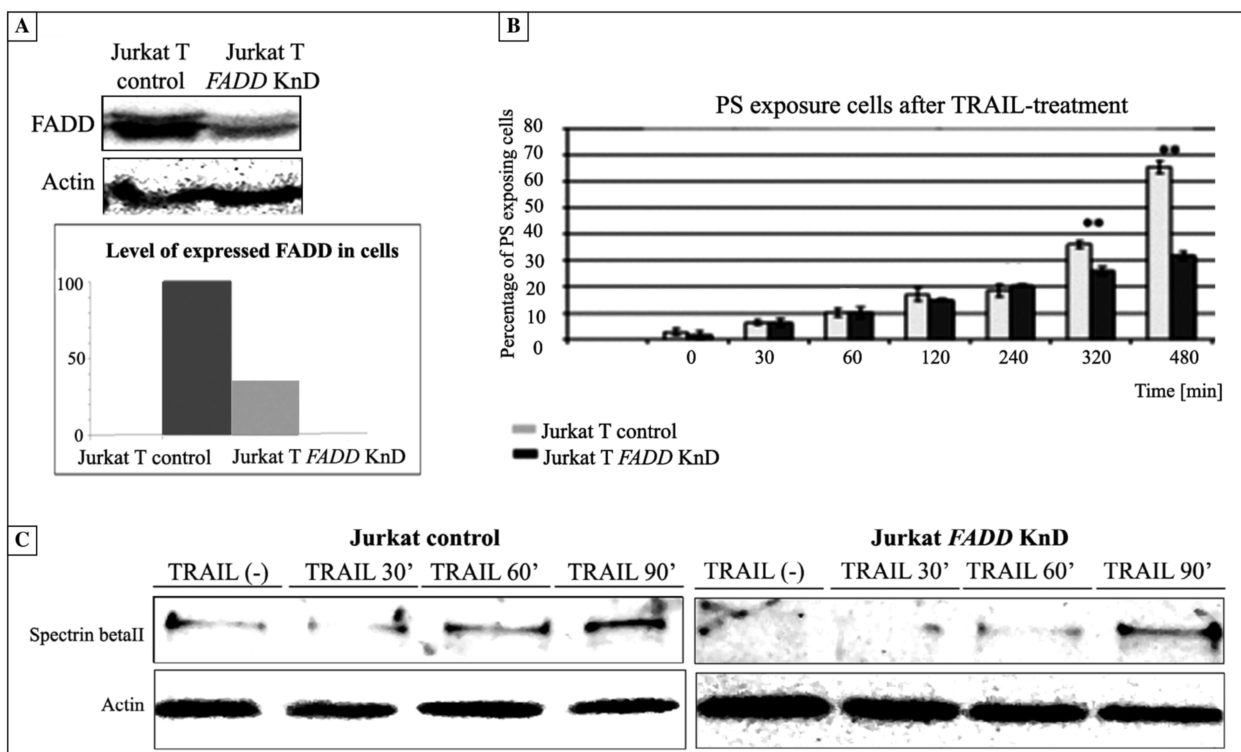

Figure 2. Aggregation of spectrin in Fas-associated death domain protein (*FADD*) knockdown (KnD) Jurkat T cells upon tumor necrosis factor-related apoptosis-inducing ligand (TRAIL-induced apoptosis). **A.** Level of FADD protein is decreased by 70% in *FADD* KnD cells, in comparison to control Jurkat T cells. shRNA lentiviral vector was used to establish stable *FADD* KnD cell-line. Level of FADD was analyzed by immunoblotting using mouse anti-FADD and rabbit anti-actin and developed by using appropriate secondary antibodies as detailed in Table 1 in Material and methods, and evaluated by densitometry; **B.** Progression of TRAIL-induced apoptosis in *FADD* KnD cells was evaluated using an Annexin V-FITC Apoptotic Detection Kit with a flow cytometer. Note a statistically significant difference in the progression of apoptosis in *FADD* KnD cells compared to control cells within 4 h of TRAIL treatment (100 ng/mL); *,** P ≤ 0.05 and P ≤ 0.01, respectively; **C.** Spectrin aggregates were observed later (90 min of TRAIL-treatment) in *FADD* KnD cells than in control cells (60 min of TRAIL-treatment). Analysis of spectrin aggregation in *FADD* KnD cells was performed by immunoblot analysis of detergent-insoluble fractions using mouse anti-spectrin and mouse anti-actin and developed by using appropriate secondary antibodies as detailed in Table 1

