AKT1/PKBα is recruited to lipid rafts and activated downstream of PKC isotypes in CD3-induced T cell signaling

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Abstract Protein kinase (PK) Cθ and Akt/PKBα cooperate in T cell receptor/CD28-induced T cell signaling. We here demonstrate the recruitment of endogenous Akt1 and PKCθ to lipid rafts in CD3-stimulated T cells. Further we show that Myr-PKCθ mediates translocation of endogenous Akt1 to the plasma membrane as well as to lipid rafts, most likely explained by the observed complex formation of both protein kinases. In addition, in peripheral mouse T cells, the PKC inhibitor Gö6850 could partially block Akt1 activation in CD3-induced signaling, placing PKC isotype(s) upstream of Akt1. However, T cells derived from PKCθ knockout mice were not impaired in CD3- or phorbol ester-induced Akt1 activity. Taken together, the results of this study give new insights into the functional link of Akt1 and PKCθ in T cell signaling, demonstrating the co-recruitment of the two kinases and showing a novel pathway leading to Akt1 transactivation where PKC isotype(s) are involved but PKCθ is not essential.

Key words: T cell; T cell receptor signaling; Raft; Protein kinase Cθ; Akt-1; Nuclear factor κB

1. Introduction

The protein kinase C (PKC) and Akt (also known as PKB or RAC-PK) families are known to act in T cell survival and proliferation signaling, but also cell cycle progression in T cell receptor (TCR)/CD28-stimulated T cells [1]. Among all T cell-expressed PKC isotypes, PKCθ has been shown to have cell type-selective expression and function [2]. PKCθ has been reported to selectively activate nuclear factor κB (NF-κB) [2–5]. Akt1 is also known to activate NF-κB [6,7]. Along this line, PKCθ and Akt1 were shown to act additively in NF-κB [8] as well as RE/AP activation, a NF-κB-binding site-containing region within the interleukin-2 promoter [9]. Moreover, Akt1 has been shown to provide the CD28 co-stimulatory signal [9]. However, TCR stimulation is also known to trigger Akt1 transactivation [10,11]. Further, co-localization of PKCθ and Akt1 has been suggested to be crucial for NF-κB transactivation, as plasma membrane- and lipid raft-targeted mutants of PKCθ and Akt1 were observed to act synergistically in NF-κB activation in unstimulated cells [8]. Hence, the plasma membrane and/or lipid rafts, dynamic assemblies of cholesterol and sphingolipids which form microenvironments in the lipid bilayer, seem to be essential compartments for the activation of PKCθ and Akt1 and/or subsequent substrate phosphorylation, as previously postulated [8].

Clustering of lipid rafts has been suggested to be important in antigen-induced T cell activation [12], as lipid rafts cluster at the antigen-induced immunological synapse. Several studies proposed that the presence or absence of specific signaling proteins within lipid rafts controls lymphocyte signaling [12]. In the last 2 years the role of the T cell synapse has been more critically evaluated [13,14], nevertheless the contents of proteins in lipid rafts have been extensively studied. For PKCθ, there is clear evidence that it translocates to lipid rafts in antigen-stimulated or CD3-ligated T cells [15]. Furthermore, it was one of the first molecules localized in the central T cell synapse where lipid rafts cluster [16,17]. Activation of Akt1 also involves its plasma membrane translocation [18], due to its pleckstrin homology (PH) domain, which binds to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). Phosphatidylinositol 3-kinase (PI-3K), which generates PIP_3, and several PH domain-containing proteins, like VAV and Tec kinases, have been identified in lipid rafts, however lipid raft recruitment of Akt has not been reported yet. Very recently, an Akt-PH-green fluorescent protein (GFP) construct could be observed inside and outside the immunological synapse, due to elevated PIP_3 levels [19,20]. Hence, Akt might be a possible candidate as the effector kinase of PIP_3 in T cell synapse signaling.

Here we show that endogenous PKCθ and Akt1 are both recruited to lipid rafts as well as to the plasma membrane in CD3-ligated T cells. A novel mechanism of Akt1 membrane/raft recruitment and hence Akt1 activation is proposed, as PKCθ is shown to be constitutively bound to Akt1 and induce Akt1 plasma membrane/raft recruitment. Furthermore, we present data demonstrating that PKC isotypes mediate CD3-induced Akt1 transactivation, beside parallel Akt1 transactivation independently of PKC enzymatic activity. However, PKCθ is shown not to be essential in Akt1 transactivation. This study confirms and gives new insights into the recently observed PKCθ/Akt1 connection in T cell activation.

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Abbreviations: CTB, cholera toxin B; NF-κB, nuclear factor κB; PMA, phorbol myristate acetate; PH, pleckstrin homology; PI-3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; cPKC and nPKC, conventional and novel PKC subfamilies, respectively; TCR, T cell receptor

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2. Materials and methods

2.1. Reagents and plasmids

Gö6850 and LY294002 were purchased from Alexis, Lausen, Switzerland. The antibodies that recognize PKC isotypes were from Signal Transduction Labs, Upstate Biotech and Santa Cruz, respectively, the anti-(p)Ser473 Akt antibody, (p)Akt substrate antibody from Cell Signaling (New England Biolabs, USA) and anti-Akt1 monoclonal antibody (mAb) from Signal Transduction Labs, USA. The antibody used for immunoprecipitation of PKCθ was obtained from Santa Cruz, USA. The antibody used for stimulation of Jurkat T cells, anti-human CD28 mAb, clone 28.2 was from Pharmingen (San Diego, CA, USA), the CD3-specific antibodies OKT3 (human) mAb and 2C11 (mouse) mAb were a gift from A. Altman. The antibody used for mouse T cell stimulation, anti-mouse CD28, was obtained from Pharmingen. The cDNA constructs were characterized recently [8].

