Structural requirements for localization and activation of protein kinase C \( \mu \) (PKC\( \mu \)) at the Golgi compartment

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We here describe the structural requirements for Golgi localization and a sequential, localization-dependent activation process of protein kinase C \( \mu \) (PKC\( \mu \)) involving auto- and transphosphorylation. The structural basis for Golgi compartment localization was analyzed by confocal microscopy of HeLa cells expressing various PKC\( \mu \)-green fluorescent protein fusion proteins costained with the Golgi compartment–specific markers p24 and p230. Deletions of either the NH\(_2\)-terminal hydrophobic or the cysteine region, but not of the pleckstrin homology or the acidic domain, of PKC\( \mu \) completely abrogated Golgi localization of PKC\( \mu \). As an NH\(_2\)-terminal PKC\( \mu \) fragment was colocalized with p24, this region of PKC\( \mu \) is essential and sufficient to mediate association with Golgi membranes. Fluorescence recovery after photobleaching studies confirmed the constitutive, rapid recruitment of cytosolic PKC\( \mu \) to, and stable association with, the Golgi compartment independent of activation loop phosphorylation. Kinase activity is not required for Golgi complex targeting, as evident from microscopical and cell fractionation studies with kinase-dead PKC\( \mu \) found to be exclusively located at intracellular membranes. We propose a sequential activation process of PKC\( \mu \), in which Golgi compartment recruitment precedes and is essential for activation loop phosphorylation (serines 738/742) by a transacting kinase, followed by auto- and transphosphorylation of NH\(_2\)-terminal serine(s) in the regulatory domain. PKC\( \mu \) activation loop phosphorylation is indispensable for substrate phosphorylation and thus PKC\( \mu \) function at the Golgi compartment.

Introduction

The PKCs comprise a family of intracellular serine/threonine kinases which are expressed in a cell type–specific pattern. PKCs have been shown to be involved in signal transduction of a wide range of biological responses including changes in cell morphology, proliferation, and differentiation (Toker, 1998; Black, 2000). Typically, PKCs are lipid-activated kinases that can be distinguished by different lipid-dependent activation modes.

Two novel lipid-activated kinases, sharing significant homology to PKCs as well as to calmodulin-dependent kinases that can be distinguished by different lipid-dependent activation modes.

Abbreviations used in this paper: GFP, green fluorescent protein; PH, pleckstrin homology.
Matthews et al., 2000b). In accordance with these studies is the finding that PKC\(\mu\) is recruited together with the tyrosine kinase Syk and phospholipase C\(\gamma\) to the B cell receptor complex upon B cell receptor stimulation and negatively regulates PLC\(\gamma\) activity (Sidorenko et al., 1996). Our previous studies further suggested a function in antiapoptotic signaling (Johannes et al., 1998). Probably the most intriguing finding is the Golgi compartment localization of PKC\(\mu\) and involvement in constitutive transport processes in epithelial cells (Prestle et al., 1996). Indeed, very recent data point to a fundamental importance of PKC\(\mu\) in G protein–mediated regulation of Golgi organization (Jamora et al., 1999) and initiation of vesicular transport processes at the TGN (Liljedahl et al., 2001).

In accordance with cell type–specific functions, PKC\(\mu\)/PKD location and activation appears to differ in different cell types and may involve different upstream regulators, including conventional PKCs (Zugaza et al., 1996; Matthews et al., 2000b). For example, PKD activation by exogenous stimuli is mediated via a PKC-dependent pathway in murine mast cells and B cells (Matthews et al., 2000b). Localization studies in the lymphocytic cell line A20 indicated a reversible, antigen receptor–triggered membrane translocation independent of the PKD PH domain (Matthews et al., 2000a).

We have performed the present study to analyze in detail structural requirements for constitutive PKC\(\mu\) localization at the Golgi compartment using the epithelial-derived HeLa cell line. We show that the NH\(_2\)-terminal domain is essential for localization of PKC\(\mu\) at the Golgi compartment and that intrinsic kinase activity is not necessary for this localization. Golgi complex attachment of PKC\(\mu\) is required for phosphorylation of activation loop serines 738/742 and subsequent NH\(_2\)-terminal phosphorylation at different serines. Overexpression of PKC\(\mu\)–green fluorescent protein (GFP) mutants comprised of the Golgi localization domains only or of a kinase-dead variant, both acting as dominant negative inhibitors of endogenous PKC\(\mu\) function, severely affected PKC\(\mu\) localization, showing in addition to Golgi localization a localization in/at vesicle-like structures.

