The protein kinase C (PKC)-related enzyme PKC/\(\mu\)/PKD (protein kinase D) is activated by activation loop phosphorylation through PKC\(\epsilon\). Here we demonstrate that PKC\(\mu\) is activated by the direct phosphorylation of PKC\(\epsilon\). PKC\(\mu\) colocalizes with PKC\(\epsilon\) in HEK293 and MCF7 cells as shown by confocal immunofluorescence analyses. PDK1, known as the upstream kinase for several PKC isoforms, associates intracellularly with PKC\(e\) and PKC\(\eta\). PKC\(\eta\) is phosphorylated by PDK1 in vitro, leading to kinase activation as similarly reported for PKC\(\epsilon\) activation by PDK1. Coexpression of PDK1, PKC\(\epsilon\) and PKC\(\mu\) in HEK293 cells results in PKC\(\mu\) activation. In contrast, the coexpression of PDK1 and PKC\(\eta\) with PKC\(\mu\) does not activate PKC\(\eta\) or consequently PKC\(\mu\). PDK1/PKC\(\epsilon\)-triggered activation of PKC\(\mu\) inhibits JNK, a downstream effector of PKC\(\mu\), whereas upon transient expression of PDK1, PKC\(\epsilon\), and PKC\(\mu\), JNK is not affected. These data implicate PKC\(\epsilon\) as the biologically important upstream kinase for PKC\(\mu\) in HEK293 cells, regulating downstream effectors. Our results further indicate a PDK1/PKC\(\epsilon\)/PKC\(\mu\) controlled negative regulation of PKC\(\eta\) kinase activity. In this study, we show that differentially activated kinase cascades involving PDK1 and novel PKC isoforms are responsible for the regulation of PKC\(\mu\) activity and consequently inhibit the JNK pathway.

The activation of protein kinases through growth factor receptors is achieved via a complex network of intracellular signal processes involving second messengers, protein-protein interaction, and the phosphorylation/dephosphorylation of interacting proteins. Protein kinase C (PKC) family has been shown to be involved in the signal transduction of a wide range of biological responses, triggering changes in cell morphology, proliferation, and differentiation (1, 2). The PKCs comprise a family of intracellular serine/threonine-specific kinases that are, depending on the isoform typically activated by Ca\(^{2+}\), lipid second messengers and/or protein activators (1, 3). Recently, a more detailed activation model involving PDK1 as a PKC activation loop kinase preceding lipid activation has been established (4).

A group of kinases with PKC-like functional structures has been described. This kinase family consists of PKC\(\alpha\) (5), its mouse homologue termed PKD (6), PKC\(\gamma\) (7), and PKD2 (8). These kinases share structural homology to PKCs with respect to the catalytic domain and to the presence of amino-terminal cysteine fingers, defining the structural basis for lipid binding. PKC\(\mu\)/PKD differs from PKC isoforms by an acidic domain (9), a PH domain within the regulatory region (10) and the lack of a typical pseudo-substrate site.

PKC\(\mu\)/PKD activation occurs through several mediators via a PKC-dependent pathway (11). PKC\(\gamma\) and PKC\(e\) have been implicated in the activation of PKC\(\mu\)/PKD through binding to the PKC\(\alpha\)/PH domain (12). Both enzymes associate and, in the case of PKC\(\gamma\), directly phosphorylate the activation loop of PKC\(\mu\) (13). Although a precise positioning of the PKC/PKC\(\mu\)/PKD module in signal transduction pathways is currently unknown, it seems to be part of a kinase cascade that is triggered by multiple cell surface receptors. In the case of PDGF-initiated signal processes, PKC\(\mu\)/PKD has been placed downstream of phospholipase \(\gamma\) (14). This predicts that phospholipase \(\gamma\)-derived diacylglycerol generation results in PKC and consequently PKD/PKC\(\mu\) activation, triggering p42 MAPK-modulated gene expression (15).

The discovery that PKC activation depends upon an ordered series of phosphorylation events before lipids activate has led to a search for an upstream kinase identifying PDK1 phosphorylating the PKC activation loop (4, 16). PDK1 is a constitutive and mainly cytosolic localized kinase that translocates upon growth factor-induced synthesis of 3\'-phosphorylated phosphoinositides via its PH domain to the cytoplasm membrane in which it phosphorylates downstream kinases such as Akt (17). The discovery that PKCs are activated through PDK1 and that PKC activates PKC\(\mu\)/PKD implicates that PDK1 is involved in PKC\(\mu\) activation. We have carried out this study to analyze in detail the activation of PKC\(\mu\) by upstream kinases and to identify potential downstream pathways. Here we demonstrate that PKC\(e\) colocalizes with, phosphorylates, and activates PKC\(\mu\). PDK1 activates PKC\(\eta\) and PKC\(e\) in vitro, but upon transient expression in HEK293 cells, PDK1 acts only via PKC\(e\) and not via PKC\(\eta\) to activate PKC\(\mu\), leading to an inhibition of JNK activity.