2.2. Cells and transfections

Jurkat TAg cells, stably transfected with the large T antigen, were used for experiments with transient overexpression, E6.1 Jurkat cells were used for experiments without overexpression, as these cells express CD28 in contrast to Jurkat TAg cells. Both cell types were maintained in RPMI medium supplemented with 10% fetal calf serum (Life Technologies). Transient transfection of cells was performed by electroporation in a BTX T820 ElectroSquarePorator (ITC, Biotech, Heidelberg, Germany) apparatus using predetermined optimal conditions: 2×10^7 cells at 450 V/cm and five pulses of 99 ms.

2.3. Purification of $CD3^+$ mouse T cells

Peripheral $CD3^+$ T cells from 6–9 week old mice were isolated from lymph nodes and spleen employing human erythrocyte lysis kit and mouse T cell enrichment columns (both R&D Systems).

2.4. Isolation of detergent-insoluble membrane (lipid raft) fractions

6×10^7 Jurkat TAg cells or 3×10^7 Jurkat E6-1 cells per assay point were transfected with 10–20 μg of the various cDNA expression plasmids, encoding wild-type (wt) or mutant PKCθ as indicated (Jurkat TAg cells), or left untransfected (Jurkat E6-1 cells). After incubation for 21 h the cells were stimulated with solid-phase IgG clones of CD3- and/or CD28-specific antibodies for 30 min at 37°C or left unstimulated. Subsequently cells were lysed in 1 ml of ice-cold MES buffer (25 mM MES pH 6.5, 5 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, 150 mM NaCl, 0.,5% Triton X-100, 50 μg/ml aprotinin and leupeptin), passing the cells 30 times through a 25G needle. Lysates were mixed with an equal volume of 80% sucrose in MES buffer and overlaid with 6 ml of 30% sucrose/MES and finally 3.5 ml of 5% sucrose/MES. Samples were centrifuged at $200\,000\times g$ for 20 h at 4°C. Tubes were removed to ice, and 12times 1-ml fractions were collected from the top. Horseradish peroxidase-cholera toxin B (HRP-CTB) staining of dot blots of each fraction revealed that fractions 4 and 5 contained lipid rafts (not shown). Fraction 12 was also retained to represent the Triton-soluble fraction. Lipid rafts were collected by centrifugation and solubilized in 1×sodium dodecyl sulfate (SDS) gel loading buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol).

2.5. Cell fractionation

1×10^7 Jurkat TAg cells per assay point were transfected with 10 μg cDNA expression plasmids, encoding mutant pEFneo Myr-PKCθ or as expression control, pEFneo GFP. After incubation for 21 h cells were stimulated with solid-phase antibodies against CD3 (Dynal IgG beads) for 20 min at 37°C or left unstimulated. Cell fractionation was performed by subsequent lysis in different MES lysis buffers (as described above; without Triton, soluble fraction; containing 0.5% Triton X-100, particulate fraction; containing 2% SDS, non-soluble fraction). 1×10^6 cell equivalents of the soluble fraction and 1×10^7 cell equivalents of the particulate and the non-soluble fraction were analyzed by immunoblotting.

2.6. Co-immunoprecipitation analysis

1×10^7 Jurkat T cells were lysed in 1 ml lysis buffer (25 mM MES pH 6.5, 5 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 50 μg/ml aprotinin and leupeptin). Lysates were precleared for 30 min at 4°C. Immunoprecipitation of PKCθ was performed at 4°C overnight. Thereafter incubation with protein G Sepharose (Amersham-Pharmacia, Vienna, Austria) for 1 h at 4°C, five times washing in lysis buffer, SDS–PAGE and immunostaining for Akt1 was performed.

2.7. Immunoblot analysis

SDS–PAGE (4–12%) was done under reducing conditions on Bis/Tris-buffered gels (Novex, San Diego, CA, USA). Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) by semi-dry blotting (90 mA, 80 min, 4°C). The primary antibodies were diluted in Tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk for incubation. Peroxidase-conjugated antibodies (Pierce, Rockford, IL, USA) served as secondary reagent (1:5000). For antigen detection enhanced chemiluminescence was used (Super Signal, Pierce).

2.8. Immunofluorescence analysis of T cells

Jurkat T cells were stimulated for 40 min with polystyrene beads (PolySciences) coated with anti-CD3. Freshly isolated mature $CD3^+$ T cells were incubated with biotinylated anti-CD3 and anti-CD28 (Pharmingen, both at 10 μg/ml) for 15 min at 4°C. Afterwards the cells were seeded on poly-L-lysine (Sigma)-coated glass coverslips and incubated with streptavidin for 30 min at 37°C. The coverslips were washed twice with phosphate-buffered saline (PBS, 4°C) and the cells fixed with Fix and Perm (An der Grub Bioresearch) for 15 min at 25°C. In a first staining step FITC-labeled CTB subunit (Sigma C1655) diluted in PBS was added to the cells and incubated for 30 min at 25°C. After extensive washing with PBS (six times) Akt1 goat antibody (Santa Cruz sc-7126) diluted in permeabilization solution (Fix and Perm) was added to the cells and incubated for 60 min at 25°C. Followed by another washing step (6×PBS) rhodamine-conjugated anti-goat IgG (Rockland code 605-700-125) diluted in permeabilization solution was added to the cells and incubated again for 60 min at 25°C. The coverslips were washed six times with PBS and afterwards antifade reagent (SlowFade Light Antifade Kit, Molecular Probes) was applied according to the manufacturer's instructions. Coverslips mounted on slides with Mowiol mounting solution (Calbiochem) were used for microscopy with an Olympus BX50 fluorescence microscope and acquisition of pseudoconfocal images was carried out with an automated Z-axis controller (E662 LVPZT Position Servo Controller, Visitron Systems) using the digital camera Microview TE/CCD1317-K/1 (Princeton Scientific Instruments) and Metamorph imaging 4.5 software (Universal Imaging Corporation). Finally the pictures (except controls) were processed with the AutoDeblur 7.5 software (Autoquant Imaging) and three-dimensional reconstruction was carried out again with the Metamorph imaging 4.5 software.