## Results

### Characterization of PKC\(\mu\)–GFP expression constructs

To analyze cellular localization of PKC\(\mu\) in living cells, a set of plasmids was constructed expressing PKC\(\mu\) mutants as COOH-terminal GFP fusion proteins. The mutants used in this study are schematically shown in Fig. 1 (for details see Materials and methods). Mutants were transiently expressed in HEK293 cells to check expression by Western blot analyses and activity pattern by in vitro autophosphorylation of PKC\(\mu\) immunoprecipitates (Fig. 2). GFP-tagged wild-type PKC\(\mu\) and the kinase-dead PKC\(\mu\)\(_{K612W}\)–GFP mutant migrated with the expected relative molecular weight of \(~140\) kD (Fig. 2, top), showing either basal autophosphorylation (wild-type) or a complete lack of kinase activity (PKC\(\mu\)\(_{K612W}\)–GFP, Fig. 2, bottom) as shown previously for wild-type PKC\(\mu\) and the K612W kinase-dead mutant (Johannes et al., 1998). Several deletion mutants lacking either the PH domain (PKC\(\mu\)\(_{A78C}\)–GFP), the cysteine finger region (PKC\(\mu\)\(_{A1C}\)–GFP, PKC\(\mu\)\(_{A2C}\)–GFP, PKC\(\mu\)\(_{A3CD}\)–GFP), or the acidic region (PKC\(\mu\)\(_{AAD}\)–GFP) were expressed and analyzed in the same way by immunoprecipitation and autophosphorylation (Fig. 2). Interestingly, deletion of the acidic domain, which was predicted to be involved in regulation of PKC\(\mu\) kinase activity (Gschwendt et al., 1997), resulted in enhancement of constitutive kinase activity (PKC\(\mu\)\(_{AAD}\)–GFP, Fig. 2, bottom) as shown previously for wild-type PKC\(\mu\) and the K612W kinase-dead mutant (Johannes et al., 1998). Several deletion mutants lacking either the first 78 amino acids, representing the hydrophobic region (PKC\(\mu\)\(_{A1.78}\)–GFP), or the 340 NH\(_2\)-terminal amino acid rich regions were deleted. PKC\(\mu\)\(_{A2C}\)–GFP and PKC\(\mu\)\(_{A3CD}\)–GFP lack the first or the second cysteine-rich region. In PKC\(\mu\)\(_{A3CD}\)–GFP both cysteine rich regions were deleted. PKC\(\mu\)\(_{A1.78}\)–GFP lacks the hydrophobic region (M1–78), whereas PKC\(\mu\)\(_{AAD}\)–GFP contains only the hydrophobic regions of wild-type PKC\(\mu\). PKC\(\mu\)\(_{A3CD}\)–GFP consists of 325 NH\(_2\)-terminal amino acids. PKC\(\mu\)\(_{A3CD}\)–GFP contains the PH domain (V409–T552). The acidic domain includes amino acids E336–D391. All mutants used in this study were expressed as COOH-terminal GFP fusion proteins as schematically indicated. Deleted domains are indicated by dashed lines. Phosphorylatable serine residues are indicated in wild-type PKC\(\mu\)-GFP. AD, acidic domain; CRD, cysteine-rich domain; WT, wild-type. K612W indicates a point mutation in the ATP-binding site.

![Figure 1. Schematic view of the PKC\(\mu\)-GFP mutants used in this study.](image)

The hydrophobic region (amino acids M1–D86) and the cysteine-rich region (CI and CII; amino acids H147–C196 and amino acids H271–C320) are located in the NH\(_2\)-terminal domain of PKC\(\mu\). The PH domain (V409–T552) is located between CI and the COOH-terminal kinase domain. PKC\(\mu\)\(_{A78C}\)–GFP lacks the PH domain, whereas PKC\(\mu\)\(_{A1C}\)–GFP and PKC\(\mu\)\(_{A2C}\)–GFP lack the first or the second cysteine-rich region. In PKC\(\mu\)\(_{A3CD}\)–GFP both cysteine rich regions were deleted. PKC\(\mu\)\(_{A1.78}\)–GFP lacks the hydrophobic region (M1–78), whereas PKC\(\mu\)\(_{AAD}\)–GFP contains only the hydrophobic regions of wild-type PKC\(\mu\). PKC\(\mu\)\(_{A3CD}\)–GFP consists of 325 NH\(_2\)-terminal amino acids. PKC\(\mu\)\(_{A3CD}\)–GFP contains the PH domain (V409–T552). The acidic domain includes amino acids E336–D391. All mutants used in this study were expressed as COOH-terminal GFP fusion proteins as schematically indicated. Deleted domains are indicated by dashed lines. Phosphorylatable serine residues are indicated in wild-type PKC\(\mu\)-GFP. AD, acidic domain; CRD, cysteine-rich domain; WT, wild-type. K612W indicates a point mutation in the ATP-binding site.
analyzed by confocal microscopy. For PKC
right lane, top and bottom; see Fig. 6 A).

nase domain, showed no autophosphorylation (Fig. 2,
protein. As expected, these fragments, which lack the ki-
1–325) were each separately expressed as a GFP fusion

PH domain and the NH

control for localization and phosphorylation studies, the

Figure 2. Expression and in vitro phosphorylation of PKC\(\mu\)-GFP
fusion proteins. HEK293 cells were transfected with the indicated
con structs. 40 h after transfection cells were lysed and PKC\(\mu\)-GFP
was immunoprecipitated using an anti-GFP antibody and subjected
to Western blotting (top) and in vitro autophosphorylation (bottom).
Shown are autoradiographs after overnight exposition.

acids (PKC\(\mu\)\(_{1–340}\)-GFP), largely covering the zinc-finger
region, were constructed and analyzed for protein expres-
sion as well as for kinase activity. PKC\(\mu\)\(_{1–78}\)-GFP displayed
a weak reduction in autophosphorylation efficiency,
whereas enzyme activity of PKC\(\mu\)\(_{1–340}\)-GFP was comparable
to wild-type kinase activity (Fig. 2, bottom). As a
control for localization and phosphorylation studies, the
PH domain and the NH\(_2\)-terminal domain (amino acids
1–325) were each separately expressed as a GFP fusion
protein. As expected, these fragments, which lack the ki-
nase domain, showed no autophosphorylation (Fig. 2,
right lane, top and bottom; see Fig. 6 A).

