We identified the multifunctional chaperon protein p32 as a protein kinase C (PKC)-binding protein interacting with PKCa, PKCζ, PKCδ, and PKCμ. We have analyzed the interaction of PKCμ with p32 in detail, and we show here in vivo association of PKCμ, as revealed from yeast two-hybrid analysis, precipitation assays using glutathione S-transferase fusion proteins, and reciprocal coimmunoprecipitation. In SKW 6.4 cells, PKCμ is constitutively associated with p32 at mitochondrial membranes, evident from colocalization with cytochrome c. p32 interacts with PKCμ in a compartment-specific manner, as it can be coimmunoprecipitated mainly from the particulate and not from the soluble fraction, despite the presence of p32 in both fractions. Although p32 binds to the kinase domain of PKCμ, it does not serve as a substrate. Interestingly, PKCμ-p32 immunocomplexes precipitated from the particulate fraction of two distinct cell lines, SKW 6.4 and 293T, show no detectable substrate phosphorylation. In support of a kinase regulatory function of p32, addition of p32 to in vitro kinase assays blocked, in a dose-dependent manner, aldolase but not autophosphorylation of PKCμ, suggesting a steric hindrance of substrate within the kinase domain. Together, these findings identify p32 as a novel, compartment-specific regulator of PKCμ kinase activity.

The protein kinases C (PKC) comprise a family of intracellular serine/threonine-specific kinases, which are implicated in signal transduction of a wide range of biological responses including changes in cell morphology, proliferation, and differentiation (1–3). The currently defined 11 members of the PKC family can be grouped into the three major classes of Ca<sup>2+</sup>-dependent classical PKCs, Ca<sup>2+</sup>-independent, novel PKCs, and Ca<sup>2+</sup>- and lipid-independent atypical PKCs as well as PKCμ and its mouse homologue PKD (4, 5), which do not conform to either one of these major classes and may thus define a new subgroup (6). PKCμ/PKD differ from the three major groups of PKC isozymes by the presence of an amino-terminal hydrophobic domain, an acidic domain (7), a pleckstrin homology domain within the regulatory region (8), and lack of a typical pseudosubstrate site. PKCμ is ubiquitously expressed, and evidence for the involvement of PKCμ in diverse cellular functions stems from reports showing enhancement of constitutive transport processes in PKCμ-overexpressing epithelial cells (9), G protein-mediated regulation of Golgi organization (10), and involvement in protection from apoptosis (11). Interestingly, PKCμ shows particularly high expression in thymus and hematopoietic cells suggesting a potential role in immune functions (12). In accordance with this is the finding that, upon B cell receptor stimulation, PKCμ is recruited together with the tyrosine kinase Syk and phospholipase Cγ to the B cell receptor complex and negatively regulates phospholipase Cγ activity (13).

In addition to lipid second messengers as regulators of PKC translocation and activation, there is increasing evidence for a role of regulatory proteins in controlling kinase activity and/or intracellular location of various PKC members. Thus, the identification of receptors of activated protein kinase C (14) as well as the binding of more general and of specific modulators such as 14-3-3 (15–17), PAR4, LIP (18, 19), and ZIP (20), respectively, points to a complex regulation of PKC-dependent intracellular pathways. Whereas the latter selectively bind to the C1 regulatory domain of the atypical PKCζ and C1 and C2 regulate kinase activity in a lipid messenger-independent manner (18), protein interacting with protein kinase Cs 1 was identified as a PKCa kinase domain-binding protein (21). By analogy, because of the ubiquitous expression of PKCμ and its apparent involvement in diverse cellular responses, the existence of cell type- and/or organelle-specific regulators of PKCμ can be postulated. Indeed, 14-3-3 proteins as well as phosphatidylinositol 4-kinases were recently identified to be associated specifically with the C1 region of PKCμ (17, 22).

To define other interacting proteins and to investigate their role in modulating kinase activity, we have used different PKCμ domains in various screening assays for PKCμ-binding proteins. The pleckstrin homology domain and the kinase domain of PKCμ were used in a yeast two-hybrid screen in order to identify new PKCμ-binding proteins. With the kinase domain as a bait, a novel PKC-binding protein was detected. We identified the multifunctional chaperon protein p32, previously described as a receptor of complement component C1q (23), the kininogen-binding protein p33 (24), and splicing factor associated protein p32 (25) as a general PKC interactor, and we describe in detail its interaction with PKCμ and the functional consequences on kinase activity.
concentrated sample buffer, subsequently fractionated on 12% SDS-PAGE carried out for 15 min at 37 °C. The reaction was stopped by adding 5 mM EDTA. The Western blot analysis of yeast lysates using a PKCμ-specific antibody. A pACT A bacterial library of human-activated B-lymphocytes was converted into vitro to plasmids (27) and used to transform the pAS1/PKCμ-expressing Y190 yeast strain according to standard procedures (26). Clones were selected on the respective medium lacking tryptophan, leucine, and histidine containing 50 mM 3-amino-1,2,4-triazole (Sigma). Upon day 4 grown colonies were analyzed by lacZ staining. Blue colonies were streaked again and confirmed by lacZ staining. PACT plasmids were recovered by bacterial transformation of yeast isolated plasmids and subjected to dideoxy sequencing of both strands.