3. Results

3.1. The plasma membrane/lipid raft-targeted mutants of PKCθ (Myr-PKCθ) and Akt1 (Myr-Akt) localize in lipid rafts of unstimulated Jurkat T cells

The recently described Myr-PKCθ and Myr-Akt cDNAs [8] and as control PKCθ wt and Akt1 wt cDNAs were overexpressed in Jurkat TAg cells. Lipid raft association was analyzed by immunoblotting of raft fractions obtained from a sucrose gradient of Triton lysates. As shown in Fig. 1 the myristoylated mutants of PKCθ and Akt1 but not or at least to a much lower extent the wt enzymes were constitutively associated with lipid rafts. This is shown to be not due to different expression levels as equal expression of wt and myristoylated enzymes is demonstrated by immunoblotting of the Triton-soluble fraction.

3.2. PKCθ and Akt1 are recruited to lipid rafts of CD3-ligated Jurkat T cells

Lipid raft isolation by sucrose gradient of Triton-solubilized Jurkat T cells was performed. Fig. 2 shows the raft fractions immunoblotted for PKCθ and Akt1 under different stimulation conditions. Cell stimulation anti-CD3 was sufficient to

induce translocation of endogenous Akt1 and PKCθ to the lipid rafts (Fig. 2A) but no recruitment could be observed in cells stimulated with CD28 alone. No recruitment could be observed in unstimulated cells either (not shown) and CD28 co-stimulation did not enhance Akt1 or PKCθ raft translocation. In addition, staining for PKCα was performed. This isotype could not be detected in the lipid raft fractions of stimulated cells. CD3-induced Akt1 translocation could also be reproduced in Jurkat TAg cells, with overexpressed Akt1 and CD28 (Jurkat TAg cells do not express endogenous CD28) (Fig. 2B). Additionally, immunofluorescence microscopy was done, investigating the raft recruitment of Akt1 and as positive control PKCθ in CD3-stimulated Jurkat T cells (Fig. 3). Consistent with the subcellular fractionation data, both PKCθ and Akt1 are recruited to lipid rafts, polarized by CD3 beads. In cells without raft polarization (cells not bound to beads) less raft localization (= co-localization with CTB) of PKCθ and Akt1 was detected.

3.3. *Complex formation of endogenous PKCθ and Akt1 in Jurkat cells*

PKCθ and Akt1 physically associate in Jurkat TAg cells, when Akt1 and CD28 are overexpressed [8]. Here we further demonstrate the constitutive association of endogenous Akt1 and PKCθ proteins by co-immunoprecipitation analysis (Fig. 4). The association was not significantly affected by CD28, CD3 or CD3/CD28 activation.

3.4. *Overexpression of the plasma membrane/lipid raft-targeted mutant of PKCθ in Jurkat T cells induces translocation of endogenous Akt1 to the plasma membrane and lipid rafts*

Jurkat TAg cells, overexpressing a plasma membrane/lipid raft-targeted PKCθ mutant (Myr-PKCθ), were investigated for changes in the subcellular distribution of endogenous

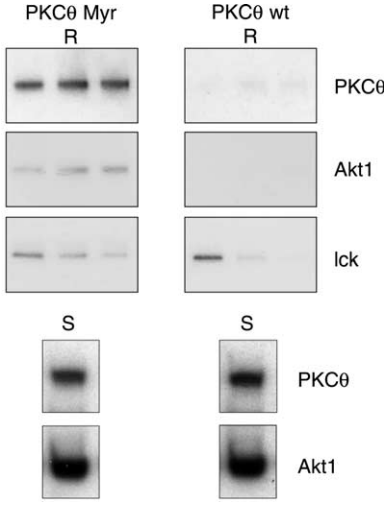

Fig. 1. The lipid raft/membrane-targeted mutants of PKCθ (Myr-PKCθ) and Akt1 (Myr-Akt) are constitutively associated with lipid rafts. Jurkat TAg cells were transfected with PKCθ wt and Akt1 wt or Myr-PKCθ and Myr-Akt cDNA, respectively. Lipid rafts were prepared by fractionation of sucrose gradients. Lipid raft fractions (R) and to control for expression the detergent-soluble fraction (S) was immunostained for PKCθ. As a marker for the raft fraction, a mAb raised against lck was used, and GM1 of each fraction was quantified in a dot blot employing HRP-CTB (not shown). A representative experiment of three independent experiments is shown.

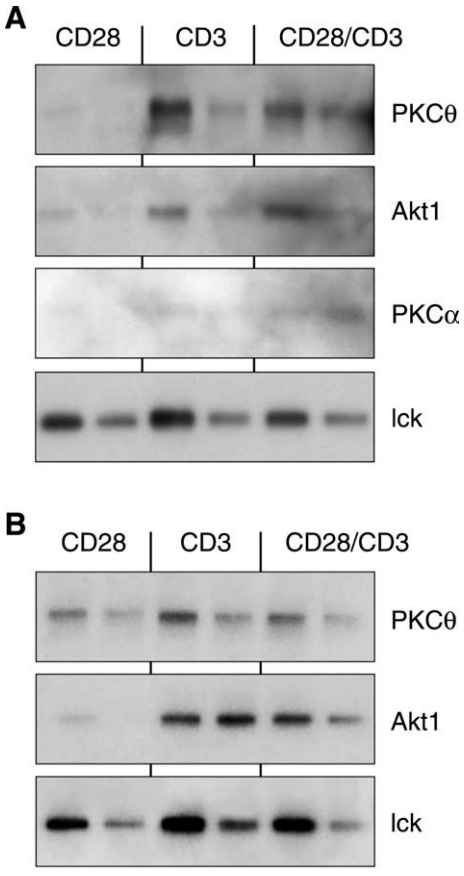

Fig. 2. Localization of PKCθ and Akt1 in lipid rafts. A,B: Jurkat TAg cells were transfected with PKCθ wt, Akt1 wt cDNA and CD28 (B), whereas for determination of endogenous PKCθ and Akt1 Jurkat T cells were left untransfected (A). The cells were stimulated for 25 min at 37°C with solid-phase IgG clones of CD3- and/or CD28-specific antibodies. Subsequently, lipid rafts were prepared by fractionation of sucrose gradients. Lipid raft fractions were immunostained for PKCθ and Akt1. As a marker for the raft fraction, lck was used, and GM1 of each fraction was quantified in a dot blot employing HRP-CTB (not shown). A representative experiment of two independent experiments is shown.