Identification of the binding domain mediating Golgi
membrane association of PKC\(\mu\).

To specify determinants and functional activities relevant for
subcellular localization, wild-type PKC\(\mu\)-GFP and kinase-
dead PKC\(\mu\)\(_{K612W}\)-GFP were expressed in HeLa cells and an-
yalyzed by confocal microscopy. For PKC\(\mu\)-GFP a diffuse
signal was revealed throughout the cell, but a clear enrich-
ment in perinuclear structures was noted (Fig. 3 A, top
rows). Staining of endogenous PKC\(\mu\) in HEK293 cells with
PKC\(\mu\)-specific antibodies verified that the PKC\(\mu\)-GFP fusion
protein does not differ in localization from endogenous
PKC\(\mu\) (Fig. 3 D). These data are in accordance with previ-
ous studies (Prestle et al., 1996; Liljedahl et al., 2001). As a
Golgi compartment–specific marker served p24, a vesicle
and Golgi compartment–associated protein (Gommel et al.,
1999). P24 appeared perinuclear and in vesicular structures
throughout the cell (Fig. 3, middle). Partial colocalization of
PKC\(\mu\)-GFP with p24-positive compartments was verified
by overlay (Fig. 3 A, right). Further, costaining with anti-
bodies specific for p230 (Kjer-Nielsen et al., 1999) and
GM130 (unpublished data), independent markers of the trans-
and cis Golgi network, respectively, verified Golgi
compartment association of PKC\(\mu\) (Fig. 3 A). Interestingly,
kinase-dead PKC\(\mu\)\(_{K612W}\)-GFP, although still partially colo-
ralized with the Golgi markers at a perinuclear region, was
found in structures dispersed throughout the cell with ap-
pearance of long tubuli and large vesicular structures (Fig. 3
A, see enlargements). Of note, these PKC\(\mu\)-positive structures
did no longer costain with any of the three applied
Golgi markers (Fig. 3 A, right).

Overexpression of a PKC\(\mu\) mutant lacking kinase activity
was shown recently to disrupt normal Golgi morphology,
pointing to an essential role of kinase activity in maintaining
Golgi structure (Liljedahl et al., 2001). Further corroborat-
ing this finding, we can show that not only expression of a
kinase-dead PKC\(\mu\)\(_{K612W}\)-GFP, but also of the Golgi binding
NH\(_2\)-terminal fragment PKC\(\mu\)\(_{1–325}\)-GFP provoked changes
in normal PKC\(\mu\) localization, with signs of tubulation and/
or large vesicle formation of PKC\(\mu\)-positive structures (Fig. 3
A). As shown in Fig. 3, A and B, suggesting a dominant negative action of
both constructs on endogenous wild-type PKC\(\mu\). Using
PKC\(\mu\)\(_{K612W}\)-GFP, we analyzed whether the observed mor-
phological changes and segregation of PKC\(\mu\) from p24-pos-
itive structures might also be associated with a relocation of
PKC\(\mu\) to different membrane compartments or other intra-
cellular structures. Costaining of PKC\(\mu\)\(_{K612W}\)-GFP with vari-
ous vesicular markers was analyzed. To this end, we could
not detect any colocalization with EEA-1, Rab5, and Rab8
as endosomal markers. Furthermore, no colocalization with
TGN38, BIP, Caveolin-1, Clathrin, or Lamp1 was detect-
able (unpublished data).

Although PH domains are frequently responsible for
membrane association (Falasca et al., 1998), the deletion
mutant of PKC\(\mu\) showed no apparent differences in intra-
cellular localization from the wild-type, and Golgi struc-
ture appeared normal (Fig. 3 B). Moreover, analysis of the
isolated PH domain expressed as a GFP fusion protein
(PKC\(\mu\)\(_{PH}\)-GFP) revealed complete segregation from p24
staining and cytosolic/nuclear location (Fig. 3 B). Contrary
to the expectations, these data show that the PH domain is
apparently not required for PKC\(\mu\) association with the
Golgi compartment. Likewise, a deletion of the acidic
domain of PKC\(\mu\) (PKC\(\mu\)\(_{AD}\)-GFP) displayed enhanced basal
kinase activity (Fig. 2) and did not interfere with Golgi
compartment localization of PKC\(\mu\) (Fig. 3 B). Together,
these data suggest that the PH and the acidic domain play
a role in negative regulation of kinase activity rather than
in localization.