MATERIALS AND METHODS

Cloning of Expression Constructs—The PDK1 expression constructs and the recombinant enzyme were gifts of Alex Toker (Harvard Medical School, Boston, MA). The construction of the PKC\(\mu\) and PKC\(\gamma\)-GFP expression vectors have been described previously (13, 18). PKC\(\gamma\)-GFP migrates in SDS-PAGE at \(\sim 110\) kDa, and PKC\(\gamma\)-GFP and PKC\(\eta\)-GFP migrate at \(80\) kDa. The PKC\(\epsilon\)-GFP expression vector was a gift of Angelika Haussler (University of Stuttgart, Stuttgart, Germany). The construction of the PKC\(\mu\)-GFP expression construct has been described previ-
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PKC\(\mu\) is a Direct Upstream Kinase for PKC\(\mu\)—We have recently shown that PKC\(\mu\) is phosphorylated and activated by PKC\(\eta\) (13). These findings together with independent studies showing association of PKD with PKC\(\eta\) and to lesser degree with PKC\(\epsilon\) (12) allow us to postulate that PKC\(\epsilon\) might also act as a direct upstream kinase for PKD/PKC\(\mu\). Earlier studies were in favor of this hypothesis, showing that PKC\(\epsilon\) similar to PKC\(\mu\) is located at the Golgi compartment (23, 24). Because PKC\(\mu\) has been demonstrated to translocate from the cytoplasm to the Golgi compartment thus leading to the appearance of an activated enzyme (19, 25), PKC\(\epsilon\) can be considered as an upstream kinase phosphorylating the activation loop of PKC\(\mu\), which has already been demonstrated for PKC\(\eta\) (13).

Using in vitro kinase assays, we have tested whether recombinant PKC\(\eta\), PKC\(\epsilon\), and PDK1 are able to phosphorylate recombinant SF9-produced purified PKC\(\mu\), leading to kinase activation. As shown in Fig. 1, left-hand panels, recombinant PKC\(\mu\) displays autophosphorylation leading to substrate (syntide 2) phosphorylation, whereas kinase-dead His-tagged PKC\(\mu\)K612W shows no evidence of autophosphorylation at all (Fig. 1, center panel). PKC\(\mu\)K612W phosphorylation is enhanced by adding purified SF9-expressed PKC\(\eta\) (positive control) and also, to a significantly higher degree, by adding purified PKC\(\epsilon\) (Fig. 1, upper panel), thus indicating a direct phosphorylation as shown for PKC\(\eta\). In contrast to the phosphorylation of kinase-dead PKC\(\mu\) by PKC\(\epsilon\), the phosphorylation of wild-type PKC\(\mu\) leads to a significant enhancement of syntide 2 phosphorylation (Fig. 1, lower panel). SF9-expressed purified PKC\(\eta\) and PKC\(\epsilon\) do not phosphorylate syntide 2 significantly but do show autophosphorylation (Fig. 1). As a control for nonspecific phosphorylation, purified PDK1 was used to phosphorylate PKC\(\mu\). No enhancement of PKC\(\mu\) phosphorylation by recombinant PDK1 was detected, demonstrating the specificity of PKC\(\eta\)- and PDK1-mediated PKC\(\mu\) activation.

PKC\(\mu\) Co-localizes with PKC\(\epsilon\)—PKD has been shown to be associated with PKC\(\epsilon\) using biochemical techniques such as in vitro pull-down assays using glutathione S-transferase fusion proteins and coimmunoprecipitations (12). Based on these data, we questioned whether both enzymes colocalize in HEK293 cells. Immunofluorescence analysis was performed after transient coexpression of a PKC\(\mu\)-GFP fusion protein with PKC\(\epsilon\) in HEK293 cells. As shown in Fig. 2, PKC\(\epsilon\) was localized in perinuclear structures (Fig. 2, center panel) similarly as PKC\(\epsilon\) (left-hand panel). An overlay of both pictures shows colocalization indicated by the yellow color (Fig. 2, upper row, right-hand panel).