Akt1. Employing cellular fractionation and lipid raft preparation by sucrose gradient, Akt1 is shown to translocate to the particulate fraction (containing plasma membrane) (Fig. 5A,B) and to the lipid raft fraction (Fig. 5C,D) in Jurkat TAg cells, expressing the Myr-PKCθ mutant. Consistently, phorbol ester stimulation of Jurkat cells mediated translocation of Akt1 to the plasma membrane (data not shown). Employing an antibody which recognizes phosphorylated Akt substrates, recently described by Zhang et al. [21], we also detected Akt substrates in the lipid raft fraction of Myr-PKCθ-expressing Jurkat cells (Fig. 5D). This Akt substrate transphosphorylation site antibody was tested for its specificity by comparing it with a (p)PKC substrate antibody and a (p)PKA substrate antibody, which recognized different phosphorylation patterns of cellular proteins (not shown). CD3 stimulation is shown to be more effective in translocating Akt1 to the plasma membrane (Fig. 5A,B). However, a likely explanation may be that protein overexpression by electroporation only affects about 25–30% of the cells, as CD3 cell stimulation activates all cells.

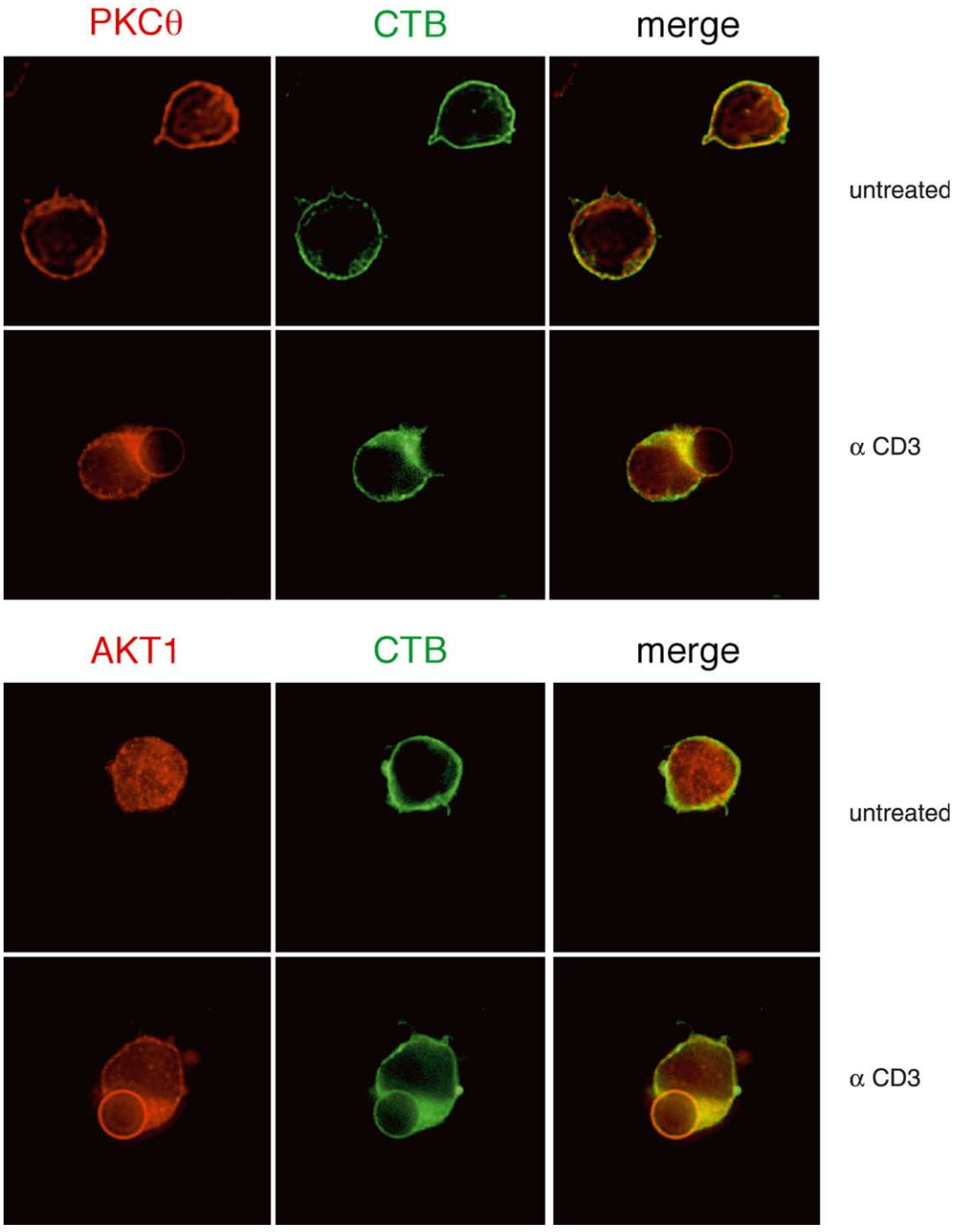

Fig. 3. Localization of PKCθ and Akt1 in polarized lipid rafts by anti-CD3 ligation (pseudoconfocal immunofluorescence). Jurkat T cells were stimulated for 40 min with polystyrene beads coated with anti-CD3. Akt1, PKCθ and lipid raft aggregation toward beads was analyzed by subsequent staining with isotype-specific antibodies (Akt1 or PKCθ/TRITC-conjugated anti-IgG) and FITC-conjugated CTB, respectively. A representative aggregate is shown.