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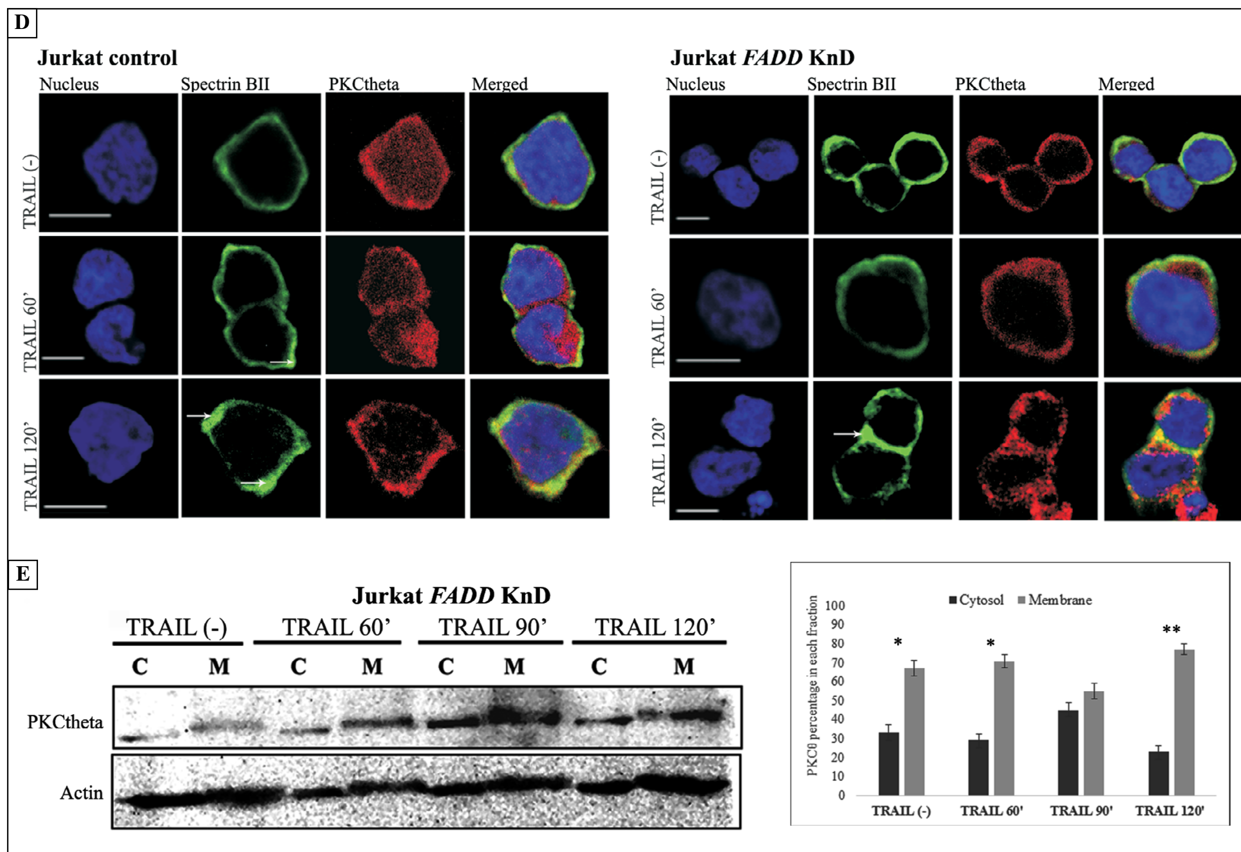

Figure 2. (cont.) Aggregation of spectrin in *FADD* KnD Jurkat T cells upon TRAIL-induced apoptosis. **D.** Dynamics of spectrin and PKC-θ aggregation in *FADD* KnD cells compared to control Jurkat T cells. Note: delayed aggregation of spectrin (green fluorescence) in Jurkat T control and *FADD* KnD cells upon TRAIL-induced apoptosis, compared to unstimulated Jurkat T control cells. A delayed translocation of protein kinases C theta (PKC-θ) (red) is also seen in *FADD* KnD cells. Blue fluorescence = nuclei (DAPI), yellow = superposition of red and green fluorescence (colocalization of spectrin betaII and PKC-θ). Immunofluorescence was performed using mouse anti-spectrin and rabbit anti-PKC-θ and developed by using appropriate secondary antibodies as detailed in Table 1. Scale bar 5 μm. For details see Material and methods **E.** A simplified subcellular fractionation experiment and Western blot analysis shows that PKC-θ is located in the membrane, even in untreated *FADD* KnD cells. TRAIL-induced apoptosis accelerates the level of PKC-θ in the membrane. All experimental details are presented in Material and methods. Densitometric analyses of membrane and cytosolic fractions are also presented in the right panel in section E. Graph represents means of biological triplicates, with error bars being standard deviations. C — cytosol fraction, M — membrane fraction. Western blot was performed using antibodies as above, for details see Table 1; *,** $P \leq 0.05$ and $P \leq 0.01$, respectively

Silencing of PRKCQ expression accelerates spectrin aggregation

Based on our published results [2] and the data presented above, we postulated that PKC-θ was involved in the regulation of spectrin aggregation. To assess the role of PKC-θ in spectrin aggregation in early apoptosis, we generated *PRKCQ* KnD cells, in which PKC-θ level was reduced by more than 50% (Figure 3A). We assessed the dynamics of early apoptosis induced by TRAIL in these cells. PS exposure (as an apoptosis marker) was not significantly different from that observed in control cells up to 4 hours post TRAIL induction (Figure 3B). We observed that spectrin aggregates appeared after 10 min of TRAIL-R activation in *PRKCQ* KnD cells, but not until 1 hour in control Jurkat T cells (Figure 3C, D).