PKCs are activated through a complex cascade involving lipids and the phosphoinositide-dependent kinase PDK1 (4, 16, 26). Therefore, we were interested in analyzing the role of PDK1 in PKC/PKC\(\mu\) activation. First, we tested whether cel-
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![Image of a diagram](http://www.jbc.org/)

**FIG. 3.** PKC\(\mu\) coprecipitates with PKC\(\eta\). HEK293 cells were cotransfected with c-myc-tagged PKD1, kinase-dead PKD1 (PKD1\(_{K112W}\)), and the indicated PKC\(\eta\) mutants. 40 h upon transfection, cells were lysed. PKD1 was immunoprecipitated using an anti-c-myc monoclonal antibody and proceeded to Western blot analysis using an anti-PKC\(\eta\) rabbit antiserum for detection (upper panel). Aliquots of the PKD1 immunoprecipitates were stained with an anti-c-myc monoclonal antibody as a control (center panel). PKC\(\eta\) expression is shown by Western blot analysis of total cell lysates (TCL). All blots shown were visualized through an alkaline phosphatase detection system. IP, immunoprecipitate.

Previous studies demonstrated phosphorylation of selected PKC isoforms by PKD1 at the activation loop as well as the presence of a complex between PKC and PKD1 (4, 16). Therefore, we further analyzed the association between PKD1 and PKC\(\eta\) using biochemical techniques. PKC\(\eta\) wild type and mutants were coexpressed with c-myc-tagged PKD1 and a kinase-dead mutant PKD1\(_{K112W}\). From the obtained cell lysates, 90% were used to immunoprecipitate PKD1 with an anti-c-myc antibody to detect PKD1 (Fig. 3, upper panel) and to detect associated PKC\(\eta\) (Fig. 3, center panel). 10% of the lysates were used to estimate PKC\(\eta\) expression (Fig. 3, lower panel). Immunoprecipitation efficiency of PKC\(\eta\) was monitored using the same amount of cell lysate as used for coimmunoprecipitations (Fig. 3, right lane, upper panel). There was no endogenously expressed PKC\(\eta\) detectable in anti-c-myc immunoprecipitates, whereas kinase-dead PKC\(\eta\)\(_{K/R}\), constitutive active PKC\(\eta\)\(_{K/R}\), as well as transiently expressed wild-type PKC\(\eta\) were readily detected in PKD1 immunoprecipitates (Fig. 3, upper panel). Interestingly, kinase-dead PKD1 showed a faster migration in SDS-PAGE as compared with wild-type PKD1, which may be attributed to the lack of autophosphorylation (Fig. 3, center panel). In addition, PKC\(\eta\) migration showed a shift in PKD1 immunoprecipitates, which is probably because of PKD1-dependent phosphorylation and which is absent in PKD1\(_{K112W}\) immunoprecipitates (Fig. 3, upper panel). PKD1/PKC\(\eta\) association is independent of phosphorylation/autophosphorylation as coimmunoprecipitates of kinase-dead mutants coprecipitate with the same efficiency as the wild-type enzymes. Expression levels of PKC\(\eta\)\(_{WT}\) and of the mutants used were monitored by Western blot detection (Fig. 3, lower panel).

**PKD1 Phosphorylates and Activates PKC\(\eta\)—**As shown in Fig. 3, the differential migration of PKC\(\eta\) in PKD1 immunoprecipitates (upper panel) indicated a phosphorylation of PKC\(\eta\) through PKD1. Therefore, we further analyzed these findings using in vitro kinase assays of PKC\(\eta\) immunoprecipitates. PKC\(\eta\) was expressed as a GFP-fusion protein in HEK293 cells, migrating at a different size in SDS-PAGE as PKDI. This expression clearly enables a distinction to be made between PKC\(\eta\) phosphorylation from the PKD1 autophosphorylation signal. PKC\(\eta\) was immunoprecipitated using a COOH-terminal antiserum and in vitro phosphorylated with SF9-produced pu-
FIG. 4. PDK1 activates PKC\(\eta\). A, PKD1 phosphorylates PKC\(\eta\). Immunoprecipitates of PKC\(\eta\) were in vitro phosphorylated with purified recombinant PDK1 from Sf9 cells, fractionated by SDS-PAGE, transferred to a membrane, and exposed to autoradiography (upper panel). PKC\(\eta\) immunoprecipitates were visualized by Western blot detection. As a negative control, PKD1 was incubated with immunoprecipitates from vector-transfected cells. As a positive control, PDK1 was incubated with immunoprecipitates from kinase-dead PKC\(\eta\)-H9262 form SF9 cells. B, time course of PKC\(\eta\) activation by PDK1. Immunoprecipitates of PKC\(\eta\) were in vitro autophosphorylated with (lower panels) or without (upper panels) purified PDK1. Loading controls were performed by Western blot analysis. MBP was included to measure substrate phosphorylation. Western blots were visualized by alkaline phosphatase.

