Protein Kinase C μ Is Negatively Regulated by 14-3-3 Signal Transduction Proteins*

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Recent studies have documented direct interaction between 14-3-3 proteins and key molecules in signal transduction pathways like Ras, Cbl, and protein kinases. In T cells, the 14-3-3 isoform has been shown to associate with protein kinase C θ and to negatively regulate interleukin-2 secretion. Here we present data that 14-3-3 interacts with protein kinase C μ (PKCμ), a sub-type that differs from other PKC members in structure and activation mechanisms. Specific interaction of PKCμ and 14-3-3 can be shown in the T cell line Jurkat by immunoprecipitation and by pulldown assays of either endogenous or overexpressed proteins using PKCμ-specific antibodies and GST-14-3-3 fusion proteins, respectively. Using PKCμ deletion mutants, the 14-3-3 binding region is mapped within the regulatory C1 domain. Binding of 14-3-3 to PKCμ is significantly enhanced upon phorbol ester stimulation of PKCμ kinase activity in Jurkat cells and occurs via a Cbl-like serine containing consensus motif. However, 14-3-3 cannot be a substrate of PKCμ. In contrast 14-3-3 strongly down-regulates PKCμ kinase activity in vitro. Moreover, overexpression of 14-3-3 significantly reduced phorbol ester induced activation of PKCμ kinase activity in intact cells. We therefore conclude that 14-3-3 is a negative regulator of PKCμ in T cells.

Members of the protein kinase C (PKC) family of intracellular serine kinases play critical roles in the regulation of a variety of intracellular signaling processes. Much attention has been focused on the role of PKCs in T cell signaling (for review see Refs. 1–3). Phorbol ester responsive PKCs in general have particular high expression in thymus and hematopoetic cells (20). PKCμ displays, in addition to the conserved kinase and regulatory domains in common to all PKC isoforms, structural features like a hydrophobic amino-terminal domain, an acidic regulatory domain (21), and a pleckstrin homology domain (22). The recruitment of signal transducers like Cbl (10) and phosphoinositide 3-kinase (9) in T cells further supports a potential role of 14-3-3 dimers in the assembly and/or regulation of signaling complexes. Evidence for an active regulatory function of 14-3-3 proteins stems from the finding that 14-3-3 binding to PKCθ negatively affects the stimulation of the interleukin-2 promoter and prevents PKCθ translocation to the membrane (18), supporting a role of 14-3-3 proteins in the regulation of PKC activity in T cells.

We have recently described a novel PKC isotype termed PKCμ (19), which, although ubiquitously expressed, shows particularly high expression in thymus and hematopoetic cells (20). PKCμ, by contrast to PKCθ, is a regulator of PKC activation in T cells.

During T cell signaling events evidence of an involvement of members of the 14-3-3 proteins, an abundant group of acidic proteins originally found in brain extracts (6–8), has been obtained. For example, it has been demonstrated that the 14-3-3 isoform interacts with the catalytic subunit of the phosphoinositide 3-kinase (9) and the Cbl protooncogene (10), affecting Ras-dependent T cell receptor-mediated signaling leading to NF-AT activation (11). Besides T cell-specific functions 14-3-3 proteins have been shown to be involved in mitogenic pathways of other cells as well, affecting regulation of the Raf kinase (7, 12), cell cycle (13), and anti-apoptotic pathways (14–16). The mechanism, by which 14-3-3 influences Raf is still unresolved, as recent data suggest that activation of Raf by 14-3-3 may in fact be due to stabilization of an activation complex rather than a direct stimulation of Raf activity (17). A stabilizing role in the formation of signaling complexes can be deduced from the capacity of 14-3-3 isoform to form dimers in vitro (10). The recruitment of signal transducers like Cbl (10) and phosphoinositide 3-kinase (9) in T cells further supports a potential role of 14-3-3 dimers in the assembly and/or regulation of signaling complexes. Evidence for an active regulatory function of 14-3-3 proteins stems from the finding that 14-3-3 binding to PKCμ negatively affects the stimulation of the interleukin-2 promoter and prevents PKCθ translocation to the membrane (18), supporting a role of 14-3-3 proteins in the regulation of PKC activity in T cells.