These data suggest that the level of PKC-θ expression may regulate spectrin aggregation under apoptotic conditions.

Spectrin phosphorylation during apoptotic aggregation

The above results suggest that PKC-θ plays a role in spectrin aggregation during the early stages of apoptosis. To assess the mechanism of spectrin-aggregate formation, we tested whether PKC-θ triggers this process by affecting spectrin phosphorylation status. By Western blotting and probing with an antibody against phosphoserine/phosphothreonine residues,

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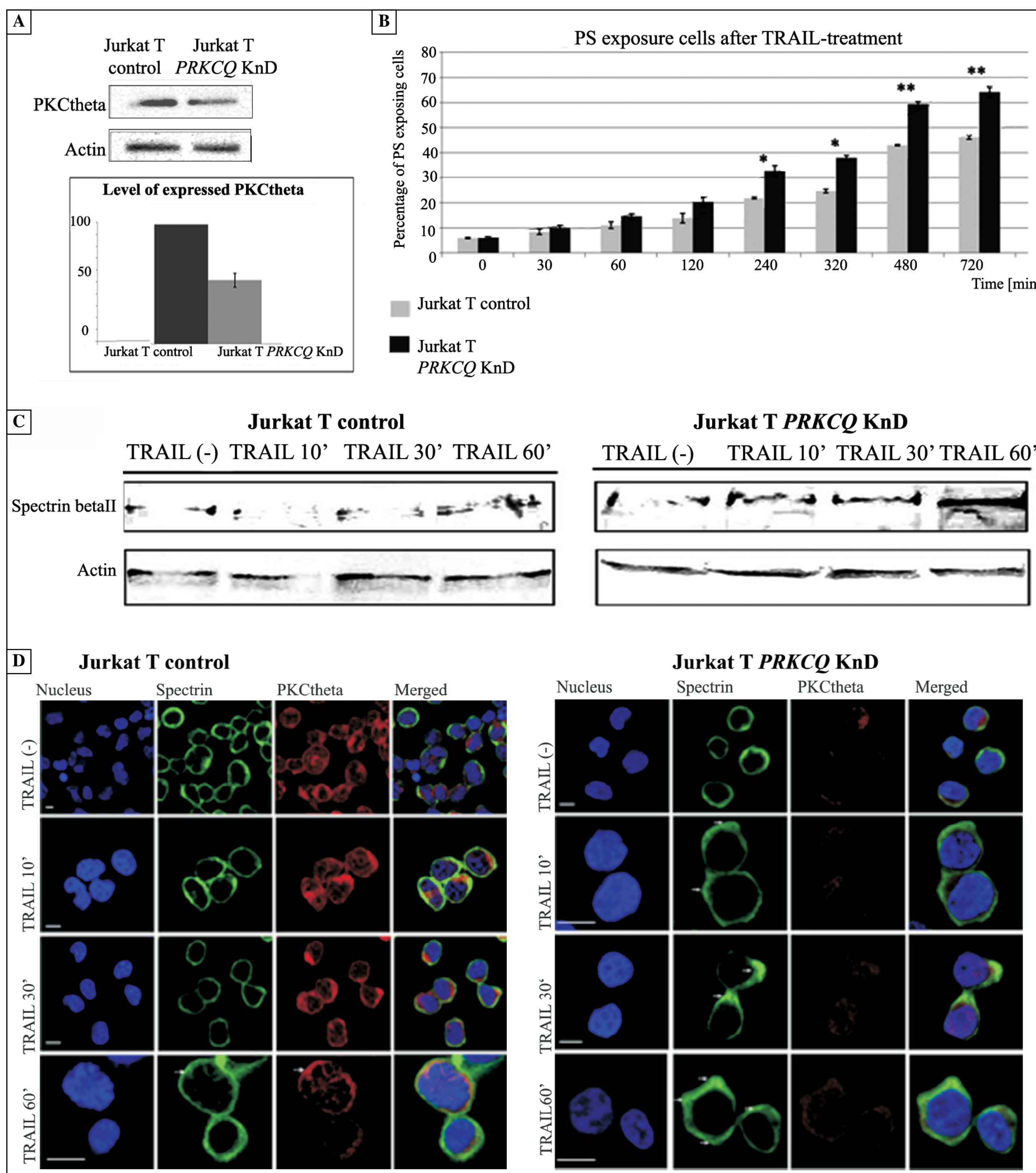