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FIG. 5. PDK1-triggered PKC activation differentially activates PKC\(\mu\). The indicated expression constructs were transiently expressed in HEK293 cells. A, 40 h upon transfection, cells were lysed and aliquots were removed for PKC\(\mu\) immunoprecipitation (left-hand panels) or measurement of transgenic expression (right-hand panels) (data not shown). Immunoprecipitates (IP) were in vitro autophosphorylated (upper panels) or, in the case of PKC\(\mu\), used to phosphorylate the substrate syntide 2 (lower left-hand panel). As a loading control, PKC\(\mu\) immunoprecipitates were visualized by Western blot detection with an alkaline phosphatase-based detection system (center left-hand panel). B, 40 h upon transfection of cells, PKC\(\eta\) or PKCe was immunoprecipitated and used for in vitro phosphorylation of MBP. C, phosphorylation of the amino-terminal PKC\(\mu\) domain. Immunoprecipitates of PKC\(\mu\)-1325GFP were in vitro phosphorylated with the indicated kinases. Shown is an autoradiograph upon quantitative phosphorimaging analysis.

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PKC\(\eta\)-mediated PDK1 phosphorylation could be shown approximately upon 5-min incubation, decreasing within a 1-h period. Under the same experimental conditions, the addition of purified PDK1 significantly enhances the substrate phosphorylation capacity of PKC\(\eta\), which shows high activation levels after just 3 min decreasing to approximate basal levels within a period of 60 min (Fig. 4B, lower panel). Using similar amounts of PKC\(\eta\) immunoprecipitates, a significant enhancement in substrate phosphorylation efficiency can be demonstrated upon incubation with PDK1, which demonstrates an in vitro activation of PKC\(\eta\) through PDK1 in accordance with the phosphorylation data.

PKD1 Differentially Affects PKCe and PKC\(\eta\)-Triggered PKC\(\mu\) Activation—PKD1 acts as an upstream kinase for PKC\(\eta\) (Fig. 4 and PKCe (4). Both enzymes activate PKC\(\mu\), predicting that a three-level activation cascade amplifies input signals and consequently enhancing PKC\(\mu\) substrate phosphorylation. To test this hypothesis, we coexpressed PKD1, PKC\(\eta\)\(\epsilon\), and PKC\(\mu\) to measure PKC\(\mu\) activation. The coexpression of PKC\(\eta\) or PKCe with PKC\(\mu\) leads to stronger syntide 2 phosphorylation of PKC\(\mu\) immunoprecipitates (Fig. 5A, lower panel) than from
PKC$\mu$-transfected HEK293 cells. Coexpression of PDK1, PKC$\epsilon$, and PKC$\mu$ also leads to the enhancement of syntide 2 phosphorylation of PKC$\mu$ immunoprecipitates and similarly of immunoprecipitates from PKC$\epsilon$/PKC$\gamma$- and PKC$\mu$-transfected cells. The expression of kinase-dead PDK1 with PKC$\epsilon$ and PKC$\mu$ reversed this effect, showing an inhibition of PKC$\mu$ substrate phosphorylation (Fig. 5A, lower panel). PDK1 acts completely differently on PKC$\eta$-triggered PKC$\mu$ activation in HEK293 cells. The coexpression of PDK1 with PKC$\eta$ and PKC$\mu$ leads to a significant reduction in syntide 2 phosphorylation of PKC$\mu$ immunoprecipitates (Fig. 5A, lower panel). Coexpressing PKC$\eta$ with kinase-dead PDK1 K112W largely does not affect PKC$\mu$ activation. These data suggest a PDK1- and PKC$\epsilon$-dependent pathway leading to PKC$\mu$ activation. The coexpression of PDK1 and PDK1K112W does not notably influence the substrate phosphorylation efficiency of PKC$\mu$ immunoprecipitates (data not shown).

Under the experimental conditions used here, i.e. coexpression of PKC$\mu$ with the indicated upstream kinases, the phosphorylation state of PKC$\mu$ immunoprecipitates only showed weak changes (Fig. 5A, upper panel), representing most probably a basal autophosphorylation. Expression levels of PKC$\mu$ immunoprecipitates (Fig. 5A, center panel) and transgenic expression (data not shown) were verified using Western blot analysis. Kinase activity and protein expression of all expression constructs used for cotransfection experiments were analyzed through in vitro kinase assays and immunoblots (Fig. 5A, right-hand panels).