Recombinant PKCμ, Phox Domain Constructs, and Cell Lines—The production and purification of PKCμ from Sf158 insect cells overexpressing PKCμ has been described (28). To produce GST-p32 fusion proteins the cDNA fragment was amplified from the pACT-p32 plasmid using primers to introduce a BamHI site 5′ of the ATG and cloned in frame in pGEX-3X. The fusion proteins for precipitation analysis were prepared according to standard procedures. The construction of the GFP-p32 construct (29) and the c-Myc-tagged PKCμ expression plasmid has been described previously (22, 28). The human SKW 6.4 B cell line (ATCC) was cultured in RPMI medium supplemented with 5% fetal calf serum. 293T cells were obtained from ATCC. 293T cells were cultured in RPMI medium supplemented with 5% fetal calf serum. SKW 6.4 and Sf158 cells were lysed at 4 °C in lysis buffer (20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, pH 7.4, 1 mM NaF, 1 mM Na3P, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 1 mM p-nitrophenol phosphate, 1 mM μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml phenylmethylsulfonyl fluoride). After 60 min cell lysis the cells were centrifuged (10,000 × g, 15 min, 4 °C), and immunoprecipitation was performed as described (31). GST fusion protein pull-down assays were performed by incubation of 1 ml of lysate (representing 500,000 Sf158 cells or 50 × 106 SKW 6.4 cells) with the indicated amounts of GST fusion proteins coupled to glutathione-Sepharose for 60 min at 4 °C. Immunocomplexes or GST complexes were washed three times and applied to SDS-PAGE followed by transfer to nitrocellulose membrane. Western blot detection of PKCμ or p32 was performed according to standard procedures.

In Vitro Kinase Assays and Cellular Fractionation—80 ng of purified recombinant PKCμ from Sf158 cells were preincubated as indicated with different amounts of GST or GST-p32 in phosphorylation buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 2 mM dithiothreitol) for 10 min at room temperature. Kinase reaction in the absence or presence of palmitoylphosphatidylserine/PtdIns(4,5)-bisphosphate (PtdIns(4,5)P2) was started by addition of 4 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech) in 10 μl of kinase buffer, and incubation was carried out for 15 min at 37 °C. The reaction was stopped by adding 5× concentrated sample buffer, subsequently fractionated on 12% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed upon autoradiography. A fluorographic band was visualized using PhosphorImager analysis (Molecular Dynamics). After immunoprecipitation PKCμ substrate and autophosphorylation were determined in vitro kinase assays as described (17). For cellular fractionation 4 × 105 SKW 6.4 and 3 × 106 293T cells were resuspended in lysis buffer containing no detergent and homogenized by applying 15 strokes with a “very tight-fitting” 5-ml Dounce homogenizer (Braun, Melsungen, Germany). Cellular debris was removed by centrifugation (800 × g, 5 min). The remaining lysate was centrifuged at 100,000 × g. The supernatant containing the cytosolic fraction was defined as the soluble fraction. The pellet was dissolved in lysis buffer containing 1% Triton X-100 and defined as the non-soluble fraction.
expressing PKC\(\mu\) control precipitates using up to 16 proteins. PKC\(\mu\) Y190 pSE1111/pSE1112 as a positive control. Clone 138 encoding p32 was identified in the two-hybrid screen described here.

SKW 6.4 cells were subjected to PKC\(\mu\)-specific antibody (Fig. 2A, top right panel), indicating direct molecular interaction of PKC\(\mu\) with p32. The reciprocal precipitation was carried out with extracts from the B cell line SKW 6.4, which expresses p32 in significant amounts (see Figs. 2C and 4) and a purified GST fusion protein of the PKC\(\mu\) kinase domain (GST-\(\mu\)Kin). Precipitates were analyzed by immunoblotting using a p32-specific rabbit antibody (Fig. 2B, top panel) or a GST-specific antibody to estimate GST loads (Fig. 2B, bottom panels). To demonstrate the specificity of the association, excessive amounts of GST protein (10-fold over GST-\(\mu\)Kin) served as a negative control, which resulted in only a very weak staining (Fig. 2B, right lanes). Thus, the data presented here provide clear evidence of specific association of p32 with the kinase domain of PKC\(\mu\).

