Regulation of intermediary metabolism by the PKCδ signalosome in mitochondria

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ABSTRACT **PKCδ has emerged as a novel regulatory molecule of oxidative phosphorylation by targeting the pyruvate dehydrogenase complex (PDHC). We showed that activation of PKCδ leads to the dephosphorylation of pyruvate dehydrogenase kinase 2 (PDK2), thereby decreasing PDK2 activity and increasing PDH activity, accelerating oxygen consumption, and augmenting ATP synthesis. However, the molecular components that mediate PKCδ signaling in mitochondria have remained elusive so far. Here, we identify for the first time a functional complex, which includes cytochrome *c* as the upstream driver of PKCδ, and uses the adapter protein p66Shc as a platform with vitamin A (retinol) as a fourth partner. All four components are necessary for the activation of the PKCδ signal chain. Genetic ablation of any one of the three proteins, or retinol depletion, silences signaling. Furthermore, mutations that disrupt the interaction of cytochrome *c* with p66Shc, of p66Shc with PKCδ, or the deletion of the retinol-binding pocket on PKCδ, attenuate signaling. In cytochrome *c*-deficient cells, reintroduction of cytochrome *c* Fe^{3+} protein restores PKCδ signaling. Taken together, these results indicate that oxidation of PKCδ is key to the activation of the pathway. The PKCδ/p66Shc/cytochrome *c* signalosome might have evolved to effect site-directed oxidation of zinc-finger structures of PKCδ, which harbor the activation centers and the vitamin A binding sites. Our findings define the molecular mechanisms underlying the signaling function of PKCδ in mitochondria.—Acin-Perez, R., Hoyos, B., Gong, J., Vinogradov, V., Fischman, D. A., Leitges, M., Borhan, B., Starkov, A., Manfredi, G., Hammerling, U. Regulation of intermediary metabolism by the PKCδ signalosome in mitochondria. *FASEB J.* 24, 5033–5042 (2010). www.fasebj.org**

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Among the cellular compartments where protein kinase Cδ (PKCδ) localizes, the mitochondria are especially significant. There, PKCδ first came to the attention of researchers investigating its apoptogenic properties (1). Subsequently, PKCδ was linked to disease processes, such as ischemia reperfusion damage in heart infarct and stroke (2–7). While these pathobiology studies indicate an important role in disease, signal pathways for PKCδ in normal mitochondria have yet to be clarified in detail. Recent studies from our laboratories on the role of vitamin A in cell survival (8) implicated PKCδ in the regulation of oxidative phosphorylation. We identified a signal chain unique to mitochondria that controlled respiration by regulating the pyruvate dehydrogenase complex (PDHC) (9).

A unique feature for the PKCδ signal chain was its dependence on the presence of vitamin A. Like other members of the serine/threonine family of kinases, PKCδ contains 2 vitamin A binding sites located in the PKC zinc-finger domains (10–12). Occupancy of these sites by vitamin A (retinol) rendered PKCδ permissible for redox activation (9). We and others had proposed previously that oxidation of cysteines in the zinc-finger domains was the critical initiation step for redox activation of PKC (13, 14).

While many questions remain unanswered on the function of PKCδ in mitochondria (*e.g.*, the targeting to the PDHC, the precise signal pathway, and the possible crosstalk with other PKC isoforms), none is more obscure than the upstream signals activating PKCδ. Diacyl-glycerol (DAG) and phorbol ester, its pharmacomimetic, were shown to bind the PKC zinc-finger domains (15–18). The local hydrophobic patch generated in this reaction was thought to guide PKC to membrane sites where the lipid environment promoted unfolding of the molecule and substrates awaited site-specific phosphorylations (19). However, the alternate mechanism of PKC activation by oxidation

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(20, 21) put the "hydrophobic patch" hypothesis into question by showing membrane translocations and substrate specificity similar to those achieved with lipid second messengers but without the need for lipid adducts. Although experimentally well supported, the redox mechanism hypothesized to underlay PKC activation contained some gaps. Neither was the source of the oxidizing agents, nor was the chemistry of how oxidation could confer enzymatic competence, known. One means of safeguarding such a requirement is by protein–protein interactions in the form of the "handshake" mechanism (22), as commonly transacted by kinases and phosphatases.

That *in vivo* redox activation of PKC involved the modification of specific sites was implied by our finding that vitamin A was essentially a redox "catalyst" (10). This cofactor bound PKC specifically at the same domains (*i.e.*, the zinc-finger domains) where DAG, phosphotidyl-serine, or phorbol ester bind as well (11, 12). In the case of PKCδ, the replacement of its 2 vitamin A binding domains with the nonbinding C1b domains of PKCα resulted in the loss of redox responsiveness, without affecting responsiveness to phorbol ester, strongly suggesting that site-specific oxidation was the key initiating event (9).

We searched for an oxidoreductase system in which PKCδ might be oxidized by protein–protein interactions in a site-specific handshake mechanism, providing spatial and temporal specificity. The literature localizes PKCδ to the intermembrane space of mitochondria (1), where it partners with the adaptor protein, p66Shc, and with cytochrome *c* (23, 24). As mentioned above, retinol binds PKCδ (12) and thus qualifies as a third partner. We report here that *in organello* PKCδ activation results from its interaction with the oxidized form of cytochrome *c*, is dependent on retinol bound to the zinc-finger domain of PKCδ, and requires the presence of p66Shc.

Our results indicate the existence of a signalosome that gauges the redox state of cytochrome *c*, responds by enzymatic activation, and transmits an activating signal to the PDH complex for increased flux of fuel into the Krebs cycle. This is the first demonstration of a redox signaling mechanism in mammals that involves cytochrome *c* and works by protein–protein interaction. It also implicates retinol as an electron bridge enabling the site–specific oxidation of PKCδ.