In the present study, we demonstrate by binding studies and pulldown assays as well as by transient expression in the T cell line Jurkat that PKCμ specifically associates in vitro and in vivo with 14-3-3 proteins. The 14-3-3 binding site within PKCμ could be located to the C1 regulatory region. 14-3-3 interacts preferentially with the activated, phosphorylated PKCμ and down-regulates kinase activity, suggesting that 14-3-3 is a regulator of PKCμ functions in T cells.

EXPERIMENTAL PROCEDURES

Recombinant PKCμ, Plasmid Constructs, and Cell Lines—The production of SF158 insect cells overexpressing PKCμ (25) and the construction of 14-3-3 and θ glutathione S-transferase (GST) fusion proteins has been described previously (9). The human T lymphoma cell line Jurkat-TAg (26) was maintained in RPMI 1640 medium supple-

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‡ The abbreviations used are: PKC, protein kinase C; GST, glutathione S-transferase; Pdbu, phorbol 12,13-dibutyrate; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.
mented with 10% fetal calf serum. GST fusion proteins were isolated according to the manufacturer’s instructions (Amersham Pharmacia Biotech). In brief, fusion proteins were bound to glutathione-Sepharose and quantitated according to Coomassie staining by densitometric scanning, calibrated against an albumin standard. PKCμ deletion mutant PKCμ was generated by digesting pBpl4 (19) with Xhol and SfiI. Overhanging 5’ and 3’ ends were filled with the Klenow enzyme, and the 2.9 kilobase PKCμ fragment was isolated and ligated in EcoRI-digested pCDNA3 (Invitrogen). PKCμ1-79 was constructed by cutting pCDNA3/PKCμ1-79 with HindIII, isolating a 800-base pair HindIII fragment followed by religating the vector/PKCμ portion. Additionally these sequences were cloned into other expression vectors and verified by transient expression (27). PKCμ point mutations (serine to alanine) were created using a polymerase chain reaction approach according to a manufacturer’s instructions (Quickchange site-directed mutagenesis, Stratagene) and were verified by dideoxy sequencing. COS transfectants stably overexpressing PKCμ were generated by transfecting COS Cells with PKCμ wild type cloned in the expression vector pCDNA3 followed selection of transfectants in neomycin (400 μg/ml) containing media for a period of 20 days. Single colonies were analyzed for PKCμ overexpression by Western blot analysis.

Immunoprecipitation by Antibodies and GST-14-3-3 Precipitation of PKCμ-Sf158 or Jurkat-TAg cells were lysed at 4 °C in lysis buffer (20 mM HEPES, 7.5 mM MgCl2, 1% NP-40, 1 mM PMSF, 10 mM NaF, 1 mM Na3VO4, 150 mM NaCl, 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM nitrophenylphosphate) using an sonifier. After centrifugation of cell debris (15 min, 10,000 rpm, type 5403, Eppendorf) GST precipitation was done by incubation with the indicated amounts of GST fusion proteins coupled to glutathione-Sepharose in 1-ml lysisate portions (500 000 Sf 158 cells or 60 000 Jurkat-TAg cells) for 90 min at 4 °C. For immunoprecipitation of PKCμ from Jurkat-TAg cells, a PKCμ antisera was used as described earlier (28). Immunocomplexes were harvested by incubation with protein G-Sepharose (Pharmacia, 30 μg/2 × 106 cell equivalents) for 30 min at 4 °C. Immunocomplexes or GST complexes were washed three times in lysis buffer and applied to SDS-PAGE following transfer to a nitrocellulose membrane. Western blot detection of PKCμ or 14-3-3 was performed according to standard conditions using monoclonal antibodies as described earlier (18, 28). GST was detected using an anti-GST mAb (Santa Cruz). Visualization for all Western blots shown was performed using an alkaline phosphatase-based detection system according to standard conditions.