3.5. *Akt1 is downstream of PKC isotypes in CD3/CD28-induced signaling in peripheral mouse T cells*

To test whether PKCθ and/or other PKC family members physiologically act upstream of Akt1 in CD3/CD28-induced signaling, as suggested above, we looked at $CD3^+$ peripheral mouse T cells, as Jurkat cells reveal constitutive Akt1 activity, due to loss of phosphoinositol phosphatase and tensin homolog, a phosphatase-degrading PIP_3 [22]. Akt1 activity was determined by immunostaining for Akt (Ser473) phosphorylation, a phosphorylation site of Akt1 known to be critical for Akt1 kinase activity [23]. Additionally, staining for phosphorylated Akt substrates [21] in whole cell lysates of mouse T cells was performed. The most prominent CD3/CD28-induced Akt phosphorylation was a 30 kDa protein substrate (p30), recently identified as S6 ribosomal protein [21]. Of note, phorbol myristate acetate (PMA)-induced phosphorylation of p30 is dependent on PI-3K activity, as the PI-3K inhibitor LY294002 can decrease its phosphorylation, further confirm-

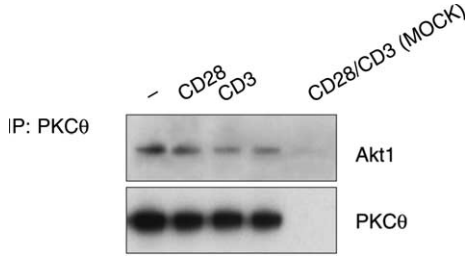

Fig. 4. Complex formation of Akt1 and PKCθ. Co-immunoprecipitation analysis of PKCθ and Akt1. Jurkat T cells were stimulated for 20 min at 37°C with solid-phase IgG clones of CD3- and/or CD28-specific antibodies. Afterwards, cell extracts were immunoprecipitated (IP) with normal goat serum (Mock) or a polyclonal anti-PKCθ antibody. The immunoblots were stained for PKCθ (lower panel) and Akt1 (upper panel). A representative experiment of three independent experiments is shown.

ing the specificity of this Akt substrate (Fig. 6A), consistent with a PI-3K/Akt-dependent phosphorylation [18]. The same substrate was phosphorylated by CD3 stimulation in the absence of a CD28 stimulus (not shown). Employing both methods for determination of Akt1 activity we were able to show that inhibition of all conventional and novel PKC subfamilies (cPKC, nPKC) by Gö6850 (also known as GF109203X and used at 100 nM) decreases CD3/CD28-induced Akt1 activity ((p)Ser473: ~45%; (p)p30: ~54%). Consistently, the phorbol ester PMA, an activator of the c and nPKCs, is able to induce Akt1 activity, which can again be abolished by preincubation with the PKC inhibitor Gö6850 ((p)Ser473: ~100%; (p)p30: ~80%), excluding pleiotropic effects of non-PKC phorbol ester receptors, and placing c and/or nPKC isotype(s) upstream of Akt1 (Fig. 6B,C).