MATERIALS AND METHODS

Biological reagents and expression vectors

The PKC antagonist, GO6976, was obtained from Calbiochem (San Diego, CA, USA), Phorbol myristoyl acetate (PMA) was from Sigma-Aldrich (St. Louis, MO, USA). Horse heart cytochrome *c* was purchased from Sigma-Aldrich. Holo-RBP and His-tagged holo-CRBP-I were expressed in *Escherichia coli*, using vectors generously donated by Silke Vogel (Columbia University, New York, NY, USA). RBP was purified as described (25), and His-tagged CRBP-I was purified by affinity chromatography on Ni column (Invitrogen Life Sciences, Carlsbad, CA, USA) following instructions of the manufacturer. Horse heart cytochrome *c* was purchased from Sigma-Aldrich. It was converted to cytochrome c^{2+} by reduction with ascorbic acid.

The following Western blot antibodies were used: anti-PDHE1, anti-COXIV and anti-VDAC (Invitrogen); anti-hsp60 (Stressgen, Ann Arbor, MI, USA); anti-GAPDH (Abcam, Cambridge, MA, USA); anti-PKCδ, anti-PKCε, anti-p66Shc, anti-cytochrome *c*, and anti-Tim23 (BD Biosciences, San Jose, CA, USA); anti-phospho-PKCδ (Thr505) (Cell Signaling, Boston, MA, USA); and anti-phospho-PDHE1 (Ser-293 (Novus Biologicals, Littleton, CO, USA). The pBABE-puro and MigR1 retroviral mammalian expression vectors were purchased from Addgene (Cambridge, MA, USA). The expression vector encoding mutant PKCδ Y332F was generously donated by Ushio Kikkawa (Kobe University, Kobe, Japan) (26). Mutations at E132⇒Q and E133⇒Q of *p66Shc*, to generate a cytochrome *c* nonbinding *p66Shc*, were performed by Quickchange (Stratagene, Inc., Santa Clara, CA, USA), as suggested in Giorgio *et al.* (24). 11,12-Dihydroretinol (DH-Rol) was synthesized as described previously (27).

Mouse strains

p66Shc$^{-/-}$ mice were maintained at Sloan-Kettering Institute from founders originally donated by Guiseppe Pelicci (University of Milan, Milan, Italy). C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

Cell lines

Mouse embryonic fibroblasts (MEFs) were derived from 13.5-d-old embryos of C57BL/6 or p66Shc$^{-/-}$ mice. The PKCδ$^{-/-}$ MEF cell line was provided by M.L. For cytochrome *c* (somatic) knockdown, the lentiviral vector pLKO.1-puro with the target sequence CCGGGCAGACCTAATAGCTTATCTTCTCGAGAAGATAAGCTATTGGTCTGCTTTTTG was used. Viral particles generated in HEK293T cells by cotransfection with packaging plasmids pMD2 and psPAX2 were used for transduction of MEFs. Puromycin (4 μg/ml) resistant cells were expanded and analyzed for cytochrome *c* expression levels by quantitiative PCR using a 1-step SYBR Green kit (Invitrogen Life Sciences), and by Western blot. The levels of cytochrome *c* transcript and protein were normalized against GAPDH.

Cell culture and transfection

MEFs were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, 1 mM pyruvate, and 4.5 g/L glucose. For vitamin A depletion, cells were incubated for 18 h in serum-free TLB medium (DMEM supplemented with 4.5g/L glucose, 0.05% bovine serum albumin, 5 mg/L transferrin, 1 μM linoleic acid, and 2 mM glutamine).

Reintroduction of full-length wild-type (wt) PKCδ gene and the retinol nonbinding mutant PKCδ has been reported previously (9). Reintroductions of the mutant PKCδ Y332F, p66Shc wt, and the double-mutant E132Q;E133Q p66Shc were performed using pBABE retroviral vector, essentially as described for wt PKCδ. Recipient cells were the respective knockout cell lines.

Measurements of oxidative phosphorylation in cells and isolated mitochondria

Intact cells (1.5×10^6) were used for O_2 consumption measurements in an oxygraph equipped with a Clark electrode.

Mouse liver mitochondria were isolated as described previously (28), and state III O_2 consumption driven by specific respiratory chain complexes was measured on 75–100 μg of mitochondrial protein, as described previously (29). All reagents were purchased from Sigma-Aldrich.

Pyruvate dehydrogenase activity was determined spectrophotometrically in isolated mitochondria (100–300 μg of protein) by measuring the increase in absorbance at 340 nm of a reaction medium containing 20 mM HEPES; 0.2 mM $MgCl_2$; 0.05 mM $CaCl_2$; 0.3 mM cocarboxylase; 0.5 mM NAD^+; 1 mM DTT; 5 mM pyruvate, and 0.24 mM coenzyme A.

ATP synthesis in isolated mitochondria (15–25 μg of protein) or in cells permeabilized with digitonin (1×10^6 cells) was measured using the kinetic luminescence assay described by Vives-Bauza *et al.* (30).

Titration of retinoids

Dose-response relations of DH-Rol and retinol in isolated mitochondria were determined by incubating mitochondria with graded concentrations of DH-Rol, retinol, or both for 10 min at 37C, using albumin as carrier. ATP synthase activity was measured as above. In some experiments retinol was delivered with titered doses of holoRBP or holoCRBP, respectively.

Mitoplast preparation for PKCδ localization within mitochondria

Mitoplasts from crude liver mitochondria were prepared as described previously (31). Briefly, mitochondria 500 μg (1 mg/ml) were resuspended in MS-EGTA (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4). Water (1:10, vol/vol) and digitonin (1 mg digitonin/5 mg mitochondrial protein) were added, and the mixture was incubated on ice for 45 min. Then, KCl (150 mM) was added, followed by incubation for 2 min on ice, and centrifugation at 18,000 g for 20 min at 4°C. The pellet containing the mitoplast fraction was resuspended at 1 mg/ml in 300 mM Tris-HCl and 10 mM $CaCl_2$, pH 7.4. The supernatant containing the postmitoplast fraction was precipitated with 12% TCA and centrifuged at 18,000g for 15 min at 4°C. The pellet was resuspended in 500 ml acetone and centrifuged at 18,000 g for 15 min at 4°C. The final pellet was then suspended in 50 μl of 2× Laemmli sample buffer.