In parallel to the GST-p32 precipitation assays (Fig. 2, A and B), association between PKC\(\mu\) and p32 was independently demonstrated by reciprocal communoprecipitation analysis using p32- and PKC\(\mu\)-specific antisera (Fig. 2C). The somewhat weaker signal of p32 observed in PKC\(\mu\) immunoprecipitates might be due to a steric hindrance by the PKC\(\mu\) antibody, which is directed against carboxyl-terminal epitopes and thus could be in proximity to the p32 binding region. Phorbol ester treatment of cells or addition of phosphatidylserine/phorbol ester micelles to in vitro pull-down assays did not enhance p32 binding to PKC\(\mu\) (data not shown), suggesting a constitutive, lipid-independent association of p32 in SKW 6.4 cells.

In order to assess the selectivity of p32 interaction with PKC\(\mu\), other PKC isotypes were analyzed by pull-down assays and communoprecipitation analyses using three different recombinant PKC isotypes representing the three major PKC subgroups. By using GST-p32, in addition to PKC\(\mu\), a specific binding of PKC\(\alpha\), PKC\(\xi\), and PKC\(\delta\) was noted (Fig. 3A). As a control, precipitation analysis of the c-Jun amino-terminal kinase (JNK) from lysates of 293T cells was performed. As shown in Fig. 3A (bottom panel), no binding of JNK could be detected. These results indicate a PKC-selective association of p32. Communoprecipitation analyses using PKC-specific antibodies further confirmed interaction of p32 with members of the different PKC subgroups (Fig. 3B).

p32 Colocalizes with PKC\(\mu\) in Mitochondria in SKW 6.4 Cells—The association of PKC\(\mu\) and p32 was further analyzed by confocal laser scanning microscopy. The literature on the cellular distribution of p32 is controversial, reporting p32 either localized at the cell membrane (33), intracellularly (34), or at mitochondria (29), which may reflect cell-specific differences. Therefore, we investigated the intracellular localization of p32 in the SKW 6.4 B cell line. As shown in Fig. 4, in these cells p32

Western blot analysis in GST-p32 precipitates using as little as 1 \(\mu\)g of fusion protein, whereas no signal was revealed in control precipitates using up to 16 \(\mu\)g of GST protein (Fig. 2A, left panels). GST 14-3-3\(\mu\) was precipitated with GST-p32 or GST proteins. PKC\(\mu\)/GST-fusion protein complexes were harvested by incubation with glutathione-Sepharose beads and subjected to Western blot analysis using a PKC\(\mu\)-specific antibody (top panels) as described under “Materials and Methods.” GST (26 kDa) or GST-p32 fusion proteins (50 kDa) were detected using a goat anti-GST antibody (Fig. 2A, bottom left panel). The experiments were performed three times with similar results.

FIG. 1. The kinase domain of PKC\(\mu\) associates with p32. A, schematic drawing of PKC\(\mu\) functional domains used for cloning of the kinase domain in the yeast expression vector (pAS1\(\mu\)Kin). The fusion protein of GALA binding domain and PKC\(\mu\) kinase domain was expressed in Y190 yeast strain, transfected with a B cell library containing GAL4 activating domain fusion proteins (see “Materials and Methods”). B, growth of yeast strains on rich media (YPEG, left panel) and selection media (HTL- and 50 mm aminotriazole, right panel). Y190 served as a negative control and Y190 pSE1111/pSE1112 as a positive control. Clone 138 encoding p32 was identified in the two-hybrid screen described here.