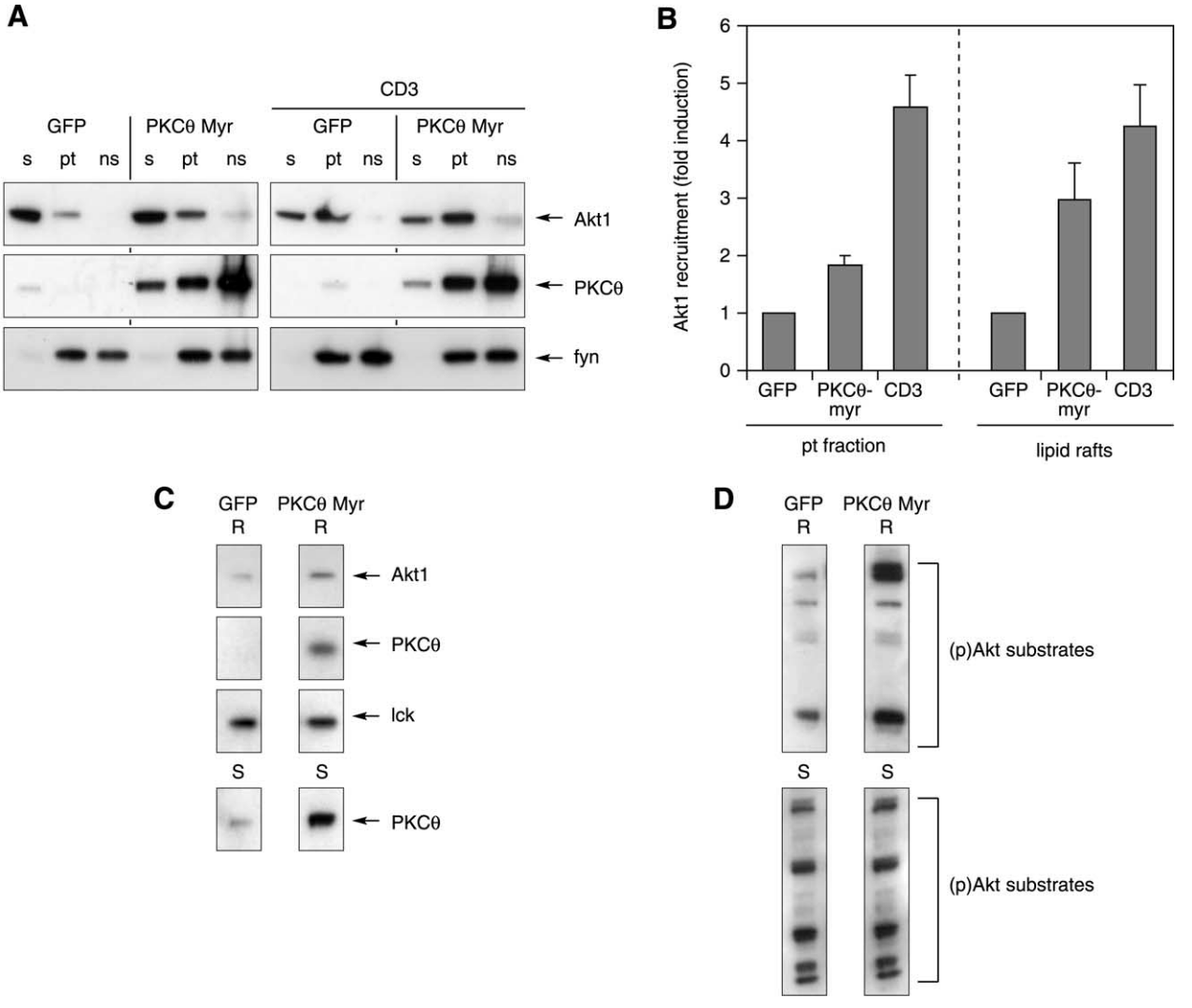

Fig. 5. Translocation of Akt1 to the plasma membrane and lipid rafts by overexpression of Myr-PKCθ. Jurkat TAg cells were transfected with Myr-PKCθ or GFP. After 21 h cells were stimulated with solid-phase CD3 for 20 min or left unstimulated, as indicated. A: Subcellular distribution of endogenous Akt1 was determined by immunoblotting. The cell fractions are defined as the soluble (s) fraction, the particulate (pt) fraction and the Triton X-100 non-soluble (ns) fraction, which were prepared as described in Section 2. The fyn protein was detected to control for cell fractionation. B: Statistical analysis of Akt1 recruitment to the pt fraction and to the lipid raft fraction. Mean values ± S.E.M. of two or three independent experiments are shown. C: Lipid rafts (R) were prepared by fractionation of sucrose gradients. As a marker for the raft fraction, staining for lck was performed. As expression control, the detergent-soluble fraction (S) was immunostained for PKCθ. D: The same blot as in C was also stained for phosphorylated Akt substrates.

3.6. PKCθ is dispensable in CD3/CD28- or PMA-induced Akt1 activation

To test whether PKCθ is the specific PKC isotype upstream of Akt1 in the signaling cascade, we investigated T cells derived from PKCθ knockout mice (Pfeifhofer et al., J. Exp. Med., submitted). However, employing the same experimental approach as in Fig. 6, T cells deficient in PKCθ did not reveal any decrease in CD3/CD28- (Fig. 7A) or in PMA- (Fig. 7B) induced Akt1 activity compared with wt mouse T cells. Additionally, we stimulated the cells with CD3 in the absence of CD28, but no difference could be observed either (data not shown).

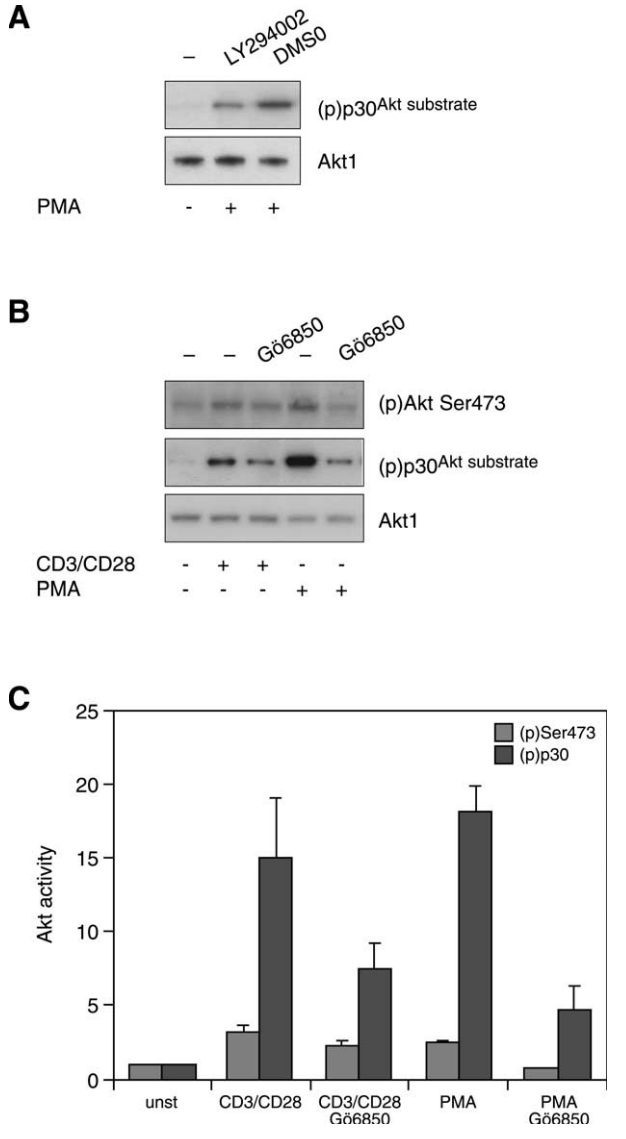

Fig. 6. Akt1 activation is regulated by PKC isotypes in peripheral mouse T lymphocytes. A,B: CD3$^+$ mouse lymphocytes, purified from lymph nodes and spleen, were treated with LY294002 (10 μM), Gö6850 (100 nM) and DMSO buffer control, as indicated. Subsequently, cells were stimulated with solid phase CD3/CD28 or PMA (30 ng/ml) for 20 min at 37°C, or left unstimulated. Akt1 activity was determined in an immunoblot, employing staining for (p)Ser473 Akt and for (p)p30, recognized by a Akt substrate site-specific antibody, respectively. C: Statistical analysis of Akt1 recruitment to the particulate (pt) fraction and to the lipid raft fraction. Mean values ± S.E.M. of two or three independent experiments are shown.