Mitoplast preparation for cytochrome *c* import

Mitoplasts for cytochrome *c* import were prepared from MEFs that were cultured for 18 h in serum-free TLB medium. Mitochondria were isolated by homogenization of the cell pellets in buffer A (0.32 M sucrose, 1 mM EDTA, and 10 mM Tris–HCl, pH 7.4) followed by centrifugation at 1000 g for 5 min. Supernatant containing mitochondria was then centrifuged at 20,000 g for 10 min, and mitochondria were resuspended at 1 mg/ml in MAITE buffer (10 mM Tris-HCl, pH 7.4; 25 mM sucrose; 75 mM sorbitol; 100 mM KCl; 10 mM K_2HPO_4; 0.05 mM EDTA; 5 mM $MgCl_2$; and 1 mg/ml BSA). Digitonin (1 mg digitonin/5 mg mitochondrial protein) was added, and the mixture was incubated on ice for 45 min. Then, cytochrome *c* (20 mM) or the combination of 20 mM cytochrome *c* plus 2 μM retinol was added to the mitoplasts and incubated at 37°C for 10 min. Pyruvate + malate-driven respiration was measured using 50 μg of the mitoplast preparation.

Immunoblot analyses

To determine the phosphorylation levels of PKCδ and PDHE1 in mitochondria and mitoplasts, 10 μg of protein was separated by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE), electroblotted onto PVDF filters (Bio-Rad, Hercules, CA, USA), and immunoblotted with the appropriate antibodies. To determine the levels of cytochrome *c* after silencing, 50 μg of total cell lysate was separated under the same conditions as described above, electroblotted, and detected using the appropriate antibodies. For coimmunoprecipitation analyses, 100 μg of lysates of purified mitochondria was treated with anti-cytochrome *c* antibody, and immune complexes were collected on protein G beads overnight. Antigens were eluted from beads with Laemmli sample buffer and analyzed by immunoblot as above.

Analysis of the phosphoproteome of mitochondria

For isoelectric focusing of mitochondrial samples, 100 μg of protein was processed with a Ready Prep 2D Cleanup kit (Bio-Rad) and resuspended in 125 μl of rehydration buffer (Bio-Rad). Samples were applied to 3–10 IPG strips (Bio-Rad) and incubated overnight at room temperature. Isoelectric focusing and 2-D SDS-PAGE were run under standard conditions, and proteins were transferred to PDVF membranes.

Statistical analyses

Comparisons between groups were made using 1-way ANOVA. Pairwise comparisons were made by *post hoc* Fisher PLSD test. Differences were considered statistically significant at $P < 0.05$. Data analyses were performed using the statistical program StatView (Adept Scientific, Bethesda, MD, USA). In all figures, error bars indicate SD.

RESULTS

PKCδ forms a complex with p66Shc in the cytosol and membrane compartments (26), and cytochrome *c* associates with p66Shc in mitochondria (23, 24). On the basis of susceptibility of PKCδ to proteolysis by proteinase K in mitoplasts, we localized this isoform in the intermembrane space of mitochondria (IMS) where p66Shc and cytochrome *c* are known to reside as well (**Fig. 1*A***). Immunoprecipitation experiments revealed the existence of a trimeric PKCδ/p66Shc/cytochrome *c* complex in the IMS of MEFs (Fig. 1*B*). Of note, immunoprecipitation with anti-cytochrome *c* antibody coprecipitated p66Shc and PKCδ, reducing the risk of contaminating mitochondria with cytoplasmic complexes. PKCε, another isoform known to localize to mitochondria (32), was excluded from this complex (data not shown). The interacting face on p66Shc, where cytochrome *c* binds, was defined by Giorgio *et al.* (24). When we mutated the respective contact amino acids, E132Q and E133Q, the trimeric complex was reduced to dimeric PKCδ/p66Shc. Similarly, mutating the Shc recognition site on PKCδ, Y332 to F (26), disrupted the trimeric complex (Fig. 1*C*). However, mutation of the retinol binding sites on PKCδ by domain exchange with the nonbinding C1b domain of

PKCα, which disrupts redox activation of PKCδ (9), did not interfere with formation of the trimeric complex (Fig. 1*C*).

The principal role of members of the shc family of adapters is to assemble signal complexes. We compared wt with *p66Shc*$^{-/-}$ mitochondria for the ability to sustain respiration at levels commonly observed in vitamin A-sufficient states. As shown in **Fig. 2*A, B***, isolated wt liver mitochondria showed prompt up-regulation of oxygen consumption and ATP synthesis when vitamin A was supplied at the physiological concentration of 2 μM with albumin as carrier. PMA up-regulated ATP synthase activity independently of retinol. Ablation of *p66Shc* resulted in reduced respiration and ATP production. In absence of p66Shc, liver mitochondria proved unresponsive to retinol (Fig. 2*A–E*), but this was partially corrected by PMA. Since PDH is a target of PKCδ signaling (9), we assayed *wt* and *p66Shc*$^{-/-}$ mitochondria for enhanced PDH output in response to retinol (Fig. 2*C*). While *wt* liver mitochondria showed a 20% increase in the PDH enzyme rate in the presence compared to the absence of retinol, no such enhancement was observed in *p66Shc*$^{-/-}$ mitochondria. Therefore, p66Shc was necessary, but not sufficient, to increase PDH activity *via* activation of the PKCδ pathway, in the presence of retinol.

Oxidative phosphorylation is fundamental to mammalian cells, and hence regulatory circuits are likely to be shared by many cell types. Because MEFs contained the tertiary PKC/p66Shc/cytochrome *c* complex, we tested these cells for responsiveness to retinol. As described previously, the rates of both oxygen consumption and ATP synthase activity were increased in vitamin A-sufficient cells, compared to vitamin A-depleted cultures (Fig. 2*D, E*), but this difference disappeared in *p66Shc*$^{-/-}$ cells. When activated, PKCδ caused the dephosphorylation of the regulatory E1 subunit of the PDH complex (33), as evidenced by the increase in the isoelectric point. However, this shift toward higher pI was not observed in *p66Shc*$^{-/-}$ cells (Fig. 2*F*).