The deletion of NH\(_2\)-terminal regions affected Golgi
compartment localization of PKC\(\mu\). As shown in Fig. 3
C, expression of PKC\(\mu\)-GFP mutants lacking either 78
(PKC\(\mu\)\(_{1–78}\)-GFP) or 340 (PKC\(\mu\)\(_{1–340}\)-GFP) NH\(_2\)-terminal
amino acids led to a complete cytosolic distribution of
PKC\(\mu\). No colocalization with p24-staining structures was
detectable (Fig. 3 C). Deletion of the complete kinase
domain did not affect Golgi compartment localization (unpub-
lished data). An NH\(_2\)-terminal PKC\(\mu\) fragment (PKC\(\mu\)\(_{1–86}\-
GFP) was found to be located completely in the cytosol,
whereas the entire NH$_2$-terminal region covering both cysteine fingers (PKC$\mu$-1–325-GFP) showed partial colocalization with p24 staining structures (Fig. 3 C). These data already suggest that the NH$_2$-terminal hydrophobic region itself is not sufficient, but might be required in concert with the cysteine-rich domains to mediate Golgi complex association of PKC$\mu$. The supposed important role of the cysteine-rich region was verified by expressing the respective deletion mutants. Deletion of either the second cysteine finger (PKC$\mu$-CII-GFP) or the complete cysteine rich region (PKC$\mu$-CRD-GFP); each resulted in cytosolic and nuclear distribution. In the case of PKC$\mu$-CII-GFP, an exclusive nuclear localization was detected (Fig. 3 C). These data identify the NH$_2$-terminal hydrophobic domain and the adjacent zinc finger regions, together covering amino acids 1–325, as the Golgi compartment binding domain of PKC$\mu$ and demonstrate that intrinsic PKC$\mu$ kinase activity is not required for association with Golgi membranes.

**Activation loop phosphorylation of PKC$\mu$ requires localization at the Golgi compartment**

The data described above show the importance of the PKC$\mu$ NH$_2$-terminal region for Golgi complex localization. As kinase-dead mutants of PKC$\mu$-GFP remain associated with Golgi region (Fig. 3 A) and other intracellular membranes (Liljedahl et al., 2001), and complete inhibition of kinase activity of wild-type PKC$\mu$-GFP by H89 did not result in a relocation to the cytosol (unpublished data), it appears that autophosphorylation is not required for membrane recruitment of PKC$\mu$. However, as upstream kinases appear to be involved in PKC$\mu$ activation, it was necessary to analyze in detail individual phosphorylation sites in PKC$\mu$ with recurrent methods. The data presented in Figure 3 provide evidence that PKC$\mu$ is recruited to the Golgi compartment in a phosphorylation-dependent manner.
spect to their role in Golgi localization and activation of the kinase.

To correlate localization with the phosphorylation state, the various PKC \( \mu \)-GFP constructs used in this study were expressed in HEK293 cells and monitored for expression level as well as for in vivo PKC \( \mu \) phosphorylation using PKC \( \mu \) phosphosite-specific antibodies. As expected, constitutive PKC \( \mu \) kinase activity was detected by pSer910-specific antibodies (Fig. 4 A, middle). As a negative control, PKC \( \mu \)K612W-GFP was included. No autophosphorylation was detectable with pSer910 antibodies. The pSer738/742 antibody detected the PKC \( \mu \)K612W-GFP mutant, pointing to PKC \( \mu \) kinase independent, constitutive phosphorylation of this site by an upstream kinase.

Deletion mutants of the PH domain, the acidic region, or deletions of either the first, second, or both cysteine-rich regions showed phosphorylation of Ser910. As the NH2-terminal deletion mutants are cytosolic, while the former two are Golgi bound (Fig. 3), Ser910 autophosphorylation appears localization independent. In contrast, only the PKC \( \mu \)ΔPH-GFP and the PKC \( \mu \)ΔAD-GFP mutant exerted significant phosphorylation at Ser738/742 (Fig. 4 A, bottom), whereas deletion mutants localized in the cytosol or in the nucleus show only weak phosphorylation at Ser738/742.

Five phosphorylation sites in PKC \( \mu \)/PKD have been described recently (Vertommen et al., 2000). As well as three phosphorylation sites in the COOH-terminal region, two phosphorylation sites at Ser205 (equivalent with Ser203 in PKD) and Ser249 (Ser255 in PKD) were reported. The NH2-terminal phosphorylation sites are likely to contribute to PKC \( \mu \) activation and/or regulation of PKC \( \mu \).