PKD1 is able to phosphorylate and activate PKC$\eta$ in vitro (Fig. 4) in a similar way as PKC$\epsilon$ (4). In contrast, upon transient expression of PDK1 with PKC$\gamma$/PKC$\epsilon$ and PKC$\mu$, only PKC$\epsilon$ is activated, thus leading to PKC$\mu$ phosphorylation. Therefore, we measured the activation state of PKC$\epsilon$ and PKC$\eta$ upon coexpression with PDK1 and/or PKC$\mu$. As shown in Fig. 5B by the autophosphorylation and MBP phosphorylation of PKC$\mu$ immunoprecipitates, PKD1 activates PKC$\epsilon$ and PKC$\eta$. Upon additional coexpression of PKC$\mu$, PKC$\epsilon$ kinase activity is unaffected, whereas PKC$\eta$ kinase activity is reduced. Expression levels of PKC immunoprecipitates used for in vitro kinase assays as well as transgenic expression were monitored using Western blot analysis (data not shown).

Furthermore, we analyzed whether PKC$\eta$ phosphorylates PKC$\mu$ at amino-terminal regulatory serine residues, serving as a binding domain for regulatory proteins such as 14-3-3 (27). This could potentially result in the inhibition of PKC$\mu$ kinase activity, e.g. by binding of 14-3-3 proteins. The amino-terminal domain of PKC$\mu$ was transiently expressed as a GFP fusion protein and immunoprecipitated. The immunoprecipitates of constitutive active PKC$\eta$-GFP, kinase-dead PKC$\eta$-GFP (as a negative control), and PKC$\mu$ were used to phosphorylate PKC$\mu$-1–325GFP. As shown in Fig. 5C, strong phosphorylation of PKC$\mu$-1–325GFP could be obtained using PKC$\mu$ immunoprecipitates but not with PKC$\eta$-GFP immunoprecipitates. Immunoprecipitates were analyzed by Western blot for the presence of the respective enzymes and fusion proteins (data not shown). In accordance with experiments showing phosphorylation-dependent shifts (19) upon coexpression of PKC$\eta$ or PKC$\mu$ with PKC$\mu$-1–325GFP (data not shown), our data indicate that PKC$\eta$ does not phosphorylate amino-terminal serine residues of PKC$\mu$. These findings show that the inhibition of PKC$\eta$ upon coexpression of PDK1 and PKC$\mu$ must occur via a different mechanism.

Coexpression of PDK1, PKC$\epsilon$, and PKC$\mu$ Inhibits JNK Activation—Because in living cells, both novel PKC isoforms act differentially on the activation state of PKC$\mu$, downstream effectors may be similarly influenced. Several laboratories have shown that PKC$\mu$/PKD activation negatively interferes with the activation of the JNK pathway (13, 28, 29). JNK has been shown to colocalize with PKD upon overexpression in COS cells (30).

Therefore, we tested whether the PKC$\mu$ regulation of JNK activity is triggered via PKD1/PKC$\eta$ or PKD1/PKC$\epsilon$. JNK activation was measured upon transfection of the indicated expression constructs (Fig. 6) by the in vitro phosphorylation of glutathione S-transferase c-jun (lower panel). Transgenic expression of PKC$\epsilon$ and PKC$\mu$ were monitored by Western blot analysis of total cell lysates (center panels). TNF, tumor necrosis factor.

As shown by Western blot analyses, endogenous PKC$\epsilon$ is significantly expressed, but endogenous PKC$\eta$ cannot be detected in HEK293 cells (Fig. 6, center panels). These findings clearly indicate PKC$\epsilon$ as the biological relevant upstream kinase in HEK293 cells, triggering PKC$\mu$ downstream signaling pathways such as the JNK activation. At the same time, a PKC$\epsilon$-triggered pathway exists in HEK293 cells independently of PKC$\mu$ triggering JNK activation. The results of this study including published data (13) are shown in diagrammatic form in Fig. 7.