Knowing that p66Shc was obligatory in mitochondria (Fig. 2*A, B*), we ascertained that it needed to interact with PKCδ in order to up-regulate respiration. We showed that PKCδ was required since its genetic ablation resulted in significantly reduced baseline oxidative phosphorylation (**Fig. 3*A, B***). Reintroduction of the wt gene restored respiration to normal levels (Fig. 3*A*). We then mutated the requisite binding site on PKCδ, Y332, recognized by the p66Shc SH2 domain (26). The mutant *PKCδ Y332F* gene did not complement the *PKCδ* $^{-/-}$ phenotype (Fig. 3*B*).

P66Shc partners with cytochrome *c* (24). To test whether an association of cytochrome with p66Shc was required for PKCδ activation, we expressed the mutant p66Shc E132Q;E133Q, which is unable to bind cytochrome *c* (24), in p66Shc$^{-/-}$ MEFs. Mitochondria of these knock-in mutant cells, like those of p66Shc-null cells, displayed suppressed baseline respiration. This condition was not up-regulated by retinol, whereas reintroduction of wt *p66Shc* restored the PKCδ signal pathway (Fig. 3*C*). Measurements of ATP synthase rates yielded concordant results (Fig. 3*D*). These findings, together with the need for retinol to occupy specific binding pockets on PKCδ

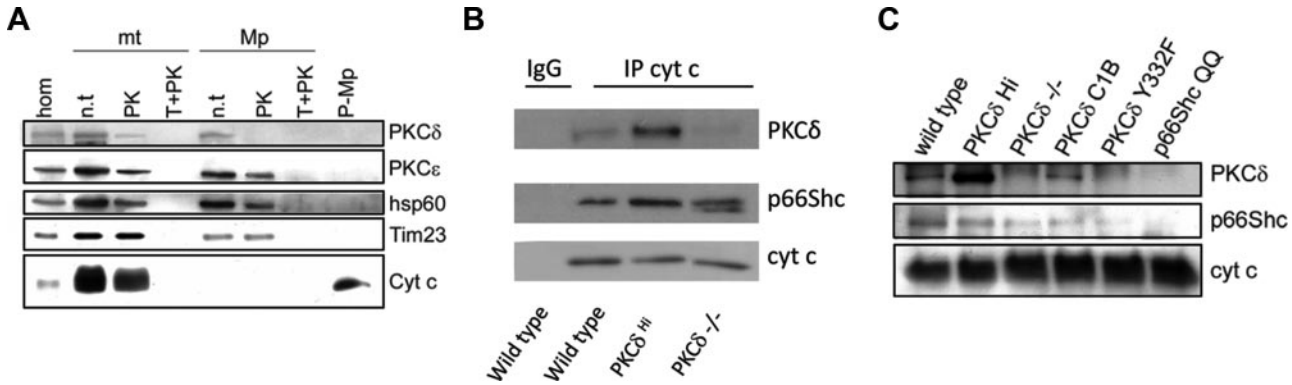

Figure 1. *A*) Location of PKC isoforms. Mitochondria (mt) and mitoplasts (Mp) of mouse embryo fibroblasts were treated with protease K (PK) or PK after Triton solubilization of the membranes, or left untreated (n.t.). These were immunoblotted for PKCδ, PKCε, Hsp60, Tim23, and cytochrome *c* (cyt c), along with mitochondria total lysate (hom) and supernates of postmitoplast preparation (P-Mp). PKCδ was present in both mt and Mp, but was sensitive to PK treatment in the latter but not the former, indicating location in the intermembrane space. PKCε resisted PK digestion in Mp and mt and hence resided in the matrix. *B*) PKCδ, p66Sch, and cyt c form a trimeric complex. Extracts of wild-type (WT) MEFs, MEFs overexpressing PKCδ (PKC^{Hi}), or PKCδ-null MEFs (PKCδ$^{-/-}$) were immunoprecipitated (IP) with anti-cyt c antibody (or control IgG), and the precipitates were immunoblotted (IB) for PKCδ, p66Shc, or cyt c. Coprecipitation of PKCδ and p66Shc with cyt c indicated binding to the same complex. *C*) PKCδ *Y332F* and p66Shc *QQ* mutants fail to form trimeric complex. Mitochondria were purified from the following cell lines: WT MEFs, PKCδ-overexpressing MEFs (PKCδ Hi), PKCδ$^{-/-}$ MEFs, PKCδ$^{-/-}$ MEFs reconstituted with retinol nonbinding PKCδ(PKCδ C1b), PKCδ$^{-/-}$ MEFs reconstituted with PKCδ Y332F mutation (PKCδ Y332F), and p66Shc$^{-/-}$ MEFs reconstituted with p66Shc E132Q;E133Q mutation (p66Shc QQ). Lysates were immunoprecipitated with anti-cyt c antibody, and precipitates were analyzed by Western blotting with anti-PKCδ, anti-p66Shc, or anti-cyt c antibodies.