Figure 3. Protein kinase C theta (PKC-θ) down-regulates spectrin aggregation in Jurkat T cells upon tumor necrosis factor--related apoptosis-inducing ligand (TRAIL-induced apoptosis). **A.** Level of PKC-θ is decreased by 50% in *PRKCQ* (PKC-θ gene) knockdown (KnD) cells in comparison to control Jurkat T cells. Level of PKC-θ was analyzed by immunoblot (antibodies used: rabbit anti-PKC-θ and rabbit anti-actin, details in Table 1) and evaluated by densitometry; **B.** Progression of apoptosis in *PRKCQ* KnD cells was evaluated as described in Figure 2 legend. Note a significant difference in the progression of apoptosis in *PRKCQ* KnD cells, compared to control cells, appears within 4 h of TRAIL treatment (100 ng/mL); *,** $P \leq 0.05$ and $P \leq 0.01$, respectively; **C.** Insoluble spectrin was observed as soon as 10 min of TRAIL-treatment in *PRKCQ* KnD cells. Analysis of the spectrin aggregation process in early apoptotic cells in immunoblot analysis of detergent-insoluble fractions following TRAIL-induced apoptosis at 10, 30 and 60 minutes. A Western blot was performed using mouse anti-spectrin and rabbit anti-actin and developed by using appropriate secondary antibodies as detailed in Table 1; **D.** Dynamics of the spectrin aggregation process in *PRKCQ* KnD cells, compared to control Jurkat T cells. Localization of spectrin (green) and protein kinase PKC-θ (red) in control and *PRKCQ* KnD cells of TRAIL-induced apoptosis, compared to noninduced Jurkat T control cells, shows that spectrin aggregates faster in *PRKCQ* KnD cells. Scale bar 10 μm

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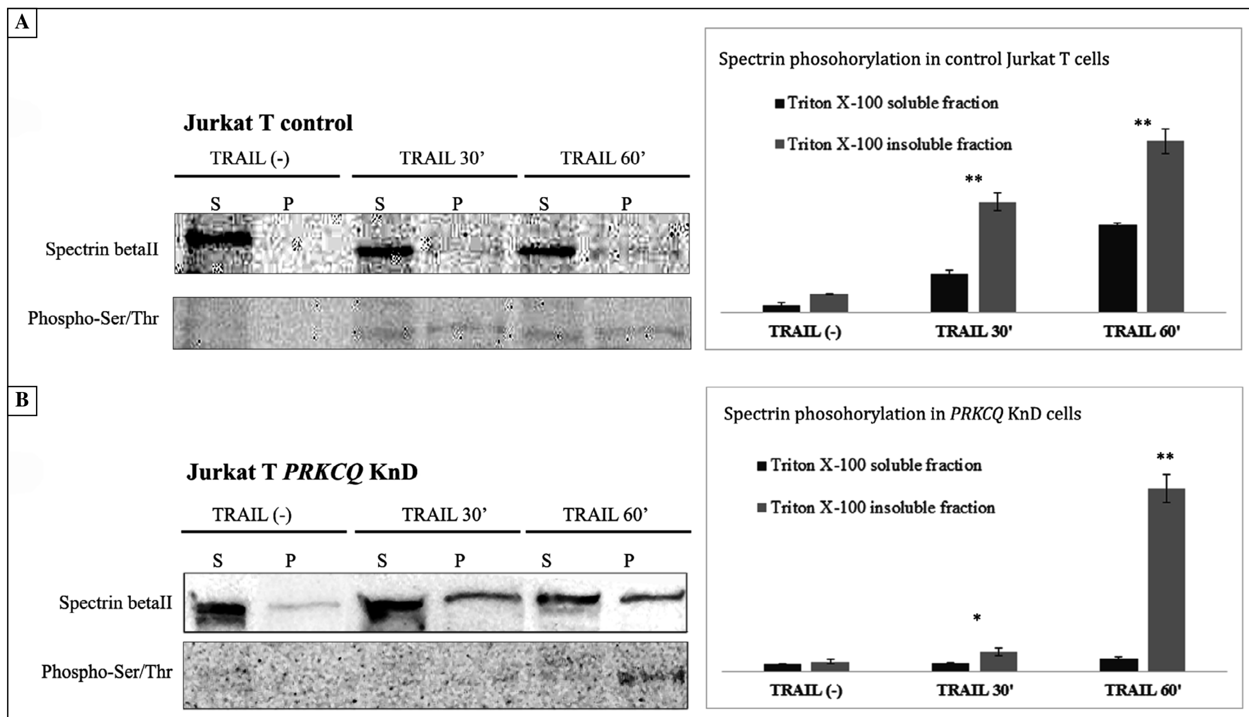