**DISCUSSION**

In this study, we have demonstrated that PKC$\epsilon$ acts as an upstream kinase in a similar way as previously shown for PKC$\eta$, directly phosphorylating PKC$\mu$ in vitro and enhancing its substrate phosphorylation efficiency. Using confocal analysis, we have also shown an intracellular colocalization of PKC$\epsilon$ and PKC$\mu$ in different cell types and a colocalization of the upstream kinase PDK1 with PKC$\epsilon$ and PKC$\eta$. The latter results were independently confirmed by biochemical coprecipitation. Although we are able to show a direct phosphorylation...
and activation of PKC\(\eta\) through PDK1 in vitro, PDK1 activation of PKC\(\eta\) does not lead to PKC\(\eta\) activation in vivo in HEK293 cells. In contrast to PKC\(\eta\), PDK1-mediated PKCe activation triggers in vivo PKC\(\mu\) activation, resulting in an inhibition of JNK. This indicates a PKD1/PKCe/PKC\(\mu\) activation cascade in HEK293 cells.

In a recent study, we have demonstrated that PKC\(\eta\) acts as an activation loop kinase for PKC\(\mu\) (13). These findings in accordance with earlier data from other laboratories (11, 12) indicate a direct phosphorylation of PKC\(\mu\) by PKC\(\eta\) and PKCe. In consequence, using purified PKCe/PKC\(\eta\) and PKC\(\mu\) enzymes, we are able to demonstrate the direct phosphorylation of PKC\(\mu\), leading to an enhanced enzymatic activity (Fig. 1) (13). Similarly to PKC\(\eta\), PKCe also colocalizes intracellularly with PKC\(\mu\) (Fig. 2), indicating a functional relationship with the Golgi compartment as both enzymes were reported to be localized and involved in Golgi-specific functions (23, 24).

The discovery of the intracellular colocalization of both PKC isotypes with PDK1 is of interest in terms of their functional relationship. For PKCe and PDK1, colocalization and activation has already been published previously (4). In this study, we demonstrate the biochemical colocalization for PKC\(\eta\) and PDK1 that is independent of either PKD1 or PKC\(\eta\) kinase activity (Fig. 3). The finding that kinase-dead PKC\(\eta\) migrates with a slower relative molecular weight in PDK1-immunoprecipitates already indicates a shift attributed to PDK1 phosphorylation. Consequently, we have demonstrated a direct phosphorylation of PKC\(\eta\) by PDK1, which in turn activates PKC\(\eta\) (Fig. 4).

PKC\(\mu\) activation is thought to be triggered by the initial phosphorylation of PDK1 at the activation loop followed by autophosphorylation in the hydrophobic motif, finally allowing cellular activation through lipids (for review see Ref. 17). The data published until now predict that PDK1 acts as the activation loop kinase either for conventional PKCs (16), atypical PKC (32), or novel PKCs (4), which is corroborated by our data showing activation loop phosphorylation of PKC\(\eta\) (Fig. 4). These findings are not unexpected as the primary structure of the activation loop of PKC isotypes is significantly conserved (17).

Fig. 7. Model of PDK1-mediated signaling leading to PKC\(\mu\) activation and triggering of downstream signaling pathways in HEK293 cells. PDK1 activates PKC\(\eta\) and PKCe. Activation of PKC\(\mu\) by PKC\(\eta\)/PDK1 leads to inhibition of PKC\(\eta\) (dotted lines). Activation of PKC\(\mu\) by PKCe/PDK1 inhibits JNK. EGF receptor, EGFR, EGF receptor.

The spatio temporal aspects of PKC\(\mu\) activation are currently unclear. As activated enzyme is located at the Golgi, activation could occur by recruiting PKCs and PDK1 to the Golgi. PKCe has been reported to be localized via the C1 domain at the Golgi (23). PDK1 could be recruited via direct binding to PKC (Fig. 4) (4) or by binding to Golgi-generated phosphatidylinositol phosphate 2 (33), which has been shown to bind the PH domain of PDK1 (34).

Taking into account the in vitro data showing activation of PKC\(\eta\) by PDK1, it is of interest to note that upon transient expression of PDK1 with PKCe or PKC\(\eta\) and PKC\(\mu\), activation of PKC\(\mu\) occurs only through PKCe (Fig. 5). Upon coexpression of PDK1 and PKC\(\eta\) with PKC\(\mu\), PKC\(\mu\) is inhibited (Fig. 5B), which points to a negative regulation through PDK1 and PKC\(\mu\). As PKC\(\mu\) is downstream PKC\(\eta\), a negative feedback loop regulated by PKC\(\mu\) can be postulated. We could further exclude the inhibition of PKC\(\mu\) via direct phosphorylation of amino-terminal serines through PKC\(\eta\) (Fig. 5C). These serines are considered as binding domains for negatively regulatory proteins as 14-3-3 (27, 35).