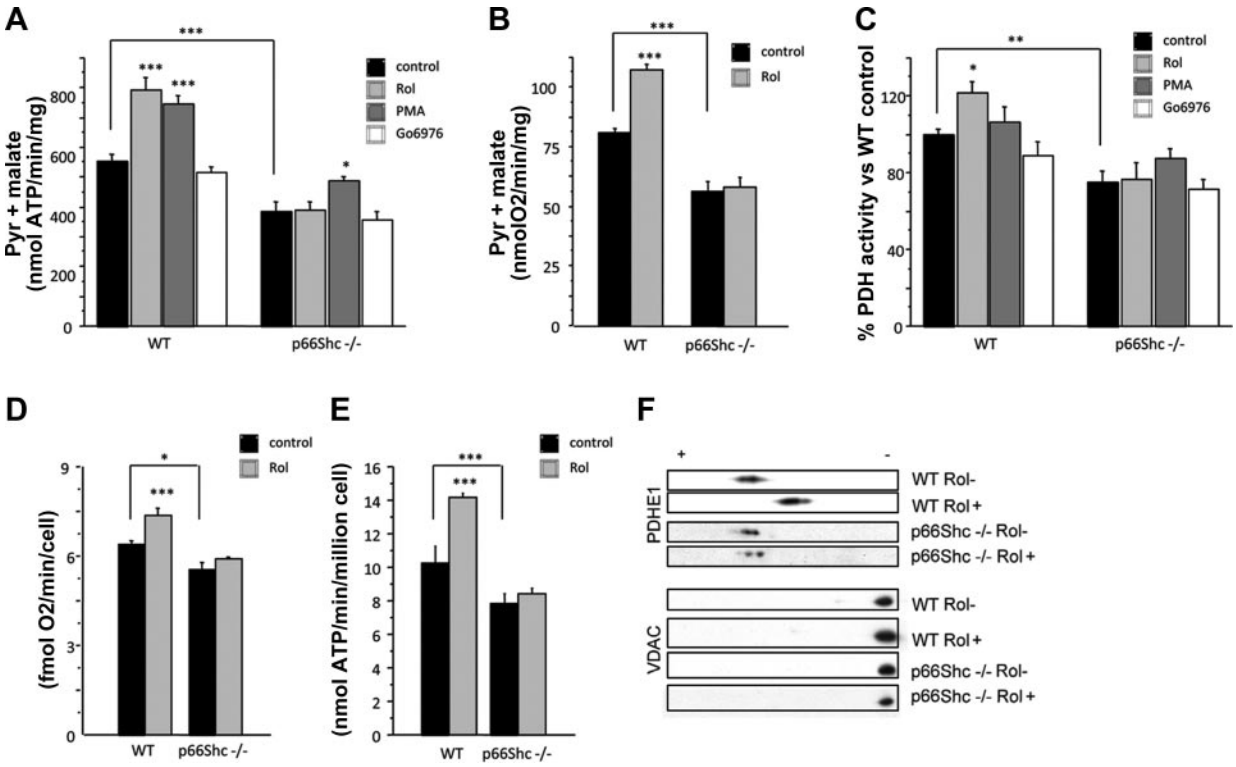

Figure 2. P66Shc is required for PKCδ-dependent signaling to PDH. *A–E*) Pyruvate/malate-dependent ATP synthase rate (*A*), respiration (*B*), or PDH activity (*C*) are up-regulated by retinol-dependent activation of PKCδ in liver mitochondria of wild type, but not p66Shc$^{-/-}$ mice, or in intact WT, but not p66Shc$^{-/-}$ MEFs (*D, E*). Baseline levels of respiration, ATP synthesis, and PDH activity were significantly reduced in p66Shc$^{-/-}$ compared to WT liver mitochondria or MEFs. Treatment with phorbol ester (PMA) partially overrode dependence on p66Shc. *F*) Phosphorylation patterns revealed by 2-D gel analysis indicated that presence of retinol favored the dephosphorylated species of pyruvate dehydrogenase regulatory subunit E1 (PDHE1), compared to retinol-deficiency, while genetic deletion of *p66Shc* attenuated dephosphorylation by retinol action. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(9), suggested that a quarternary complex, PKCδ/retinol/p66Shc/cytochrome *c*, formed the functional core of a signalosome controlling PDH function.

To investigate this concept further and to determine whether the oxidized form of cytochrome *c* was involved in PKCδ activation, we generated MEFs in which 50–90% of the cytochrome *c* pool was depleted by shRNA knockdown (**Fig. 4*A***). Mitochondria of these cells displayed low respiratory capacity but contained a fully functional PDH complex. When oxidized cytochrome *c* was reintroduced in the IMS by permeabilizing the membranes with a mild digitonin treatment, respiration was restored to wt levels, and retinol produced an additional increase in oxygen consumption. The latter effect was dependent on PKCδ, since mitoplasts of MEFs deficient in both cytochrome *c* and PKCδ did not respond to retinol (Fig. 4*B*).

To test whether cytochrome *c* needed to bind p66Shc, we tested mitoplasts (*i.e.*, permeabilized mitochondria) of MEFs with dual p66QQ-knock-in and cytochrome *c*-knockdown mutations. We observed neither normalization of oxygen consumption by cytochrome *c* addition nor up-regulation by retinol, suggesting that complex formation of cytochrome *c* with p66Shc was necessary for signaling to PDH (Fig. 4*C*).

We further showed that the phosphorylation patterns of both PKCδ and PDHE1 were appropriately modified when oxidized cytochrome *c* was given to cytochrome *c* knockdown mitoplasts, revealing increased phosphorylation of PKCδ T505, and decreased phosphorylation of PDHE1 (Fig. 4*D*, *E*). However, in the absence of the cytochrome *c* docking site on p66Shc, PDHE1 remained largely phosphorylated (Fig. 4*E*), suggesting that no activation occurred.

To confirm that the oxidized—and not the reduced—form of cytochrome *c* was the activator of this signalosome, we took advantage of the G6930A cell line, which carries an homoplasmic mutation in cytochrome *c* oxidase subunit I, and completely lacks cytochrome *c* oxidase activity (34). Therefore, cytochrome *c* is not oxidized by the enzyme and accumulates in its reduced form. Addition of oxidized cytochrome *c* together with retinol to mitoplasts from G6930A cells showed a reduction in the amount of phosphorylation of PDHE1, due to activation of the signalosome. However, with reduced cytochrome *c*, no change in phosphorylation status occurred (Fig. 4*G*).