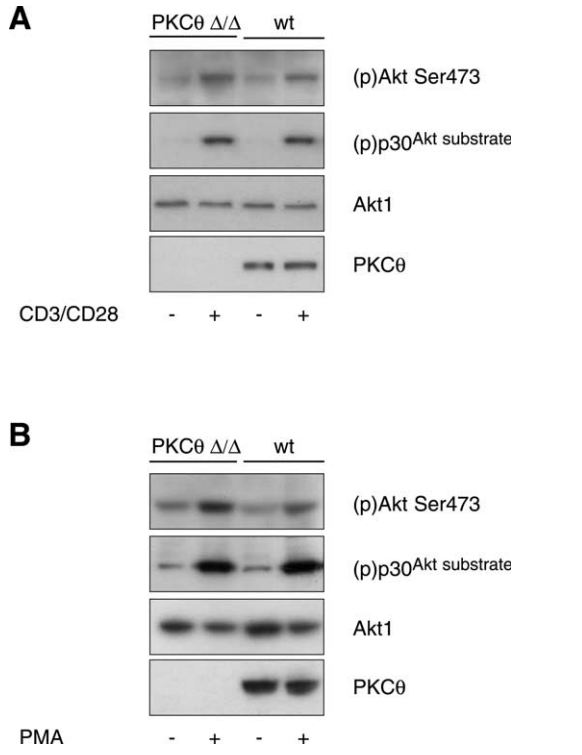

Fig. 7. Enzymatic activity of Akt1 is equal in T lymphocytes derived from PKCθ-deficient or wt mice, stimulated with CD3/CD28 or PMA. CD3$^+$ mouse lymphocytes, purified from lymph nodes and spleen, were stimulated with solid phase CD3/CD28 and PMA (30 ng/ml) for 20 min at 37°C, or left unstimulated. Akt1 activity was determined by immunostaining for (p)Ser473 Akt and for a phosphorylated Akt substrate, p30, recognized by a (p)Akt substrate site-specific antibody. A representative experiment of three independent experiments is shown.

3.7. CD3/CD28 crosslinking induces Akt1 capping in primary mouse T cells

Importantly, endogenous Akt also localizes the CD3/CD28 crosslinking-induced caps in primary T cells. Consistent with the subcellular fractionation data of Jurkat cells, Akt1 (similarly to PKCθ, [15]) is recruited to lipid rafts, polarized by CD3/CD28 crosslinking (Fig. 8). In cells without raft polarization (unstimulated control) virtually no raft localization (= co-localization with CTB) of Akt1 was detected.

4. Discussion

We here demonstrate that endogenous PKCθ and Akt1 are recruited to the plasma membrane as well as to lipid rafts in CD3-ligated Jurkat T cells. The PH domain of Akt is known to bind PIP_3, generated by activated PI-3K. Akt1 membrane localization was found to be a primary determinant of its subsequent activation by PDK1, which also contains a PIP_3-binding PH domain [24]. PKCθ has also been reported to translocate by a PI-3K-dependent mechanism [25]. However, this is the first study demonstrating recruitment of Akt1 to lipid rafts. Several studies have suggested a role for lipid rafts in Akt signaling. Recently, a novel, raft-associated Akt1 Ser473 kinase activity has been identified [26]. Ligation of the T cell co-receptor molecule CD38 has also been reported to involve lipid rafts in Akt transactivation [27]. Further, LAT is a raft-associated transmembrane adapter molecule, which

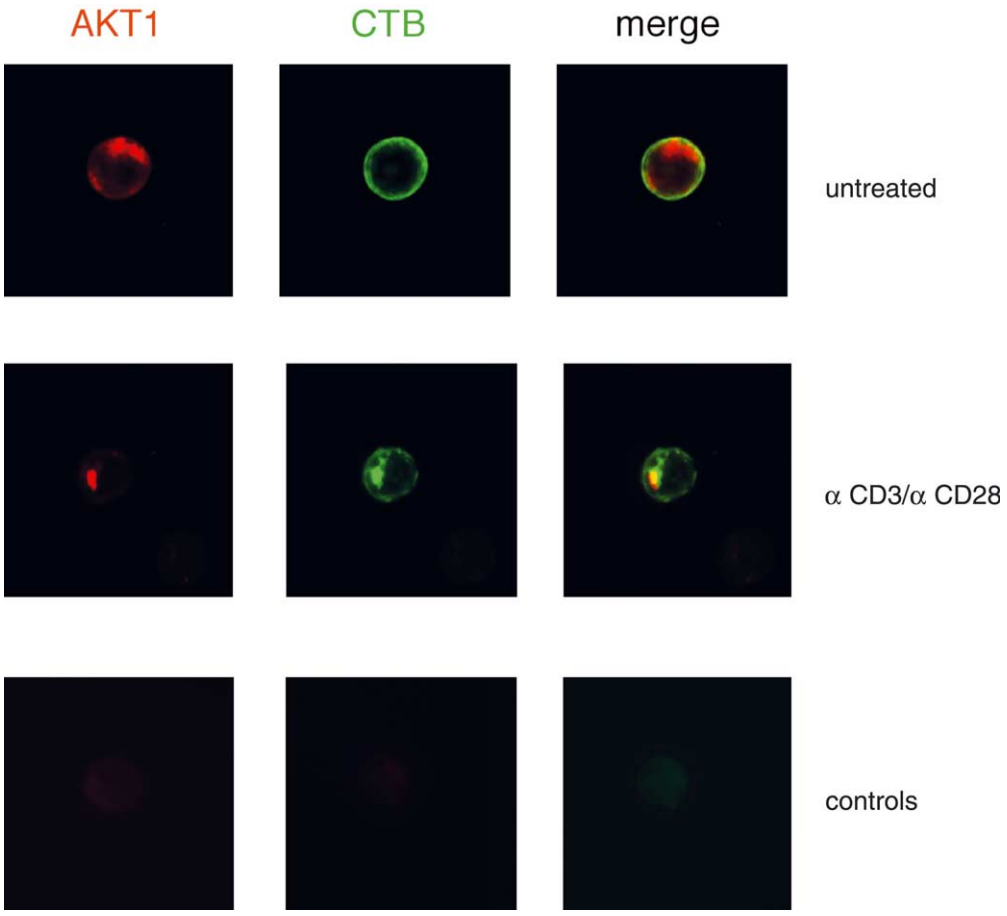

Fig. 8. Localization of endogenous Akt1 in lipid rafts of primary $CD3^+$ mouse T cells (pseudoconfocal immunofluorescence). Freshly isolated mature $CD3^+$ mouse T cells were incubated with biotinylated anti-CD3 and anti-CD28 for 15 min at 4°C and subsequently stimulated with streptavidin for 30 min at 37°C. Lipid raft fractions were immunostained employing FITC-CTB. A representative experiment is shown.