Transfections—7.5 × 10⁶ Jurkat-TAg cells were seeded per 80-mm diameter dish in 5 ml of RPMI supplemented with 10% fetal calf serum and transfected with 5 μg of DNA and 20 μl of Superfect reagent (Qiagen) according to the manufacturer’s protocol. Cells were harvested and analyzed 48 h upon transfection by immunoprecipitation analysis as described above. In the case of 14-3-3 overexpression experiments, PKCμ was immunoprecipitated and in vitro autophosphorylated as described below. Exponentially growing 293 cells, 40–90% confluent, were transfected with the indicated plasmids containing 2 μg of DNA and 60 μl of Superfect reagent for each well of a 6-well plate or 10 μg of DNA and 60 μl of Superfect reagent for a 100-mm plate. Extracts from one well were used for each immunoprecipitation and GST 14-3-3 precipitation of PKCμ.

In Vitro Kinase Assays—Jurkat-TAg cells were stimulated with phorbol 12,13-dibutyrate (PdBu, 100 nM) for the indicated times, lysates were prepared, and PKCμ was immunoprecipitated. PKCμ autophosphorylation was determined in an in vitro kinase assay as described previously (28). In brief, the immunoprecipitates were washed twice in lysis buffer and once in phosphorylation buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 2 mM dithiothreitol). The immunocomplexes were mixed with 10 μl of phosphorylation buffer containing 0.2 μl of γ³²P]-ATP (Amersham Pharmacia Biotech) and incubated for 10 min at 37 °C. The reaction was stopped by adding 5× SDS-PAGE sample buffer, fractionated by SDS-PAGE followed by transferring to a nitrocellulose membrane, and visualized by phosphoimaging (Molecular Dynamics). For the in vitro inhibition assays, 80 ng of purified PKCμ enzyme from Sf158 cells (25) was used with the indicated amounts of GST 14-3-3 or GST 14-3-3B added.

Phosphatase Treatment and Far Western Blot Analysis—PKCμ was immunoprecipitated from 5 × 10⁶ Sf158 cells using 4 μl of a rabbit antisera raised against a carboxy-terminal epitope. Protein G-Sepharose bound immune complexes were in vitro phosphorylated as described above and washed twice to remove nonincorporated [γ³²P]-ATP. Bound PKCμ was eluted in a final volume of 100 μl upon adding 50 μl of immunizing peptide (1 mg/ml) by incubating 30 min at 4 °C. PKCμ was incubated with Phosphatase 2A (0.4 units) for the indicated times. Equal aliquots were subjected either to GST 14-3-3 precipitation followed by autoradiography to detect PKCμ immunodetection or to direct immunoblot analysis to compare precipitation efficiencies. For Far Western analysis, PKCμ from Sf158 or Jurkat-TAg cells was immunoprecipitated as described. Aliquots of immunoprecipitates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. PKCμ detection was carried out using a PKCμ mAb. 14-3-3 binding to activated PKCμ was analyzed essentially as described (29). Detection of bound 14-3-3 was carried out by a 2-h incubation with 10 μg/ml GST 14-3-3 fusion protein and visualized using an alkaline phosphatase-coupled anti-GST secondary antibody.

RESULTS

14-3-3 Specifically Associates with PKCμ in Vivo—14-3-3 has been recently reported to associate with PKCθ, which is highly expressed in T cells (4, 5). To test whether 14-3-3 would also interact with another T cell expressed isoform, PKCμ, we analyzed recombinant PKCμ for potential 14-3-3 association. GST 14-3-3 fusion proteins were used to precipitate PKCμ expressed in Sf158 cells. As shown in Fig. 1A, in GST pulldown assays a 14-3-3 dose-dependent binding of PKCμ can be detected by immunoblot analysis (upper panel), showing best detection using 4 μg of 14-3-3 GST fusion protein. 14-3-3 binding to PKCμ is specific because no binding to the respective amount of GST proteins was detectable. Only a fraction of total recombinant PKCμ was precipitated with 14-3-3 GST protein, as shown by comparison with PKCμ immunoprecipitation by PKCμ-specific polyclonal antibodies (Fig. 1A, left lane), even when the GST 14-3-3 concentration was increased to 20 μg (data not shown).