FIG. 2. PKC\(\mu\) associates with p32 in vitro. A, Sf158 cell extracts expressing PKC\(\mu\) were incubated with the indicated amounts of GST-p32, GST as a negative control, or GST-14-3-3\(\mu\) as a positive control (left panels). In vitro binding of purified PKC\(\mu\) to p32 is shown (right panels). 80 ng of purified PKC\(\mu\) were precipitated with GST-p32 or GST proteins. PKC\(\mu\)/GST-fusion protein complexes were harvested by incubation with glutathione-Sepharose beads and subjected to Western blot analysis using a PKC\(\mu\)-specific antibody (top panels) as described under “Materials and Methods.” GST (26 kDa) or GST-p32 fusion proteins (50 kDa) were detected using a goat anti-GST antibody (bottom panels). B, precipitation analysis of SKW 6.4 cells. 5 \(\times\) 10\(^6\) SKW 6.4 cells were lysed and incubated with 1 \(\mu\)g of GST-\(\mu\)Kin or 10 \(\mu\)g of GST bound to glutathione-Sepharose beads as a negative control. Bound proteins were separated by 12% SDS-PAGE and subjected to immunoblot analysis using a p32 antibody (top panels) or a GST anti-GST antibody (bottom panels). C, communoprecipitation of PKC\(\mu\) with p32. 5 \(\times\) 10\(^6\) of SKW 6.4 cells were subjected to PKC\(\mu\) or p32 immunoprecipitation using rabbit antibodies. Immunocomplexes were subjected to SDS-PAGE preceded by Western blot analysis using PKC\(\mu\)-specific (top left panel) or p32-specific antibodies (bottom left panel). The experiments were performed three times with similar results.
PKC is partially located at mitochondria in 293T cells (Fig. 5), indicating that PKC$\mu$ is localized predominantly at intracellular compartments (Fig. 4, top row, left panel). Costaining with antibodies against cytochrome c (Fig. 4, top row, middle panel) resulted in a nearly identical staining pattern, which was confirmed by overlay analysis indicated by the blue color shown in the top right panel. In SKW 6.4 cells, PKC$\mu$ shows a broad speckled distribution throughout extranuclear regions of the cell (Fig. 4, middle row, left panel). Overlay of PKC$\mu$- and p32-specific staining verifies a partial colocalization of both proteins (Fig. 4, middle row, right panel). A double staining with PKC$\mu$ (Fig. 4, bottom row, left panel) and cytochrome c (Fig. 4, bottom row, middle panel)-specific antibodies confirmed that PKC$\mu$ is partially located at mitochondria in SKW 6.4 cells (shown in blue at Fig. 4 in the bottom row, right panel). In 293T cells (Fig. 5, upper panel) and in SKW 6.4 cells, only a weak colocalization signal with p24 was revealed (Fig. 5, bottom panels), which is in accordance with an enrichment of PKC$\mu$ at mitochondria in the latter cell line. The data presented here thus indicate a cell type-specific compartmentalization/enrichment of PKC$\mu$ either at mitochondria, in the B cell line SKW 6.4 (Fig. 4), or at Golgi structures in 293T cells (Fig. 5).

p32 Affects PKC$\mu$ Kinase Activity—Since in in vitro studies p32 specifically binds to PKC$\mu$ and appears to be constitutively associated with the kinase in the B cell line SKW 6.4, we investigated whether it affects PKC$\mu$ kinase activity in vitro. Incubation of PKC$\mu$ with GST-p32 led to a slight enhancement of autophosphorylation (Fig. 6). We analyzed further whether substrate phosphorylation is affected by GST-p32 binding to PKC$\mu$ also. As shown in Fig. 6A, phosphorylation of the well known in vitro substrate aldolase (7, 17) was significantly inhibited over a 10-fold range (0.1–1 $\mu$g of p32). PKC$\mu$-mediated aldolase phosphorylation was not affected in the presence of 1 $\mu$g of GST protein (Fig. 5B, right lane), indicating that inhibition of aldolase phosphorylation was not due to unspecific effects of the GST moiety. Quantitative analysis revealed, in the presence of 1 $\mu$g of GST-p32 an approximately 70% inhibi-
PKC\(\mu\) is regulated by the multifunctional protein p32

In this study, we identified by yeast two-hybrid screening a novel PKC\(\mu\)-interacting protein, the previously described protein p32 (Fig. 1), which has been associated with multiple, chaperon-like functions. p32 may serve as a compartment-specific regulator of PKC\(\mu\) kinase activity. Cellular colocalization of PKC\(\mu\) and p32 at mitochondria was shown in the B cell line SKW 6.4 by confocal immunofluorescence microscopy (Fig. 4). Functional interaction of both proteins was shown by precipitation analysis with GST fusion proteins (Fig. 6), these findings indicate a p32-dependent regulation of compartmentalized PKC\(\mu\) kinase activity and suggest a new mechanism of regulation of kinase activity via kinase domain interacting proteins, identifying an as yet unrecognized functional role of p32 in this process.