recruits several signaling molecules, including PKC and Akt1 activating proteins, like phospholipase Cγ1 and PI-3K, upon T cell activation. SHIP, a phosphatase of PIP_3, has been described as an inhibitor of Akt in B cells [28,29] and has also been found to translocate to lipid rafts in inhibitory BCR signaling [30,31]. The function of recruitment of Akt1 to lipid rafts and the role of lipid rafts in T cell activation in general are not clear. It has been shown that lipid rafts cluster at the T cell synapse in antigen-induced T cells. Of note, the PH domain of Akt has been shown to localize to the synapse in living cells [19,20], suggesting Akt as an effector kinase of synapse signaling. Alternatively, PKCθ and Akt1 additive signaling could be a prerequisite for synapse formation and/or lipid raft clustering, proposing a functional link with cytoskeletal reorganization. TCR-induced lipid raft clustering as well as Akt1 activation is impaired in p110δ PI-3K transgenic mice [32]. However, in T cells derived from PKCθ knockout mice, CD3-induced lipid raft clustering seems to be normal (data not shown). Alternatively, lipid rafts could not be essential for T cell activation at all. Substrate phosphorylation could also take place at the plasma membrane outside the rafts. Recently, a study demonstrated that CD3-dependent TCR activation occurs independently of cholesterol extraction [33]. The plasma membrane contains several phosphorylation PKC substrates in CD3-ligated, phorbol ester-stimulated or Myr-PKCθ-expressing Jurkat T cells (B. Bauer, unpublished observation). However, a membrane-recruited mutant of PKCθ (PKCθ-CAAX), which was not detectable in lipid rafts, was not active in NF-κB signaling nor in c-Jun N-terminal kinase 2 signaling and revealed no functional synergy with Myr-Akt (data not shown). This was not due to conformational inactivation, as PKCθ-CAAX revealed normal kinase activity in vitro (data not shown). Moreover, Myr-PKCθ mediated phosphorylation of Akt substrates in the lipid raft fraction (Fig. 5D). It is possible, therefore, that these distinct compartments, the plasma membrane and lipid rafts, act in different signaling cascades and biological functions but this has to be further clarified.

We here further suggest a functional link in the membrane/raft recruitment of PKCθ and Akt1. We suggest membrane/raft recruitment of Akt1, partially independent of its PIP_3-induced membrane translocation, as also proposed for the Akt-binding oncoprotein TCL1, which acts as an Akt co-activator by Akt membrane recruitment [22]. We show that PKCθ is able to mediate Akt1 translocation, most probably by complex formation. Hence, the recently observed slightly elevated NF-κB activation induced by the Myr-PKCθ mutant [8] could be due to this translocation of endogenous Akt1. Consistently, the signal is enhanced in cells co-expressing both Myr-PKCθ and Myr-Akt recombinant proteins. However, the Myr-Akt mutant, known to be constitutively active [34], is inactive in NF-κB transactivation without Myr-PKCθ co-expression, indicating that PKCθ has additional function(s) in NF-κB signaling than just inducing Akt1 mem-

brane/raft translocation. For instance, PKCθ could mediate recruitment of e.g. Akt1 substrates and/or effectors. PKCθ has been reported to bind to the inhibitor of κB factor kinase complex, which is also recruited to lipid rafts in CD3/CD28-activated T cells [35].

Employing peripheral T cells this study further demonstrates a novel, PKC-dependent transactivation pathway of Akt1 in CD3/CD28- or only CD3-stimulated T cells. Phorbol ester-induced Akt1 transactivation in T cells has been reported previously [36] and is also demonstrated in Fig. 6B,C. In CD3-ligated naive T cells activation of c and nPKC isotypes is upstream of Akt1 transactivation and downstream signaling, as a specific inhibitor of c and nPKCs can decrease Akt signaling function (Fig. 6B,C). In this PKC-inducible signaling pathway leading to Akt1 transactivation, PI-3K is involved downstream of PKC isotypes, as an inhibitor of PI-3K can significantly albeit only partially inhibit PMA-induced Akt1 transactivation and downstream signaling (Fig. 6A). However, PKCθ is shown not to be essential employing T cells derived from PKCθ knockout mice (Fig. 7). This could be due to functional redundancy and/or no functional requirement of PKCθ in Akt1 transactivation. Additionally, in CD3/CD28-ligated cells, Akt1 transactivation is mediated by a PKC-independent pathway, seen by the only partial reduction of Akt1 activity by the c and nPKC inhibitor Gö6850. Much higher inhibition by Gö6850 could be achieved in PMA-induced Akt1 activation (Fig. 6B,C).

In summary, this study investigates the subcellular location of PKCθ and Akt1, demonstrating their co-recruitment to the plasma membrane including lipid rafts in CD3-activated T cells. A novel mechanism of Akt1 membrane/raft recruitment is proposed, as PKCθ is shown to induce Akt1 plasma membrane/raft recruitment, most likely explained by the observed complex formation of both protein kinases. We here further demonstrate the existence of parallel signaling pathways, partially regulated by c and/or nPKCs, leading to Akt1 transactivation and downstream signaling in CD3-ligated peripheral mouse T cells. PKCθ is shown not to be essential for this. This multi-regulation of Akt1 is not unexpected for this crucial survival kinase.

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