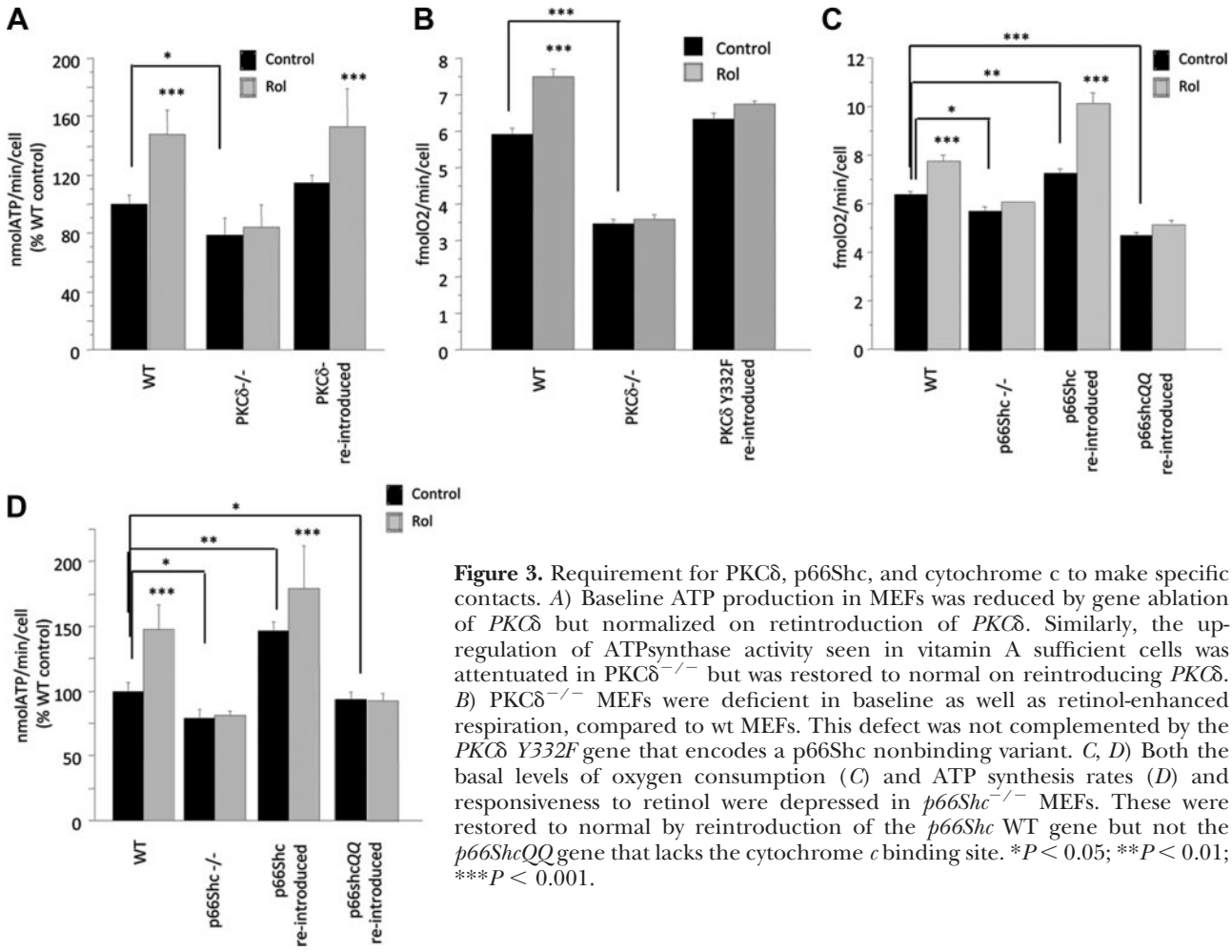

Figure 3. Requirement for PKCδ, p66Shc, and cytochrome c to make specific contacts. *A*) Baseline ATP production in MEFs was reduced by gene ablation of *PKC*δ but normalized on retintroduction of *PKC*δ. Similarly, the upregulation of ATPsynthase activity seen in vitamin A sufficient cells was attentuated in PKCδ$^{-/-}$ but was restored to normal on reintroducing *PKC*δ. *B*) PKCδ$^{-/-}$ MEFs were deficient in baseline as well as retinol-enhanced respiration, compared to wt MEFs. This defect was not complemented by the *PKC*δ *Y332F* gene that encodes a p66Shc nonbinding variant. *C*, *D*) Both the basal levels of oxygen consumption (*C*) and ATP synthesis rates (*D*) and responsiveness to retinol were depressed in *p66Shc*$^{-/-}$ MEFs. These were restored to normal by reintroduction of the *p66Shc* WT gene but not the *p66ShcQQ* gene that lacks the cytochrome *c* binding site. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Retinol is transported in plasma under physiological conditions by retinol binding protein (RBP) and intracellularly by cellular retinol binding protein (CRBP). Albumin efficiently substituted for RBP and CRBP. **Figure 5*A*** shows that at 2 μM (the retinol dose optimum; ref. 9) albumin/retinol, holoRBP, and holoCRBP activated the PKCδ signal path with equal efficiency in intact MEFs and isolated mitochondria, respectively.

Why was retinol needed at all for the redox activation of PKC? One of the attractive possibilities is that the retinol molecule, owing to its conjugated double-bond system, might serve as a bridge funneling electrons from redox-sensitive cysteines of PKCδ to oxidized cytochrome *c*. Of the pair of electrons in need of being relayed from PKCδ, cytochrome c^{3+} can absorb only one at a time, inevitably leaving the other stranded, forming a radical. This radical has to be somehow stabilized until a second cytochrome c^{3+} molecule is deployed to accept it; the conjugated double-bond system of retinol might be suited for this task. To test this hypothesis, we substituted DH-Rol for retinol. We found that interrupting the π-electron system of retinol no longer allowed for PKCδ activation (Fig. 5*B*). However, since DH-Rol bound PKCδ through its intact β-ionone ring, as did retinol (11), it was readily displaced by excess retinol, restoring the PKCδ/PDH pathway.

DISCUSSION

Our previous work established that PKCδ signals pyruvate dehydrogenase kinase 2 (PDK2) to upregulate PDHC activity (9). This is accomplished by dephosphorylation, hence inactivation, of PDK2 by an as yet unidentified phosphatase. Since PDK2 functions as a negative regulator of PDH, reduced PDK2 kinase activity translates into augmented output of PDH (33). The question addressed here concerns the upstream signals and the biochemical mechanisms that control PKCδ activation. The important lead from the literature that PKCδ partnered with p66Shc (23, 26) was confirmed by us and extended to mitochondria. The pivotal discovery of the partnership between p66Shc and cytochrome *c* was another piece of the puzzle (24). It suggested the possibility of redox activation of PKCδ as an alternative to the classic PKC activation by lipid second messengers. The results presented here support the concept that redox activation of PKCδ is indeed central to the mechanism. While oxygen stress was