Figure 4. Spectrin phosphorylation upon apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). **A.** Western blot analysis of detergent-soluble and insoluble fractions isolated from control Jurkat T cells within 30 and 60 min post-induction indicate phosphorylation of spectrin on S/T residues in the same fractions as was detected immunoblotting with mouse anti-spectrin and rabbit anti-phosphoserine/threonine antibodies, as described in Table 1; **B.** Western blot analysis of detergent-soluble (S) and insoluble (P) fractions isolated from *PRKCQ* (PKC-θ gene) knock-down (KnD) Jurkat T cells after the same as above time intervals indicate only the appearance of phosphospectrin in a detergent-insoluble fraction of spectrin within 60 min of TRAIL-treatment, even though aggregation is detectable sooner (30 min of apoptosis induced by TRAIL). S — supernatant, P — pellet. Graph represents means of biological triplicates, with error bars being standard deviations; *,** $P \leq 0.05$ and $P \leq 0.01$

we found that spectrin became phosphorylated within 30 min of TRAIL-treatment in control Jurkat T cells, and phosphorylated spectrin was found both in the detergent-soluble (S) and detergent-insoluble fractions (P) (Figure 4A). Phosphorylated spectrin constituted a higher proportion of detergent-insoluble spectrin than of detergent-soluble spectrin (Figure 4A). In contrast, detergent-soluble spectrin was not phosphorylated in the *PRKCQ* KnD cells (Figure 4B). In the detergent-insoluble fraction, phosphorylated spectrin was only observed within 60 min of apoptosis induced by TRAIL. This confirms the effect of PKC-θ signaling on early apoptotic spectrin-aggregate formation but also indicates a lack of a causative role of phosphorylation on spectrin aggregates formation.

Overexpression of the carboxyl-terminal fragment of spectrin βII affects endogenous spectrin aggregation and PKC-θ translocation

According to the NetPhosph (http://www.cbs.dtu.dk/services/NetPhos/) or UniProtKB (http://www.uniprot.org/), two serine residues, S2171 and S2359, in the C-terminal fragment of βII spectrin can be phosphorylated (Figure 5A). To determine whether an excess of this spectrin fragment would affect endogenous spectrin aggregation through its potential competition for phosphorylation on S/T residues, the GFP-tagged C-terminal fragment of spectrin βII (residues 2142–2384) was expressed in Jurkat T cells. Within TRAIL induction of apoptosis, progress of spectrin aggregation was evaluated in these cells and compared to control cells. We found that, after 30 min of TRAIL-treatment, endogenous spectrin aggregates were formed in cells overexpressing the spectrin βII fragment, whereas aggregates were absent at this time in control cells (Figure 5B). Within 30 min of apoptosis in cells overexpressing spectrin fragment, PKC-θ was localized mostly in cytosol, while in non-transfected cells PKC-θ was present in cytosol and cell membrane. This difference was not seen within 60 min of apoptosis (Figure 6). These data suggest that the C-terminal fragment of spectrin can act as a substrate or, more possibly, as a site of interactions between spectrin and PKC-θ.