Figure 4. Localization of PKC\( \mu \)-GFP at the Golgi compartment is required for phosphorylation of serines 738/742. (A) Differential phosphorylation of PKC\( \mu \)-GFP deletion mutants. HEK293 cells were transfected with the indicated plasmids. Expression of the fusion proteins was monitored by Western blot analysis using an anti-GFP antibody. PKC\( \mu \)-GFP phosphorylation was measured by phospho-specific antibodies recognizing phosphorylated Ser738/742 and Ser910. (B) Characterization of PKC\( \mu \)-GFP phosphorylation mutants. (C) PKC\( \mu \)K612W-GFP colocalizes with the Golgi compartment-specific marker p24. (D) PKC\( \mu \)-GFP with phosphorylated activation loop is exclusively recovered in the organelle fraction. HEK293 cells were transfected with PKC\( \mu \)-GFP or PKC\( \mu \)K612W-GFP and separated into soluble proteins from organelles structures sedimenting at 100,000 g. Western blot analysis was performed by anti-GFP or phosphorylation-specific antibodies.
To further determine phosphorylation-dependent influence on Golgi complex localization of PKC\(\mu\), all predicted phosphorylation sites (Ser\(_{910}\), Ser\(_{738/742}\), Ser\(_{249}\), Ser\(_{205}\)) were mutated to alanine and characterized for activation loop and COOH-terminal phosphorylation. As shown in Fig. 4 B by Western blot analysis using phosphosine-specific antibodies, mutations of NH\(_2\)-terminal serine residues (S205A; S249A) did not influence phosphorylation sites on Ser\(_{738/742}\) or Ser\(_{910}\). Mutants of either Ser\(_{738/742}\) or Ser\(_{910}\) did affect detection by the respective antibodies, but did not influence other phosphorylation sites. Mutants were further analyzed for intracellular colocalization with p24. As shown for the Ser\(_{738/742}\)Ala double mutation, Golgi complex localization (Fig. 4 C) was not affected, indicating that phosphorylation of these activation loop sites is not a prerequisite for Golgi complex localization, but instead suggests that activation loop phosphorylation requires Golgi complex localization of PKC\(\mu\). All other phosphorylation site mutants analyzed showed similar localization as wild-type PKC\(\mu\)-GFP (unpublished data).

In addition, intracellular distribution of PKC\(\mu\)-GFP and PKC\(\mu\)K612W-GFP was analyzed by biochemical methods. As shown in Fig. 4 D, after separation of soluble proteins from organelles and structures phosphorylation of PKC\(\mu\) in the activation loop was exclusively recovered in the organelle fraction, whereas PKC\(\mu\) was recovered in both fractions (Fig. 4 D). Phosphorylation of Ser\(_{910}\) was not affected by intracellular localization of PKC\(\mu\), as cytosolic and particular fractions contain approximately equal amounts of this phosphorylated species of PKC\(\mu\).

Golgi region–localized PKC\(\mu\) is recruited from the cytosolic pool and is independent of activation loop phosphorylation. As shown by FRAP experiments (Fig. 5), cytosolic PKC\(\mu\)-GFP and PKC\(\mu\)S738/742A-GFP rapidly translocate to the Golgi region. Upon bleaching of Golgi region–localized PKC\(\mu\)-GFP and PKC\(\mu\)S738/742A-GFP within the circled area (Fig. 5 A, right), specific GFP fluorescence disappears leaving only the cytosolic and vesicular pool of PKC\(\mu\) within the cell (Fig. 5 A, middle). Within a 15-min period, cytosolic PKC\(\mu\)-GFP and PKC\(\mu\)S738/742A-GFP are rapidly recruited to the Golgi region (Fig. 5 A, right). As illustrated in Fig. 5 B by the reverse experiment, i.e., bleaching of cytosolic PKC\(\mu\)-GFP and PKC\(\mu\)S738/742A-GFP, respectively, a decay of Golgi region–specific PKC\(\mu\)-GFP and PKC\(\mu\)S738/742A-GFP staining was found (Fig. 5 B). Interestingly, in addition to an assumed cytosolic redistribution, which cannot be readily detected because of dilution of the fluorescence signal, we observed a redistribution of PKC\(\mu\)-GFP, in particular structures outside of the defined region (Fig. 5 B, enlargements). Of note, no difference between wild-type and activation loop mutant PKC\(\mu\)-GFP was observed. These data clearly indicate a translocation of cytosolic PKC\(\mu\) to the Golgi region independent of its activation loop phosphorylation and point to a constitutive attachment of PKC\(\mu\) to Golgi membranes.

![Figure 5](https://example.com/figure5.png)

**Figure 5. Constitutive recruitment of PKC\(\mu\)S738/742A-GFP to the Golgi compartment.** (A) The Golgi pool of PKC\(\mu\)-GFP recovers rapidly after photobleach independent of activation loop phosphorylation. The outlined area in the prebleach image (left) was photobleached. Pictures were taken after the indicated times shown in the middle and right panels. (B) Constitutive association of PKC\(\mu\)-GFP with the Golgi compartment and membrane structures. Fluorescence outside of the marked region indicated in the prebleach image was eliminated by photobleaching. Note that the fluorescence intensity of PKC\(\mu\)-GFP at the Golgi region is saturated in all of the images to allow visualization of less bright structures. Cells were preincubated with cycloheximide (20 \(\mu\)g/ml) for 2 h.