Next, the association of 14-3-3 proteins with endogenous PKCμ was investigated. 14-3-3 GST fusion proteins were used to precipitate PKCμ from extracts of Jurkat-TAg cells. As shown in Fig. 1B, endogenous PKCμ could be specifically precipitated from lysates of Jurkat-TAg cells. Both 14-3-3 isoforms, 14-3-3σ and 14-3-3ζ (30), were equally suited to precipitate PKCμ. The respective controls, glutathione S-transferase, and as a control for nonspecific binding, the pleckstrin homology domain of PKCμ expressed as a GST fusion protein did not detectably precipitate PKCμ in pulldown assays (Fig. 1B, left lanes).

14-3-3 association with PKCμ was also shown in coprecipitation experiments using PKCμ-specific antibodies. As in 293 cells endogenous PKCμ levels are too low to detect 14-3-3 association (data not shown); cotransfection of PKCμ and 14-3-3 was performed in 293 cells. Additionally, 14-3-3 was transiently overexpressed in stable COS-PKCμ transfectants, and PKCμ was immunoprecipitated from lysates of double transfectants. In both cases, different amounts of 14-3-3 DNA were used for transfection to ensure optimum expression. As shown in Fig. 1C (left panels), in cotransfected 293 cells 14-3-3 can be readily detected in PKCμ immunoprecipitates upon appropriate expression of both cDNAs (PKCμ/14-3-3 DNA ratio 1:10). Likewise, in stably PKCμ expressing COS transfectants, 14-3-3 can also be coprecipitated with PKCμ upon transient overexpression using 10 μg of the respective 14-3-3 expression construct (Fig. 1C, lower right panel). As the subtype-specific anti-14-3-3 mAb is directed against an epitope within the potential binding site of target proteins, the reciprocal immunoprecipitation experiment was precluded.

14-3-3 Binds to a Serine-Derived Motif within the C1 Region of PKCμ—The cysteine fingers in the C1 region of PKCs have been previously reported to be the binding site for second messengers as well as for regulatory proteins affecting protein kinase activity (31–34). Fig. 2A displays the location of these

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2 Y. C. Liu, unpublished observations.
domains in PKC\(\mu\). In an attempt to identify potential binding sites of 14-3-3, we transiently overexpressed in 293 cells an amino-terminal PKC\(\mu\) deletion mutant and a mutant lacking in addition the C1 binding region. The mutants PKC\(\mu_{1–79}\) and PKC\(\mu_{1–279}\) were generated by deletion analysis initiating translation at Met-80 or Met-341 (see "Experimental Procedures") were used. Transfection of these mutants in 293 cells resulted in the expression of approximately 100- and 70-kDa variants of PKC\(\mu\) as shown by immunoprecipitation (Fig. 2B). 14-3-3\(\beta\) GST fusion proteins were used to precipitate PKC\(\mu\), and the mutants from lysates of 293 cells were transfected with the respective expression constructs. As shown in Fig. 2C, PKC\(\mu\) could be readily detected in 14-3-3\(\beta\) GST precipitates from 293 cells expressing wild type PKC\(\mu\) and the PKC\(\mu_{1–79}\) mutant. Although expressed at high level (Fig. 2B), the PKC\(\mu_{1–279}\) mutant was not detectable in 14-3-3\(\beta\) GST precipitates (Fig. 2C). Similar data were obtained by overexpressing the PKC\(\mu\)

kinase domain (data not shown). These findings indicate a binding of 14-3-3\(\tau\) approximately within the region between amino acid 80–340 containing the complete C1 regulatory domain of PKC\(\mu\).