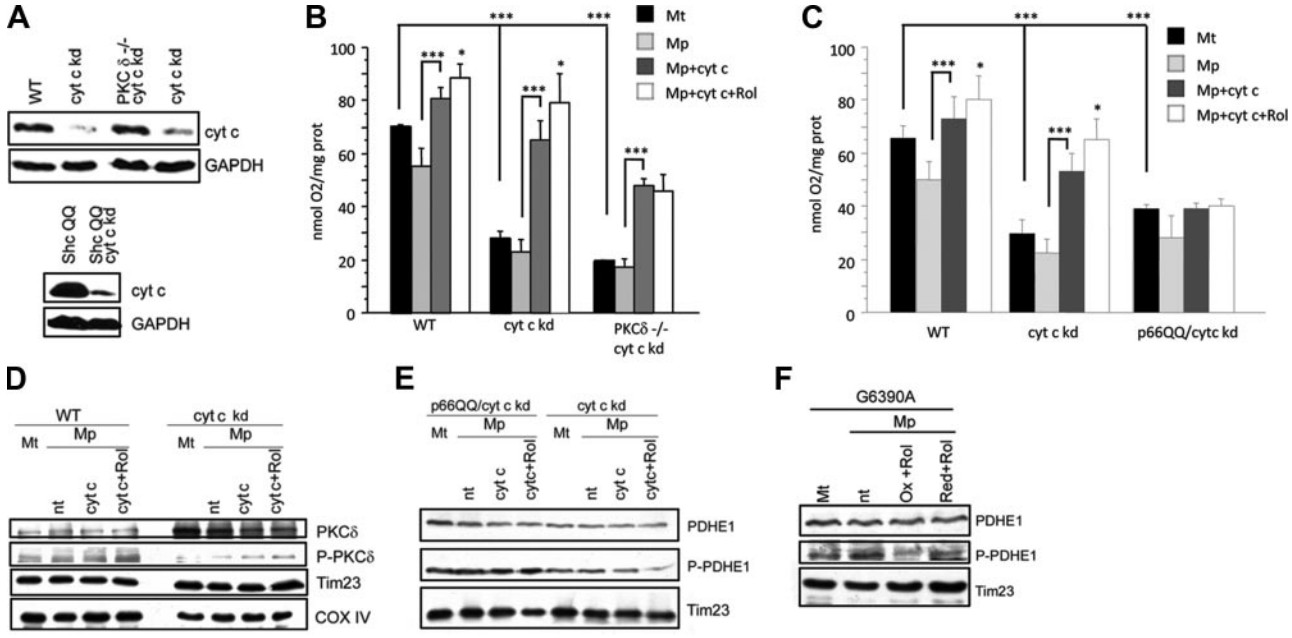

Figure 4. Cytochrome *c* (cyt c) as the upstream driver of the PKCδ signal path. *A*) Mitoplasts [MP; defined as digitonin-permeabilized mitochondria (Mt)] of MEFs of WT, PKCδ$^{-/-}$, and p66ShcQQ genotype displayed 50 to 80% reduction of their intrinsic cyt c after shRNA knockdown (cyt c kd) (90% reduction by qtPCR, data not shown). *B*) Mp of WT MEFs exhibited reduced oxygen consumption due to loss of cyt c. Addition of cyt c^{3+} normalized respiration, and retinol (Rol) enhanced this further (left). shRNA knockdown decreased baseline levels of oxygen consumption, and addition of cyt c^{3+} Mp largely recovered respiration and responsiveness to Rol (middle). PKCδ$^{-/-}$ + cyt c kd Mp treated with cyt c^{3+} recovered respiration to a lesser degree, but failed to respond to Rol (right). *C*) Mp of MEFs expressing the mutant *p66shc QQ* allele on *shc*$^{-/-}$ + cyt c kd background failed to respond to cyt c^{3+} protein, with or without Rol (right), as compared to WT and cyt c kd MEFs (left and middle). *D*) Mp of WT and cyt c kd MEFs were stimulated with cyt c^{3+} with or without Rol and probed by immunoblot for PKCδ T505 phosphorylation. Increased P-PKCδ was observed in both cell lines, indicating increased PKCδ enzyme activity. *E*) Mp of MEFs of dual *p66shc QQ/cyt c*^{kd} genotype failed to show decreased PDHE1 phosphorylation after cyt c^{3+} stimulation, compared to cyt c kd Mp that did. Costimulation with Rol resulted in further dephosphorylation in the latter but not the former Mp. *F*) Mp of the complex IV-deficient cell line G6390A exhibited hyperphosphorylation on PDHE1. Addition of cyt c^{3+} and Rol, but not cyt c^{2+} and Rol, resulted in dephosphorylation of PHE1. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

previously reported to lead to PKCδ activation (20), a precise redox mechanism was never described. We have now found an oxidoreductase system that mediates site-specific oxidation of PKCδ. Oxidized cytochrome *c* partners with p66Shc and retinol in the PKCδ signal module, fulfilling key requirements expected for an upstream activator, including residence in the IMS where PKCδ is located, physical

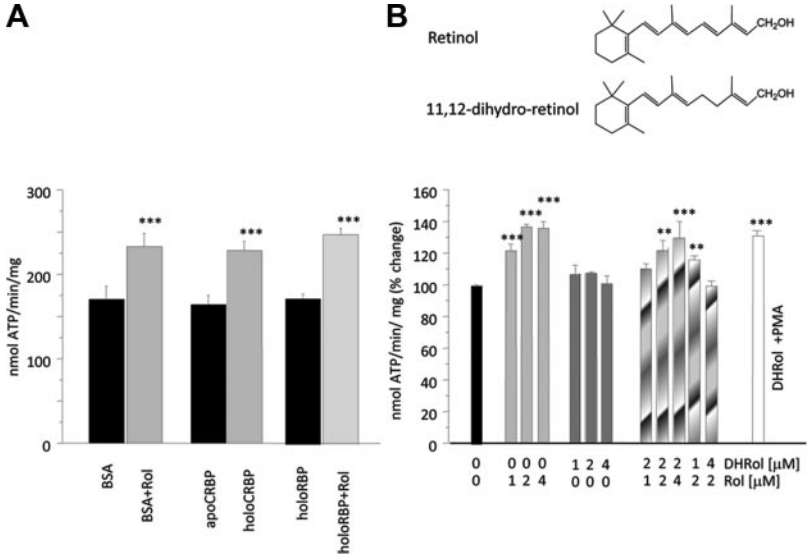

Figure 5. *A*) Delivery of retinol (Rol) is equally efficient with albumin, RBP, or CRBP. Isolated liver mitochondria were stimulated with protein carrier alone or carrier Rol complexes at 2 μM. ATP synthase rate increased by one-third within 10 min using BSA/Rol complex, holoCRBP, or holoRBP. *B*) Dependence of Rol effect on intact conjugated double-bond system. When stimulated with Rol using BSA as carrier, WT MEFs exhibited increased ATP synthase acitivity. DH-Rol failed to stimulate a similar increase. With mixtures of Rol and DH-Rol, the outcome was determined by the retinoid in most excess, indicating pharmacological reversible inhibition. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