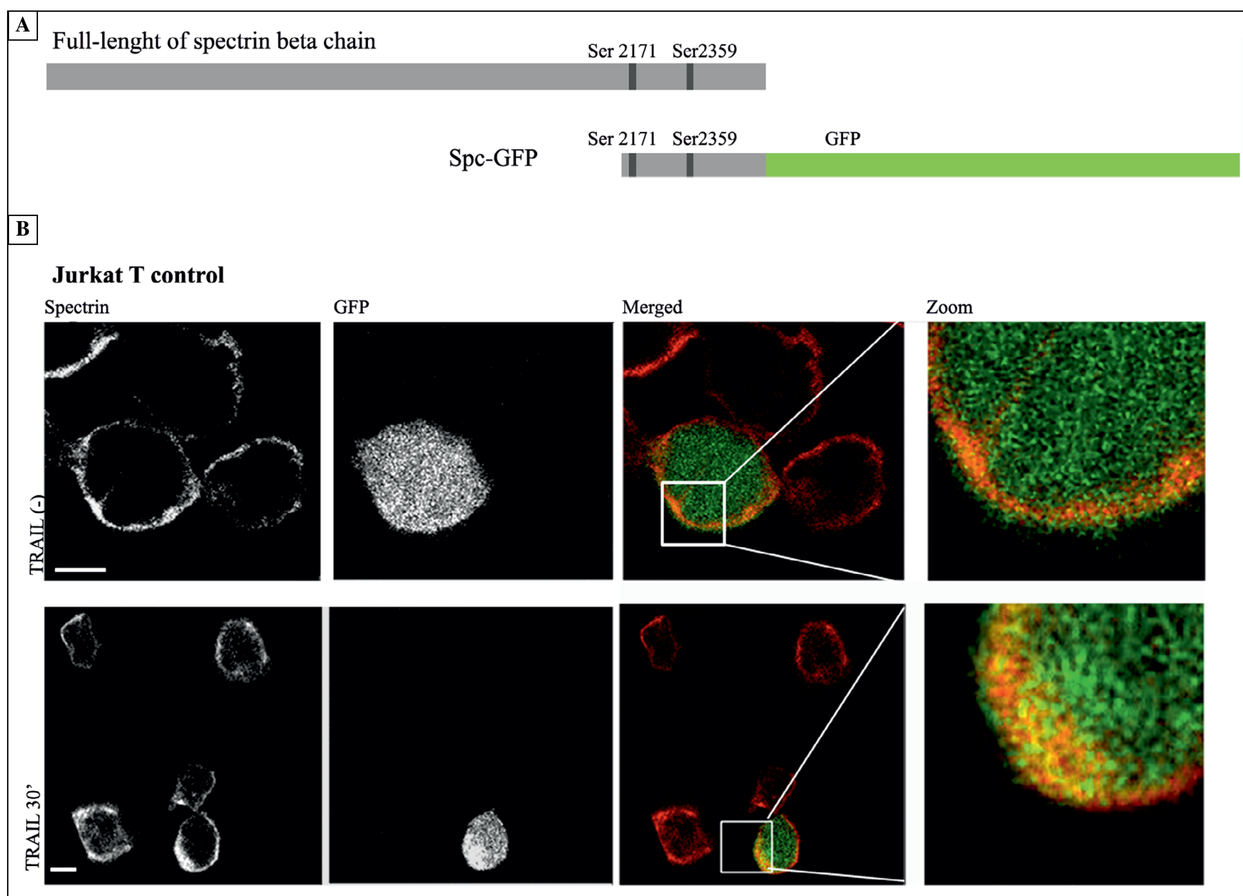

Figure 5. Overexpressed spectrin fragment accelerates aggregation of endogenous spectrin upon tumor necrosis factor-related apoptosis-inducing ligand (TRAIL-induced apoptosis) in Jurkat T cells. **A.** βII spectrin fragment (2142–2384): Spc-GFP, was cloned into a GFP-tagged construct (see Material and methods section); **B.** Endogenous spectrin (labeled in red on merged images, antibodies used mouse anti-spectrin and developed by using appropriate secondary antibodies as detailed in Table 1 accumulates below the cell membrane within just 30 min of TRAIL-treatment (100 ng/mL). Compared to untransfected cells, the overexpressed fragment of spectrin accelerates aggregation of endogenous spectrin. Scale bar 10 μm

Discussion

The phenomenon of aggregation of spectrin, the cell membrane skeletal protein, during early apoptosis induced by a range of cytostatic drugs including fludarabine, mitoxantrone, and dexamethasone was reported a decade ago [1]. Further studies showed that spectrin aggregate formation is a hallmark of early apoptosis [2, 13], with the data pointing to a regulatory role for PKC-θ in this process. Cytostatic drugs are known to activate both extrinsic and intrinsic apoptotic pathways. Our data show that spectrin is more sensitive to the extrinsic pathway of apoptosis induction *via* TRAIL. Recombinant protein used here binds to TNFRSF10A/ /TRAILR1, TNFRSF10B/TRAILR2 to induce apoptosis (according to the manufacturer). The current study assessed the roles of PKC-θ and FADD in early apoptotic spectrin aggregation induced by TRAIL.

We compared the occurrence and timing of several events associated with apoptotic spectrin aggregation in intact Jurkat T and *PRKCQ* and *FADD* KnD cell lines. Our findings are summarized in Figure 7. In control cells, spectrin aggregates within 60 min of TRAIL-induced apoptosis. Spectrin aggregation is preceded by PKC-θ translocation, which occurs only within 30 min of apoptosis. Caspase 8 activation occurs after spectrin aggregation, and is followed by PS exposure. In *PRKCQ* KnD cells, accelerated spectrin aggregation is observed, occurring within 10 min of TRAIL-treatment. This is followed by caspase 8 activation and PS exposure as in control cells. In *FADD* KnD cells, PKC-θ is present in the cell membrane before TRAIL-induced apoptosis, but aggregation of spectrin is not seen until 120 min of TRAIL-treatment. PS exposure is not seen until 720 min of TRAIL-treatment. Therefore, based on these observations, we conclude that PKC-θ is a negative regulator of TRAIL-induced and FADD mediated spectrin aggregation.