14-3-3\(\tau\) binding has been reported to involve a serine consensus motif like RSXSXP (35, 36) or RX\(R/S\)X\(R/S\) (37). Therefore, we searched for potential serines matching the predicted consensus sequences within the C1 region of PKC\(\mu\). Two serine regions, serine 205/208 (RRLNVSILT) and serine 219/223 (IRTSSAEILST; Fig. 2A), show some similarity to the predicted 14-3-3\(\tau\) binding consensus sequences. Of interest, these regions also exert homology to the predicted consensus sequence of PKC\(\mu\) substrates (38), therefore potentially representing an autophosphorylation site (see below). The indicated serine pairs were mutated to alanine (PKC\(\mu_{S205A,S208A}\) and PKC\(\mu_{S219A,S223A}\) and expressed in 293 cells (Fig. 2, A and B). The sets of mutants were further combined in another expression plasmid carrying the double mutant (PKC\(\mu_{D205D,220D}\): S205A,S208A/S219A,S223A; Fig. 2A) and, upon transient expression in 293 cells, analyzed for 14-3-3\(\tau\) binding capacity. As shown in Fig. 2A, all mutants were equally well expressed in 293 cells. In 14-3-3\(\tau\) GST precipitates, both the PKC\(\mu_{S205A,S208A}\) mutant and the PKC\(\mu_{S219A,S223A}\) mutant, were still detectable, but in contrast, the mutant lacking both serine motifs, PKC\(\mu_{D205D,220D}\), could hardly be detected in 14-3-3\(\tau\) GST precipitates (Fig. 2C). This suggests that both serine motifs, Ser-205/208 and Ser-219/223, are involved in PKC\(\mu\) binding. To investigate potential autophosphorylation of serine 205/208 or serine 219/223, the mutants were expressed in 293 cells, and in vitro autophosphorylation assays were performed. As shown in Fig. 2D, the double mutant showed significant reduction in autophosphorylation (30%) compared with PKC\(\mu\) wild type, whereas both PKC\(\mu_{S205A,S208A}\) and PKC\(\mu_{S219A,S223A}\) mutants display only weak reduction in PKC\(\mu\) autophosphorylation (data not shown). PKC\(\mu\) contains approximately 10 phosphorylation sites. Thus likely mutation of one site is probably below the detection level. Together with the data of the 14-3-3 pulldown assays, these findings, point to serine 205/208 and serine 219/223 as functional important phosphorylation sites in PKC\(\mu\).

14-3-3\(\tau\) Associates with Phosphorylated PKC\(\mu\)—As shown for the association of 14-3-3\(\tau\) with Cbl, serine phosphorylation of Cbl is essential (37). We therefore tested whether activated PKC\(\mu\), which has been shown to be exclusively phosphorylated on serine residues (28), displays enhanced binding of 14-3-3\(\tau\) GST fusion proteins. Indeed, PKC\(\mu\) could be more efficiently precipitated with 14-3-3\(\tau\) GST fusion proteins upon phorbol ester stimulation of Jurkat-TAg cells (Fig. 3A). Upon stimulation of cells with phorbol ester for 5 and 10 min, respectively, an approximately 4- and 10-fold enhancement of 14-3-3\(\tau\) binding to PKC\(\mu\) was observed (Fig. 3A). Control immunoprecipitation of PKC\(\mu\) performed in parallel from aliquots (20 \times 10^6 cells) of the culture verified approximately equal amounts of PKC\(\mu\) in each group (Fig. 3A, lower panel). Activation of PKC\(\mu\) by phorbol ester treatment of cells was assessed by in vitro autophosphorylation of immunoprecipitates (Fig. 3A, middle panel). This revealed in accordance with earlier findings (20) a moderate stimulation of kinase activity by phorbol ester, which is also evident from a shift toward slower migrating bands (Fig. 3A, middle and lower panels). Enhanced binding of 14-3-3\(\tau\) to phosphorylated PKC\(\mu\) explains its relatively weak binding to PKC\(\mu\) isolated from untreated SF158 cells (Fig. 1A) that displays only a low basal PKC\(\mu\) activity. Association of 14-3-3\(\tau\) with in vivo activated PKC\(\mu\) was further demonstrated by Far

\(^{2}\) F. J. Johannes and T. Herget, unpublished observations.
Western analysis, where binding of 14-3-3γ to PKCμ was probed with 14-3-3γ GST fusion proteins and subsequent detection by anti-GST antibodies. Although upon cellular stimulation by PdBu PKCμ was present in equal amounts in immunoprecipitates (Fig. 3B, right panel), detection of PKCμ with the 14-3-3 probe was only possible upon preactivation of PKCμ (Fig. 3B, left panel).