**NH\(_2\)-terminal phosphorylation is a consequence of activation loop phosphorylation of PKC\(\mu\) at the Golgi compartment**

The above studies already suggested a multistep process of PKC\(\mu\) activation with auto- and transphosphorylation events for the COOH-terminal–located phosphorylation sites. To decipher the sequence of phosphorylation events leading to activation and regulation of PKC\(\mu\), we established an in vitro transphosphorylation assay using several NH\(_2\)-terminal PKC\(\mu\) domains expressed as GFP fusion proteins as substrates for PKC\(\mu\)-GFP. As shown in Fig. 6 A, the PKC\(\mu\)l-86-GFP domain could be efficiently phosphorylated by PKC\(\mu\)-GFP, whereas the PKC\(\mu\)l-325-GFP domain, as well as the PKC\(\mu\)K612W-GFP domain, were not phosphorylated by PKC\(\mu\)-GFP. According to published data, the phosphorylation site was predicted to be Ser\(_{205}\) within the 14-3-3 binding site (Hausser et al., 1999) or Ser\(_{249}\) predicted to be phosphorylated by an upstream kinase (Vertommen et al., 2000). To further analyze whether the above-described NH\(_2\)-terminal homologous transphosphorylation occurs in intact cells, PKC\(\mu\)l-325 was coexpressed with wild-type or mutated PKC\(\mu\)-GFP and analyzed by shift assays indicative of poten-
tial phosphorylation within this domain. As shown by Western blot analysis (Fig. 6 B), coexpression of PKCµ1-325 together with PKCµ-GFP led to the appearance of two bands at the expected size of the fragment. The slower migrating band of PKCµ1-325 represents the phosphorylated protein which is evident from coexpression of PKCµ1-325 with kinase-dead PKCµK612W-GFP, where only the faster migrating band appeared (Fig. 6 B, top). Conversely, coexpression of constitutively active PKCµA325-GFP led to the exclusive appearance of the slower migrating band, indicating strong transphosphorylation of the NH2-terminal fragment. Interestingly, coexpression of PKCµKCRD-GFP did not result in phosphorylation of PKCµ1-325. As shown above, this mutant lacks the Golgi localization domain and is therefore not phosphorylated at the activation loop Ser738/742. Accordingly, these findings suggest a stepwise activation by phosphorylation of Ser910 and Ser738/742 followed by NH2-terminal phosphorylation of PKCµ.

To confirm this sequential phosphorylation process, mutations in known phosphorylation sites (S205A, S249A, S738/S742A, S910A) were introduced in PKCµ-GFP, expressed, and analyzed by kinase assay for auto/trans- and substrate phosphorylation. Immunoprecipitates of PKCµS738/742A-GFP did not show detectable aldolase- or PKCµ1-325-GFP phosphorylation, whereas in the case of all other mutants, auto- and substrate phosphorylation was not affected (Fig. 6 C). These data indicate that activation loop phosphorylation on Ser738/742 is essential for transphosphorylation of NH2-terminal residues.

**Discussion**

In this study, we analyzed the structural basis for Golgi compartment localization of PKCµ in epithelial cells. Using a set of deletion mutants we can show by confocal microscopy...
that NH₂-terminal residues covering amino acids 1–325 constitute the Golgi compartment localization domain. Moreover, we show that phosphorylation of PKCμ is not required for binding to Golgi membranes but rather that phosphorylation of Ser738/742 requires Golgi localization. Our data further suggest a sequence of events in which transphosphorylation of NH₂-terminal epitopes occurs subsequent to activation loop phosphorylation, whereas autoprophosphorylation at Ser910 is independent of localization and of phosphorylation of the activation loop. The findings presented in this study are illustrated in a model shown in Fig. 7.

PKCμ is comprised of several structural domains which are putatively able to mediate membrane interactions, such as a hydrophobic NH₂-terminus and two cysteine-rich zinc finger regions, highly conserved among PKC members and shown to be involved in Golgi compartment localization of PKCe (Lehel et al., 1995), as well as a PH domain considered to mediate membrane association of proteins via binding to phosphatidylinositol phosphate (Harlan et al., 1994). Biochemical studies have recently shown that the hydrophobic region of PKD does not function as a genuine transmembrane domain (Jamora et al., 1999), which is underlined by our studies showing that the expression of the human homologous fragment does not, on its own, localize to membranes. However, the analysis of the various NH₂-terminal deletion mutants of PKCμ provide direct evidence that this region is, in concert with both zinc fingers involved in Golgi compartment localization of PKCμ, whereas the PH domain, unexpected from its functional relevance for PKCμ activation at the Golgi complex, is not involved. The functional importance of the NH₂-terminal region is further stressed by Golgi complex localization of overexpressed PKCμ1–325-GFP, resulting in a similar appearance of vesicular structures as expression of kinase-dead PKCμ (Fig. 3). This points to a dominant negative effect of this mutant by competition with endogenous PKCμ for binding to Golgi membranes and thus negatively affecting structure and potential functions in Golgi complex (Liljedahl et al., 2001).

The simultaneous requirement of the three subdomains within the NH₂-terminal regulatory region for PKCμ association with Golgi membranes points to the need for multiple interactions. In addition to potential hydrophobic interactions via the NH₂-terminus and lipid messenger binding to the zinc finger regions, protein–protein interactions of this PKCμ domain with integral or associated Golgi membrane proteins are likely to be involved. Although these Golgi membrane interaction partners of PKCμ have to be identified in further studies, the NH₂-terminal region is already known to serve as a binding domain for regulatory proteins. For example, 14-3-3 proteins can bind to PKCμ and negatively regulate its kinase activity (Hausser et al., 1999). Other proteins, such as the tyrosine kinase Btk and lipid PI4- and PI4-5 kinases, were also shown to be associated with PKCμ via the NH₂-terminal region (Nishikawa et al., 1998; Johannes et al., 1999). As the PI4-5 kinase does not associate with kinase-dead PKCμ, a role of phosphorylation-triggering association with this target protein was predicted (Nishikawa et al., 1998). From the studies presented here, for PKCμ binding to the Golgi region, an essential role of phosphorylation is ruled out, as evident e.g., from Golgi membrane localization of kinase-dead, kinase domain–deficient, and activation loop–deficient PKCμ. Accordingly, a role of PI4-5 kinase in serving as a Golgi region receptor of PKCμ appears very unlikely.