Spectrin began forming apoptotic aggregates approximately within 60 min of induction, which suggests

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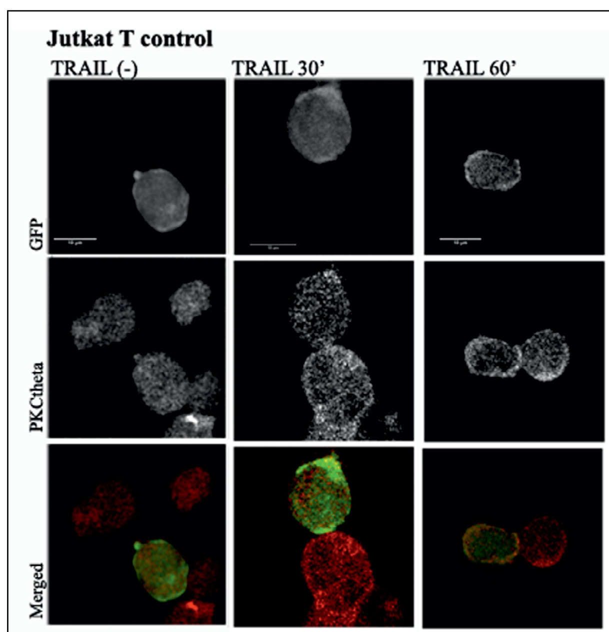

Figure 6. Effect of overexpressed spectrin fragment on protein kinase C theta (PKC-θ) translocation under apoptotic conditions. In cells with overexpressed spectrin (labeled in green, antibodies used rabbit anti-PKC-θ and developed by using appropriate secondary antibodies as detailed in Table 1) delayed PKC-θ translocation from cytosol to the cell membrane of 30 min of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) treatment (100 ng/mL) can be observed. Within 60 min of apoptosis induced by TRAIL, PKC-θ is observed in the cell membrane in both cell types. Spc-GFP, labeled in green, PKC-θ labeled in red. Scale bar 10 μm

that the translocation/activation of PKC-θ induces aggregation of spectrin. However, we found that treatment with PMA, which induces PKC-θ activation and translocation to membrane [38], did not result in accelerated spectrin aggregation. Thus, we conclude that PKC-θ translocation/activation connected with TRAIL treatment can be a direct inducer or mediator of spectrin aggregation. Our results suggest that rather the presence of PKC-θ and not its translocation/ /activation status in the membrane can affect apoptosis-induced formation of spectrin aggregates.

In PKC-θ-deficient T cells Fas-induced activation of apoptotic molecules such as caspase-8, caspase-3, and Bid was not efficient and delayed apoptosis and PKC-θ deficient T cells were resistant to apoptosis [29]. The role of PKC-θ activity in regulation of apoptosis is, however, not clear and some reports show that pharmacological inhibition of PKC-θ promoted Fas-mediated apoptosis [28]. In our PKC-θ-deficient model in Jurkat T, PRKCQ KnD cells, we observed increased sensitivity to the apoptotic factor, TRAIL, with accelerated spectrin aggregation, which is in agreement with our previous studies [2], but we have to remember that the effect of PKC-θ pharmacological inhibition in HL60 cells resulted in opposite effect, *i.e.* PS exposure and spectrin aggregation was visibly slowed down [2], therefore the effect of PKC-θ could be cell-line specific. In the same studies it was shown that formation of spectrin aggregates during early apoptosis involves PKC-θ and that the inhibition of PKC-θ by its pseudosubstrate peptide increases spectrin aggregation during the initial hours of apoptosis [2]. With these findings and our results on *PRKCQ* KnD the role of PKC-θ on spectrin aggregation is clearly demonstrable, but there is an important outstanding question as to how PKC-θ regulates spectrin aggregation. The data presented here indicate that the translocation/activation of PKC-θ does not lead to spectrin aggregation. PKC-θ is known to re-organize the cytoskeleton through phosphorylation of its components, such as adducin [39, 40]. Phosphorylation of adducin inhibits actin-capping and maintains the ability of actin to recruit spectrin attachment to actin filaments, which leads to cytoskeletal reorganization. There is no direct evidence that spectrin is a substrate for PKC-θ, but spectrin can be phosphorylated, and in its phosphorylated state affects synaptic remodeling, receptor-mediated endocytosis, apoptosis, stability of the cell membrane skeleton and the response of the renal epithelial cell to ischemic injury [30, 40–42]. Formation of spectrin aggregates in mouse lymphocytes after induction of fever-like whole body hyperthermia (WBH) was shown to involve PKC-θ and PKCβ, which were found in aggregates after 12 hours of WBH [19]. In these aggregates, β-spectrin was confirmed to be phosphorylated, and the process could be inhibited by the PKC inhibitor, bisindolylmaleimide. It was also showed that PKC-θ associated with spectrin mediates either the reorganization of the spectrin-based skeleton and/or phosphorylates specific nearby targets that are involved in apoptosis [19]. In our study, aggregated spectrin was also phosphorylated, but its phosphorylation is not crucial for spectrin-PKC-θ aggregation. In PRKCQ KnD cells, non-phosphorylated spectrin aggregated rapidly.