Binding of 14-3-3γ to PKCμ is dependent on endogenous kinase activity. A kinase dead PKCμ mutant, PKCμK612W (27, 39) displaying no detectable autophosphorylation (Fig. 4, upper panel) was tested for potential precipitation by 14-3-3γ GST fusion proteins. As shown in Fig. 4, upon overexpression of the PKCμK612W mutant, no detectable autophosphorylation and subsequently no precipitation by 14-3-3γ was detectable. In contrast, PKCμ wild type and a pleckstrin homology domain deletion mutant, which has been previously shown to exert constitutive kinase activity (40), were shown to be efficiently precipitated by 14-3-3γ GST proteins (Fig. 4, upper panel). These data provide further evidence that 14-3-3γ association requires autophosphorylation of PKCμ. In an independent approach to scrutinize phosphorylation dependence of 14-3-3γ binding, PKCμ immunoprecipitates from Sf158 cell were in
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**Fig. 3. Enhanced 14-3-3\(\tau\) binding to activated PKC\(\mu\).** A, phorbol ester stimulation enhances PKC\(\mu\)-14-3-3 interaction. Jurkat-TAg cells were stimulated with phorbol ester (100 nM) for the time indicated, and lysates were prepared. PKC\(\mu\) was either immunoprecipitated by a polyclonal PKC\(\mu\) antiserum or precipitated by 14-3-3-\(\tau\)-GST fusion protein. GST proteins served as negative control. To verify in vitro activation by PdBu, aliquots of the lysates were immunoprecipitated by an anti-PKC\(\mu\) and subjected to in vitro autophosphorylation and autoradiography (middle panel). PKC\(\mu\) was visualized by Western blot analysis (lower panel). B, Far Western analysis of 14-3-3\(\tau\)-PKC\(\mu\) interaction. Jurkat-TAg cells were stimulated with 100 nM PdBu, and PKC\(\mu\) was immunoprecipitated and subjected to SDS-PAGE as described. Detection was carried out upon preincubating blots with 14-3-3\(\tau\) GST over-night with an anti-GST antibody (left panel) or by anti-PKC\(\mu\) mAb (right panel) as described under "Experimental Procedures."

**Fig. 4. 14-3-3\(\tau\) binding to PKC\(\mu\) is dependent on PKC\(\mu\) kinase activity.** The PKC\(\mu\) kinase dead mutant PKC\(\mu\)K612R, a pleckstrin homology domain deletion mutant (PKC\(\mu\)DEL), PKC\(\mu\) wild type (PKC\(\mu\)WT), and the respective vector control were transfected in 293 cells and immunoprecipitated (IP) with a PKC\(\mu\)-specific antisera (right lanes) or precipitated with 14-3-3\(\tau\) GST fusion proteins. Both PKC\(\mu\) precipitates were subjected to in vitro autophosphorylation and exposed to autoradiography upon SDS-PAGE (upper panel) and Western blot analysis (lower panel).

**Fig. 5. 14-3-3\(\tau\) binding to PKC\(\mu\) is phosphatase-sensitive.** PKC\(\mu\) immunocomplexes from Sf158 cells were in vitro autophosphorylated. PKC\(\mu\) was eluted from the protein G beads by incubating with immunizing peptide and subjected to phosphatase 2A treatment for the indicated times. Aliquots were removed, subjected to direct SDS-PAGE and immunoblot analysis (middle panel) followed by autoradiography (top panel) or precipitated using an 14-3-3\(\tau\)-GST fusion protein (bottom panel). 14-3-3\(\tau\) GST precipitates were subjected to SDS-PAGE and PKC\(\mu\) was detected by immunoblotting with a PKC\(\mu\)-specific rabbit antiserum.