Selective activation of PKC\(\mu\) through PKCe further acts on downstream signaling cascades such as JNK. The PDK1 dependence of PKCs explains our earlier findings of expressing a constitutive active mutant of PKC\(\eta\) with PKC\(\mu\) and thus inhibiting JNK activity. This mutant (PKC\(\eta\);A,R) most probably bypasses the need for PDK1 activation, leading to PKC\(\mu\) activation and inhibition of JNK (13). The finding that PDK1 and PKCe coexpression in HEK293 cells leads to enhanced JNK activation indicates a PKCe-dependent JNK activation, which has to be distinguished from the opposite effect, namely the PKCe/PKC\(\mu\) mediated inhibition of JNK activity. PKCe is involved in several cellular functions in which the PKC\(\mu\)-mediated effect seems to be dominant via PKCe-mediated JNK activation.

A recent study reported a negative regulation of JNK through PKD by the physical interaction of activated PKD with JNK, leading to the inhibition of JNK and c-jun phosphorylation by the sterical blocking of substrate access (30). In an earlier study, direct phosphorylation of Thr-654/Thr-669 of the EGF receptor via PKC\(\mu\) has been suggested. This probably leads to negative interference with EGFR-induced JNK activation through the PDGF-mediated activation of PKC\(\mu\) (see Fig. 7) (29). Both models complement each other, implicating a PDGF-induced activation of PKC\(\mu\)/PKD via PDK1/PKCe and leading to the phosphorylation of the EGF receptor and, in parallel, the complexing of JNK with PKC\(\mu\)/PKD. This would provide a potential parallel switch effectively shutting down JNK via PKC\(\mu\) activation.

PKC\(\mu\) activation through distinct PKC subtypes probably reflects cell-type/tissue-specific functions. PKC\(\eta\) expression has been associated with the cellular differentiation of keratinocytes (36), which do strongly express PKC\(\mu\) (37). In contrast, PKC\(\eta\) is not detected in HEK293 cells, whereas endogenous PKCe is expressed significantly higher (Fig. 6, center panel), pointing to an endogenous PKC\(\mu\) activation via PKCe. Keratinocyte differentiation is dependent upon PKC\(\eta\) (38) and is accompanied by the down-regulation of EGF receptor signaling (39). These findings point to a role played by PKC\(\eta\) activated via PDK1, thus, phosphorylating the activation loop of PKC\(\mu\) and resulting in the phosphorylation of the EGF receptor and to a role in initial signal events preceding cellular differentiation in this cell type.