The fact that spectrin is a protein that can be phosphorylated, along with the observed presence of PKC-θ in apoptotic complexes, prompted us to assess the interaction between the GFP-conjugated C-terminal βII spectrin fragment (GFP-Spc) and PKC-θ. Overexpression of the GFP-Spc fragment resulted in faster aggregation of endogenous spectrin, and delayed translocation of PKC-θ to the membrane. These differences in PKC-θ localization were not seen during later stages of the apoptotic process. We conclude that the presence of PKC-θ in the membrane can delay spectrin aggregate formation, although the role of phosphorylation remains to be fully elucidated. It is possible that this part of spectrin

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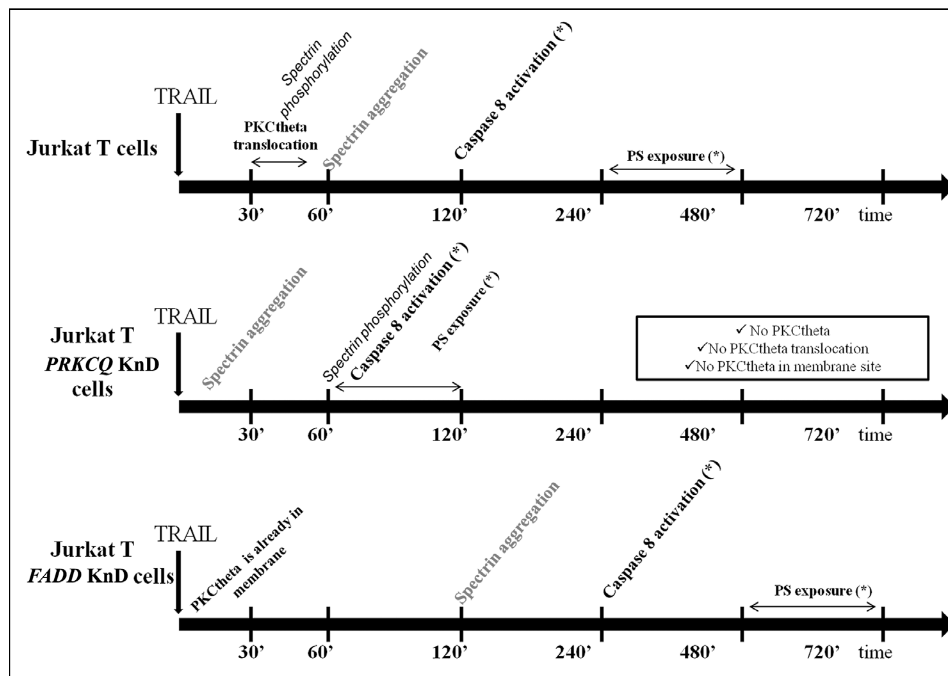

Figure 7. Sequence of events within tumor necrosis factor-related apoptosis-inducing ligand (TRAIL-induced apoptosis) in three cell lines: Jurkat T control, Jurkat T *PRKCQ* (PKC-θ gene) knockdown (KnD) and Jurkat T Fas-associated death domain protein (*FADD*). In control cells spectrin aggregates within 60 min of TRAIL-induced apoptosis, preceding protein kinase C theta (PKC-θ) translocation (30 min of apoptosis). Within spectrin aggregation caspase 8 activation takes place, and then phosphatidyleserine (PS) exposure occurs. In *PRKCQ* KnD cell line, an accleleration of spectrin aggregation is observed within 10 min of TRAIL treatment, then, as in control cells, caspase 8 activation and PS exposure take place. In control cells PKC-θ translocates to the membrane fraction upon TRAIL induction of apoptosis. In cells with lower expression of *FADD*, spectrin aggregates later, compared to control cells, but PKC-θ is already present in the cell membrane even before TRAIL-induced apoptosis. (*) assumed time where more than 30% of cells presented active caspase 8 or in other way, 30% or more cells present exposed PS

molecule is responsible for its interaction with PKC-θ and phosphorylation of other skeletal or regulatory components plays a role in this process.

Understanding the molecular mechanism(s) governing spectrin aggregation will help to elucidate the role of spectrin in the early stage of apoptosis. This additional knowledge regarding the regulation of apoptosis is likely to be relevant to studies of apoptosis in cancer cells. Our results suggest that PKC-θ plays a protective 'structural-adapter' function in spectrin-aggregation, whereas FADD is a spectrin-aggregation inducer.

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