*not affect PKC\(\mu\) protein levels (Fig. 5, middle panel). 14-3-3\(\tau\) Inhibits PKC\(\mu\) Kinase Activity in Vitro and in Vivo—14-3-3 binding to phosphorylated target proteins has been shown to modify cellular responses. For example the 14-3-3-mediated sequestration of the proapoptotic factor Bad, upon its serine phosphorylation by AKT/PKB, destroys the Bad-Bcl-2 complex and thus modifies the apoptotic response of affected cells (14–16). As 14-3-3\(\tau\) binds to serine phosphorylated PKC\(\mu\) (Fig. 4), a similar sequestration mechanism could occur. As a consequence, a reduction of PKC\(\mu\) kinase activity would be conceivable. Therefore, we tested whether the presence of 14-3-3\(\tau\) interferes with PKC\(\mu\) kinase activity. Purified PKC\(\mu\) from Sf158 cells (25) was subjected to in vitro kinase assays in the presence of various amounts of 14-3-3\(\tau\) GST fusion protein (Fig. 6, top panel). PKC\(\mu\) autophosphorylation was substantially inhibited already at a concentration of 1 \(\mu\)M 14-3-3\(\tau\) GST, and a complete inhibition was noted at approximately 20 \(\mu\)M of 14-3-3\(\tau\) GST (Fig. 6, top panel). The GST control protein did not affect PKC\(\mu\) autophosphorylation up to a concentration of 20 \(\mu\)M (Fig. 6, top panel). Autophosphorylation was also not affected in the presence of the same molar concentrations of a typical substrate-like syntide 2 (Ref. 25 and data not shown). These findings point to a specific inactivation of PKC\(\mu\) kinase upon 14-3-3\(\tau\) binding, which was corroborated by analysis of substrate phosphorylation. Similar as shown for the autophosphorylation, a quantitative inhibition of PKC\(\mu\) substrate phosphorylation was obtained in the presence of 20 \(\mu\)M 14-3-3\(\tau\) GST. A quantitative analysis of inhibition of PKC\(\mu\) autophosphorylation activity revealed an IC\(_{50}\) of approximately 4 \(\mu\)M (Fig. 6, bottom panel) for autophosphorylation and substrate phosphorylation alike. Phosphopeptide analysis of purified recombinant PKC\(\mu\) revealed 10 distinct peptides indicating phosphorylation sites. Therefore in the experiment shown in Fig. 6, inhibition of autophosphorylation activity largely reflects other than the 14-3-3\(\tau\) binding sites. Moreover, because at the position of GST 14-3-3\(\tau\), no bands were detectable in autoradiographs of SDS gels, the data further show that 14-3-3\(\tau\) is not phosphorylated by PKC\(\mu\) in vitro (Fig. 6, top panel).**
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40% reduction of PKC\(\mu\) kinase activity was revealed upon transfection of 14-3-3r in both assays, PKC\(\mu\) autophosphorylation as well as aldolase phosphorylation (Fig. 7, upper panels).

**DISCUSSION**

In this study, we identify PKC\(\mu\) as a novel 14-3-3r interacting protein and show that PKC\(\mu\) kinase activity is negatively regulated by 14-3-3r. The specificity of PKC\(\mu\)/14-3-3r interaction and its relevance is evident from (i) identification of the binding site in the C1 regulatory domain of PKC\(\mu\) containing serine motifs for autophosphorylation and 14-3-3r binding, (ii) a requirement of autophosphorylation for efficient 14-3-3r binding, and (iii) a highly effective down-regulation of kinase activity upon 14-3-3r binding in cell free assays and intact cells.

14-3-3r binding to several signal transducers (7–10) including PKC isotypes (18, 41, 42) has been reported, but controversial data exist as to the functional role of these interactions (7, 8, 41). Of relevance to the findings reported here, 14-3-3r has been described to inhibit PKC\(\theta\) regulated interleukin-2 expression in T cells by preventing its translocation to the membrane (18). Together with other studies, in which binding of 14-3-3r to the phosphoinositide 3-kinase (9) and to dictyostelium myosin II heavy chain kinase (41) was also found to cause inhibition of the respective enzymatic activities, a more general function of 14-3-3r as a negative regulator of signal transduction pathways can be assumed.