**DISCUSSION**

In this study, we identified by yeast two-hybrid screening a novel PKC\(\mu\)-interacting protein, the previously described protein p32 (Fig. 1), which has been associated with multiple, chaperon-like functions. p32 may serve as a compartment-specific regulator of PKC\(\mu\) kinase activity. Cellular colocalization of PKC\(\mu\) and p32 at mitochondria was shown in the B cell line SKW 6.4 by confocal immunofluorescence microscopy (Fig. 4). Functional interaction of both proteins was shown by precipitation analysis with GST fusion proteins (Fig. 6), these findings indicate a p32-dependent regulation of compartmentalized PKC\(\mu\) kinase activity and suggest a new mechanism of regulation of kinase activity via kinase domain interacting proteins, identifying an as yet unrecognized functional role of p32 in this process.
ments by the multifunctional protein p32. Accordingly, PKC\(\mu\) kinase regulation by p32 may not only serve as a paradigm to explain a differential, cell-, and/or compartment-specific activation of ubiquitously expressed kinases by virtue of a cell-specific intracellular location/function of regulatory molecules but also provides new insight into regulation of kinase activity toward specific substrates by kinase domain interacting proteins (Figs. 6 and 7). These data show that PKC\(\mu\) is, in addition to its regulation by lipids and 14-3-3 proteins (17), controlled by a p32-dependent mechanism that probably controls substrate access by steric hindrance.

So far, the biological role of p32 appeared rather unclear due to the diverse functions reported; p32 has been originally identified as a cell surface protein binding to the globular “heads” of the complement factor C1q (23). It has also been described as a cell surface kininogen-binding protein (24). In addition, several independent reports have described p32 as an intracellular protein (34, 37), which colocalizes in the endothelial cell line EA.hy926 with a mitochondrial marker protein (29). p32 has been shown to be important for the maintenance of mitochondrial oxidative phosphorylation (38). Mitochondrial functions of p32 are further indicated by the identification of a yeast homologue of p32, called Mam33p, that has been localized to the inner mitochondrial membrane (39). Other reports confirmed that p32 is located at mitochondria, but in addition a nuclear localization was found and a function of p32 as part of an import machinery was postulated (40). Moreover, p32 was described as part of the RNA splicing complex SP2 in HEK cells (25). p32 has further been shown to associate with many viral proteins including HIV-1 Tat (41) and Rev (42) as well as with EBNA-1 of Epstein-Barr virus (43). The latter p32 functions are all considered to modulate transcription factor activity. The participation in different biological processes like mitochondrial functions, transcription- and splicing factor modulation, and potential role in complement cascade or blood coagulation (44) suggest a typical chaperon function of p32.

In this paper, we describe a novel aspect of p32 biology with a functional role as an inhibitor of kinase activity. The presented data show that p32 binds to the kinase domain of PKC\(\mu\) and, without being a substrate, inhibits phosphorylation of aldolase, yet maintains or even enhances the level of autophosphorylation. As different phosphorylation sites trigger the activation state of PKC isoforms (45), similar mechanisms are conceivable for PKC\(\mu\). For the p32-mediated regulation of PKC\(\mu\) activity, several possibilities may be considered. First, p32 may interfere with substrate phosphorylation by steric hindrance. Second, p32 binding to PKC\(\mu\) could induce a conformational change such that endogenous auto phosphorylation sites are preferentially used over cellular substrates. Third, the phosphorylation sites critical for kinase activation are blocked by p32, disabling substrate phosphorylation, yet leaving auto phosphorylation at other serine residues of PKC\(\mu\) unaffected. Several phosphorylation sites important for PKC\(\mu\) activation have now been mapped within the catalytic domain (47), which is in accordance with the latter model of p32 interference with PKC\(\mu\) function. Together, our data presented here thus indicate that, besides regulation of PKC\(\mu\) kinase activity via the C1 domain either by activating lipid second messengers and phorbol ester (1, 3) or inactivating 14-3-3 proteins (17), other domains are involved in modulating PKC\(\mu\) activity also. Since in contrast to 14-3-3, p32 does not affect lipid-induced PKC\(\mu\) autophosphorylation (Fig. 6), we propose that PKC\(\mu\) activity is controlled by at least two independent mechanisms. Moreover, our finding that p32 binds to different PKC isoforms points to a more general p32-based mechanism of controlling PKC kinase activity.

The differential cellular localization of p32 in different cell types (29, 40) may contribute to the compartment-specific functional role of various PKC isoforms, including PKC\(\mu\). As shown here, in the SKW 6.4 cell line, p32 largely colocalized with cytochrome c, indicative of a mitochondrial localization (Fig. 4). In full accordance with the in vitro binding studies, PKC\(\mu\) partially colocalized with p32 at mitochondria, as revealed from confocal microscopy (Fig. 4) and cell fractionation studies (Fig. 7). Therefore, we propose that p32 is a part of an intracellular receptor that retains PKC\(\mu\) at intracellular compartments such as mitochondria and serves as a regulator of its kinase activity.

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