The PKCμ PH domain does not contribute to the localization at Golgi membranes. As deletion resulted in constitutive kinase activity, these data support a specific regulatory function of this domain (Iglesias and Rozengurt, 1998; Hausser et al., 2001) (Fig. 2). Of note, the PH domain has been shown to mediate the interaction with PKCδ, which is thought to play a role in PKCμ activation (Waldron et al., 1999). The participation of the PH domain of the murine PKCμ homologue, PKD, in function at the Golgi region during G-protein signaling events has been demonstrated previously (Jamora et al., 1999). Our data clearly indicate that the PKCμ PH domain serves a regulatory function, probably by coupling to upstream pathways and, in contrast to classical PH domains, does not mediate membrane localization.

Our data also shed light on the sequence of events leading to activation of PKCμ. We provide evidence that activation of PKCμ is a complex process involving auto- and trans-

![Diagram of PKCμ activation](Image)
phosphorylation events at Ser930 and Ser738/742, respectively, followed by phosphorylation of NH₂-terminal residues. The role of the NH₂-terminal phosphorylation is currently unclear. As it is performed through a homologous transphosphorylation event by activated PKCα (Fig. 6) its function might be in the generation of phosphopeptides mediating the binding of regulatory proteins such as 14-3-3 (Hauser et al., 1999) or of potential substrates such as PI kinases (Nishikawa et al., 1998). Within the domain between amino acids 200–250 a clustering of potential phosphorylation sites are located (12xSer, 4xThr). Therefore, it presently cannot be excluded that, dependent on the cellular context, different residues might be phosphorylated and thus may differentially influence activity of PKCα.

As celluarily expressed kinase-dead PKCα is phosphorylated on Ser738/742, these sides can be considered as transphosphorylation sites for an upstream kinase. This reasoning is supported by H89 inhibition of PKCα kinase, demonstrating selective inhibition of phosphorylation of Ser930 and not of Ser738/742 (unpublished data). Therefore, our data point to an H89-insensitive upstream kinase. According to published data and our own observations, PKCγ is activated by upstream PKCs (Zugaza et al., 1996). PKCγ and also PKCe were recently implicated in PKD activation (Waldron et al., 1999). PKCe has been located at the Golgi compartment and a role in Golgi region–specific functions was suggested previously (Lehel et al., 1995). The data presented here are in accordance with a participation of PKCe in Golgi region functions via activation of PKCα. In support of this, Golgi region localization domain mutants did not show phosphorylation on Ser738/742 (Fig. 4 A). On the other hand, activation loop mutants, similarly to wild-type PKCα, were localized at the Golgi region (Fig. 4 C). This reemphasizes a phosphorylation-independent localization of PKCα at the Golgi region and suggests PKCe as a candidate for an upstream kinase for activation loop phosphorylation of PKCα at the Golgi compartment.

Materials and methods

**Plasmid constructs and cell lines**

cDNA constructs containing wild-type and various mutant PKCα sequences in the pCDNA mammalian expression vector have been described previously (PKCαH147–C196, PKCαH147–C196, PKCαH147–C196, PKCαH147–C196, and PKCαH147–C196 (Johannes et al., 1998, 1999). Deletion of the C1 motif, amino acids H147–C196 (PKCαH147–C196); and the combination of both motifs (PKCαH147–C196) were generated by an overlap PCR using Taq-polymerase (MBI Fermentas). Site-specific mutations within PKCα-GFP resulting in single amino acids substitutions S205A, S249A, S738/742A, S910A) were performed by a PCR approach using the QuickChange site-directed mutagenesis system (Stratagene) according to the manufacturer’s instructions. The integrity of the PCR-amplified plasmids were verified by sequencing. Fig. 1 shows a scheme of the different mutants used in this study. The GFP-tagged wild-type and mutant PKCα expression plasmids were obtained by subcloning the respective PKCα coding sequence into the EcoRI-BamHI sites of the polylinker of the pEGFP-N1 vector from CLONTECH Laboratories, Inc. HeLa and HEK293 (American Type Culture Collection) were cultured in RPMI medium supplemented with 5% FCS.

**Antibodies and reagents**

Antibodies directed against phosphoSer930 and phosphoSer738/742 of PKD were purchased from NEB/Cell Signaling. p24-specific antibodies were provided by F. Wieland (University of Heidelberg, Heidelberg, Germany). Anti-GFP antibodies were obtained from Roche Diagnostics. Anti-p230 and anti-GM130 antibodies were purchased from Transduction Laboratories. Anti-PKCα rabbit antibody was obtained from Santa Cruz Biotechnology, Inc. Secondary alkaline phosphatase conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies were purchased from Dako or Sigma-Aldrich. The Alexa 546–conjugated goat anti–rabbit and anti–mouse antibodies were purchased from Molecular Probes. Protease- and phosphatase inhibitors were from Biomol.