Activation of conventional and novel PKC isotypes typically occurs by binding of second messengers like diacylglycerol or phorbol ester to the C1 region (28, 30–32). The C1 region further serves as a binding region for regulatory proteins, as has been shown for the atypical PKC\(\lambda\) and \(\zeta\) (33, 34). The fact that regulatory lipids and proteins can bind within the same region necessitated precise identification of the binding site of 14-3-3r in PKC\(\mu\).

Phosphoserine binding motifs for 14-3-3 proteins like RSXP and RXXpSXP have been identified by extensive screening using peptid libraries (36). These motifs are present and functional in several already known 14-3-3 binding proteins including PKC\(\varepsilon\) and PKC\(\gamma\) (36). In contrast, a novel motif has been identified in Cbl (37), displaying RX\(_1\)pSX\(_2\)pS\(_3\), which differs basically from the above motif by absence of prolines. A motif similar to the latter containing one serine (RSLS\(_1\)pS\(_2\)pS\(_3\)VE), mediating the binding to 14-3-3r, has been identified in the phosphatase protein-tyrosine phosphatase 1 (43). Two consensus sequences matching the Cbl-derived consensus motif were found to comprise two spatially related potential 14-3-3r binding regions within the PKC\(\mu\) C1 regulatory domain, located between amino acids 80–340 (Fig. 2A). The mutational analyses performed here provide direct evidence for the involvement of both the serine 205/208 (RRLS\(_{1,2}\)S\(_{3}\)SL) and serine 219/223 (IRTS\(_{1,2}\)AE\(_{1}\)SLST) motif in 14-3-3r binding, as mutation of only one motif retained, in each case, 14-3-3r binding to PKC\(\mu\), whereas the simultaneous mutation of both motifs nearly completely abrogated 14-3-3r binding (Fig. 2C). These findings suggest that PKC\(\mu\) uses a similar serine-based motif for 14-3-3r binding as Cbl (37). Of note, we obtained evidence that both of these serine motifs (Ser-219/223) serve as autophosphorylation sites of PKC\(\mu\), which is in accordance with a requirement of phosphoserines for 14-3-3r binding. This is underlined by the finding that 14-3-3r binding to PKC\(\mu\) is dramatically enhanced upon phorbol ester stimulation of PKC\(\mu\) autophosphorylation. Similar data have been reported for the interaction of 14-3-3r and Cbl, which also requires serine phosphorylation of Cbl for efficient 14-3-3r binding (37). It is further of interest to note that the two 14-3-3r binding motifs are located within the 80-amino acid spacer (19) between the two zinc fingers of PKC\(\mu\). Thus, the 14-3-3r binding site is spatially separated from the lipid...
messeion/porpholester binding site located within the cytosine-rich zinc fingers (31, 32). Both the distinct sites used for lipid and 14-3-3 binding and the prerequisite of lipid messengers-dependent autophosphorylation for efficient 14-3-3 binding clearly favor a model of a sequential action of these two PKCm regulators. We propose that 14-3-3m acts as an allosteric inhibitor of already activated PKCm rather than a competitor of activating lipid messengers. Binding of 14-3-3m to PKCm appears of functional significance as shown by a highly efficient in vitro inhibition of PKCm by micromolar concentrations of 14-3-3m (Fig. 6) and a significant reduction of PKCm activity in vivo upon moderate overexpression of 14-3-3m in T cells (Fig. 7).

In conclusion, we propose that 14-3-3m plays a role as a negative feedback regulator of PKCm, ensuring a tight control of kinase activity. Upon binding of activating second messengers to the zinc fingers, PKCm undergoes autophosphorylation and exerts enhanced kinase activity toward appropriate substrates. Serine phosphorylation of defined regions of the regulatory domain of PKCm in turn creates a high affinity binding site for 14-3-3m, which subsequently down-regulates PKCm kinase activity. As 14-3-3m is a T cell-specific isoform of this family of adapter/regulator proteins and PKCm is not only highly expressed in T cells but also participates in T cell antigen-receptor-mediated signal events, the biological significance of the PKCm-14-3-3 interaction becomes apparent.

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