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REFERENCES

1. Toker, A. (1998) *Front Biosci.* 3, D1134–D1147
2. Black, J. D. (2000) *Front Biosci.* 5, D406–D423
3. Diaz-Meco, M. T., Municio, M. M., Sanchez, P., Lozano, J., and Moscat, J. (1996) *Mol. Cell. Biol.* 16, 105–114
4. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) *Science* 281, 2042–2045
5. Johannes, F. J., Prestle, J., Eiu, S., Oberhagemann, P., and Pfizenmaier, K. (1994) *J. Biol. Chem.* 269, 6140–6148
6. Valverde, A. M., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 8572–8576
7. Hayashi, A., Steki, N., Hattori, A., Kazuma, S., and Saito, T. (1999) *Biochim. Biophys. Acta* 1450, 89–106
8. Sturany, S., Van Lint, J., Muller, F., Wilda, M., Hameister, H., Hocker, M., Brey, A., Gern, U., van den Bergh, T., and Sauer, M. (2001) *J. Biol. Chem.* 276, 3310–3318
9. Gschwendt, M., Johannes, F. J., Kittstein, W., and Marks, F. (1997) *J. Biol. Chem.* 272, 20742–20748
10. Gibson, T. J., Hyvonen, M., Muscaro, A., Saraste, M., and Birney, E. (1994) *Trends Biochem. Sci.* 19, 349–353
11. Zugaza, J. L., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1996) *EMBO J.* 15, 6220–6230
12. Waldron, R. T., Iglesias, T., and Rozengurt, E. (1999) *J. Biol. Chem.* 274, 9224–9230
13. Brandlin, I., Hubner, S., Eissler, T., Muciños, A., Henschel, A., Hauser, A., Link, G., Rupp, S., Storz, P., Pfizenmaier, K., and Johannes, F. J. (2002) *J. Biol. Chem.* 277, 6490–6498
14. van Lint, J., Ni, Y., Valius, M., Merlevede, W., and Vandenheede, J. R. (1998) *J. Biol. Chem.* 273, 7038–7043
15. Hauser, A., Storz, P., Hubner, S., Brandlin, I., Martinez-Moya, M., Link, G., and Johannes, F. J. (2001) *FEBS Lett.* 492, 39–44
16. Dutli, E. M., Toker, A., and Newton, A. C. (1998) *Curr. Biol.* 8, 1366–1375
17. Storr, P., and Toker, A. (2002) *Front Biosci.* 7, D136–D152
18. Johannes, F. J., Horn, J., Link, G., Haas, E., Sinmienski, K., Wajant, H., and Pfizenmaier, K. (1998) *Eur. J. Biochem.* 257, 47–54
19. Hauser, A., Link, G., Bamberg, L., Burzlaff, A., Lutz, S., Pfizenmaier, K., and Johannes, F. J. (2002) *J. Cell Biol.* 156, 65–74
20. Dieterich, S., Herget, T., Link, G., Bottinger, H., Pfizenmaier, K., and Johannes, F. J. (1996) *FEBS Lett.* 381, 183–187
21. Johannes, F. J., Prestle, J., Dieterich, S., Oberhagemann, P., Link, G., and Pfizenmaier, K. (1995) *Eur. J. Biochem.* 227, 303–307
22. Wajant, H., Johannes, F. J., Haas, E., Siewierski, K., Schwenzer, R., Schulz, C., Weiss, T., Grell, M., and Schur, C. (1998) *Curr. Biol.* 8, 113–116
23. Lefebvre, C., Olah, Z., Jakab, G., and Anderson, W. B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 1406–1410
24. Prestle, J., Pfizenmaier, K., Brenner, J., and Johannes, F. J. (1996) *J. Cell Biol.* 134, 1401–1410
25. Maeda, Y., Beznoussenko, G. V., Van Lint, J., Mironov, A. A., and Malhotra, V. (2001) *EMBO J.* 20, 5982–5990
26. Toker, A., and Newton, A. C. (2000) *Cell* 103, 185–188
27. Hauser, A., Storz, P., Link, G., Stoll, H., Liu, Y. C., Altman, A., Pfizenmaier, K., and Johannes, F. J. (1999) *J. Biol. Chem.* 274, 9258–9264
28. Hurdl, C., and Rozengurt, E. (2001) *Biochim. Biophys. Res. Commun.* 282, 404–408
29. Bagowski, C. P., Stein-Gerlach, M., Choidas, A., and Ullrich, A. (1999) *EMBO J.* 18, 5567–5576
30. Hurd, C., and Rozengurt, E. (2002) *Oncogene* 21, 2154–2160
31. Li, R. C., Ping, P., Zhang, J., Weid, W. B., Cao, X., Gao, J., Zheng, Y., Huang, S., Han, J., and Ren, R. (2000) *Ann. J. Pathol.* 179, H1679–H1689
32. Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S., and Toker, A. (1998) *Curr. Biol.* 8, 1069–1077
33. Jones, D. H., Morris, J. B., Morgan, C. P., Kondo, H., Irvine, R. P., and Cockcroft, S. (2000) *J. Biol. Chem.* 275, 13962–13966
34. Currie, R. A., Walker, R. S., Gray, A., Deak, M., Casamayor, A., Downes, C. P., Cohen, P., Alessi, D. R., and Lucocq, J. (1999) *Biochem. J.* 337, 575–583
35. Vernet, M., Schütt, H., Eiermann, A. M., Merlevede, W., Vandenheede, J. R., and Van Lint, J. (2000) *J. Biol. Chem.* 275, 19567–19576
36. Ohba, M., Ishino, K., Kashiwagi, M., Kawabe, S., Chida, K., Huh, H. N., and Kuroki, T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 19567–19576
37. Tomonaga, S., Ito, S., Matsui, K., Ishido, K., Ohba, M., Ohba, M., and Kuroki, T. (1999) *Science* 281, 5199–5207
38. Rennecke, J., Rehberger, P. A., Turnebender, G., Johannes, F. J., Stohr, M., Marks, F., and Richter, K. H. (1999) *Int. J. Cancer* 80, 98–103
39. Cahoon, S., Calin, C., Talora, C., Kuroki, T., Stein, P. L., and Dotto, G. P. (2000) *Cancer Cell* 1, 211–2129
40. Medema, J. P., Sark, M. W., Backendorf, C., and Bos, J. L. (1994) *Mol. Cell. Biol.* 14, 7078–7085

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Ilona Brändlin, Tim Eiseler, Rüdiger Salowsky and Franz-Josef Johannes

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