**HEK293 and HeLa cell transfections**

HEK293 and HeLa cells were maintained at 37°C in a 5% CO₂ atmosphere in RPMI medium supplemented with 5% FCS. The day before transfection, HEK293 cells were seeded at 3 × 10⁵ cells per well in a 6-well plate (for in vitro kinase assays and Western blot) or at 2 × 10⁶ cells per 150-mm dish (for immunofluorescence microscopy). DNA transfections (2 μg plasmid DNA per 3 × 10⁵ cells and 1 μg plasmid DNA per 5 × 10⁶ cells) were performed using Superfect reagent (Qiagen) according to the manufacturer’s instructions. In brief, appropriate DNA amounts were mixed with the Superfect reagent, incubated at room temperature for 10 min in order to allow the complex to form, and then directly added to the culture medium. 2–3 h later, cells were transferred to fresh RPMI supplemented medium and incubated for further 40 h at 37°C.

**Immunoprecipitation and in vitro kinase assays**

HeLa and HEK293 cells transiently expressing the indicated PKCα-GFP mutants were lysed at 4°C in lysis buffer (20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 1 mM NaF, 1 mM sodium orthovanadate, 10 μM/μl leupeptin, 0.5 mM PMSF). After 30 min cell lysis, the lysates were centrifuged (10,000 g, 15 min, 4°C), the supernatant was collected, and immunoprecipitation of GFP fusion proteins was performed with 400 ng of anti-GFP antibody. After a 1-h incubation at 4°C, 30 μl of protein G sepharose was added and the mixture was incubated at 4°C for 1 h. The sepharose pellet was then washed twice in lysis buffer and once in kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 2 mM DTT) and PKCα activity (as measured by auto- and substrate phosphorylation) was determined by incubating immunocomplexes with 10 μl of kinase buffer containing 2 μCi [γ-³²P]-ATP with or without 5 μg alydolase at 37°C for 15 min. Reactions were terminated by the addition of 5× SDS-PAGE sample buffer and analyzed by SDS-PAGE, Western blotting, and autoradiography. Autoradiographs were analyzed by quantitative phosphoshamge analysis (Molecular Dynamics).

**Western blot analysis**

For Western blot analysis, transfected HEK293 cells were treated as described in the figure legends before being lysed in 200 μl lysis buffer followed by boiling with 5× SDS-PAGE sample buffer. Equal amounts of protein were loaded on a 12.5% SDS-PAGE. Upon fractionation, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked, followed by incubation either with a monoclonal antibody against GFP (1:1,000), a mouse antiserum raised against the NH₂-terminal region of PKCα (1:1,000), or the rabbit antibodies phosphoSer744/748 and phosphoSer916 (both 1:500). Membranes were incubated with alkaline phosphatase–conjugated anti–mouse IgG or anti–rabbit IgG antibodies (1:5,000). Immunoblots were developed according to standard procedures.

For separation of soluble proteins from organelles 4 × 10⁵ HEK293 cells were transfected with 20 μg of pEGFP-N1-PKCα or pEGFP-N1-PKCαH147–C196, and 100 μl Superfect reagent (Qiagen) according to the manufacturer’s instructions. 40 h after transfection, cells were harvested and resuspended in 500 μl lysis buffer without Triton X-100. Homogenization was done by applying 20 strokes with a “very tight fitting” 5-ml Dounce homogenizer (Braun). To remove cellular debris, the cellular extract was centrifuged at 1000 g followed by centrifugation of the supernatant for 1 h at 100 000 g (TLA 100; Beckman Coulter). Soluble proteins were recovered in the supernatant, whereas organelles and structures were recovered in the pellet. The pellet was resuspended in lysis buffer. For Western blot analysis equal amounts of protein were loaded onto a 12.5% SDS-PAGE.

**Conflonal immunofluorescence analysis**

HeLa cells grown on glass coverslips and expressing the indicated GFP–tagged PKCα mutants were washed once in PBS and fixed in 3.5% paraformaldehyde (pH 7.4) for 20 min at 37°C. Fixed cells were blocked and permeabilized in 5% normal goat serum and 0.05% Tween 20 for 30 min at room temperature. Coverslips were then incubated for 2 h at room temperature with the p24 rabbit antibody (1:200) or the p230 mouse antibody (1:200). Coverslips were washed three times in PBS and incubated with an anti–rabbit or an anti–mouse IgG Alexa 546–labeled antibody.
(1:500) for 1.5 h at room temperature. Cells were washed three times in PBS and mounted in Fluoromount G (Dianova). Images were acquired using a confocal laser scanning microscope (TCS SP2; Leica) equipped with a 63×1.4 HCG PlanAPO oil immersion objective. GFP was excited with an argon laser (488-nm line), whereas Alexa 546 was excited with a helium-neon laser (543-nm line). Each image represents a two-dimensional parallel projection of sections in the Z-series taken at 0.5–1-μm intervals across the depth of the cell.

Selective photobleaching was performed on the Leica TCS SP2 using 80 consecutive scans with a 488-nm laser line at full power. Live cells were held at 37°C and 5% CO2 atmosphere.

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