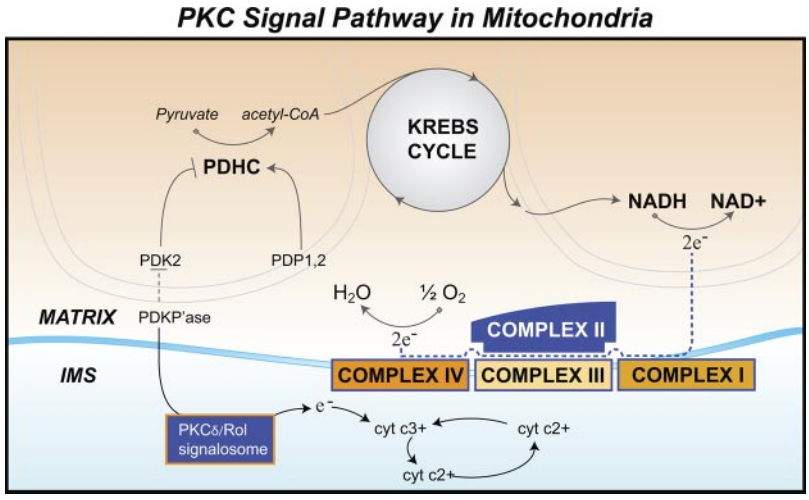

Figure 6. Diagram of the proposed PKCδ signal path. The PKCδ signalosome is redox activated by oxidized cytochrome *c* (cyt c^{3+}) and sends a forward signal to the PDHC A hypothetical phosphatase, PDKP'ase deactivates PDK2 and permits pyruvate dehydrogenase phosphatase (PDP1,2) to activate PDHC. Increased flux of acetyl-CoA into the Krebs cycle augments the reducing equivalents entering the ETC (blue stippled line). Accelerated workload of the ETC lowers the ratio of cyt $c^{3+/2+}$, thus attenuating the PKCδ signal.

association with PKCδ on the p66Shc platform, and possession of a redox potential well suited for protein oxidation. Genetic and biochemical experiments (Figs. 3*D* and 4*A–D*) support the idea that cytochrome c^{3+} acts as the upstream driver of the PKCδ signal pathway. Thus, mitoplasts of cytochrome *c*-knockdown MEFs display low baseline levels of respiration, which are readily restored to near normal levels by the reintroduction of exogenous cytochrome c^{3+}. Adding retinol plus cytochrome *c* drives oxygen consumption even higher, indicating that the entire PKCδ pathway is restored. Attempts to activate PKCδ with cytochrome c^{3+} in a cell free system were unsuccessful. This was in part explained by absence of p66Shc, but addition of p66Shc recombinant protein did not result in the *in vitro* activation of PKCδ (data not shown). The mere presence of PKCδ and p66Shc in the same mix might not suffice without appropriate tyrosine phosphorylation of PKCδ and binding to the shc SH2 domain. Furthermore, the *in organello* signal module, for instance, required not only the presence of p66Shc, as inferred from ablation of the *p66Shc* gene but also a specific orientation of p66Shc to cytochrome *c*, since the deletion of the cytochrome *c* docking site strongly attenuated PKCδ signaling to PDH (Fig. 3*A*, *B*). P66Shc behaved in mitochondria as a straightforward adapter protein, as it does in several other signal modules throughout the cell, without the need for intrinsic oxidoreductase capacity.

Whether under certain *in vitro* conditions p66Shc oxidizes reduced cytochrome *c* and elicits oxygen radicals, as reported previously (24), was not addressed in our work. However, the interaction of p66Shc with oxidized cytochrome *c* studied by us is thermodynamically unfavorable for ROS generation, thereby rendering unlikely a direct role of ROS in the redox activation of PKCδ. The main purpose of p66Shc was to provide a platform for appropriate orientation of PKCδ to cytochrome *c*. When we mutated the Y332 of PKCδ, which in its phosphorylated state forms the specific binding site for the p66Shc SH2 domain, the PKCδ signal path fell silent (Fig. 3*B*).

Binding PKCδ to one end of p66Shc and cytochrome *c* to the other suggests that the underlying purpose is to facilitate site-directed oxidation of PKCδ. Several studies have confirmed PKC redox activation (20, 21), although the requisite covalent modifications have not been described. The architecture of the trimeric p66Shc/PKCδ/cytochrome c^{3+} complex could bring an oxidizing agent into contact with PKCδ with the stereo specificity provided by selective protein-protein interaction (22). However, the identity of the oxidized sites in PKCδ remains to be established. Of note, cytochrome c^{2+}, although binding p66Shc (24), did not activate PKCδ (Fig. 4*F*).

Few precedents for redox signaling in eukaryotes are known in mechanistic detail, none involving cytochrome *c*. However, redox activation of bacterial enzymes is pertinent. In bacterial heat-shock protein 33 (Hsp33), oxidation of the zinc-finger domain leads to local conformation changes, which then progress to the large-scale rearrangements required for full chaperone activity (35, 36). In striking similarity, the activation domain of PKCδ is also organized into a zinc-finger structure (17). While zinc fingers evolved to stabilize the tertiary structure of proteins, their coherence is vulnerable to oxidation since zinc chelation depends on cysteine thiolate anions. Therefore, removal of a pair of electrons from zinc fingers, tantamount to oxidation, would by necessity result in the disassembly of the respective zinc-coordination center. We propose that, like the Hsp33 paradigm, the release of Zn^{2+} ions and local structural change of the zinc finger following loss of the thiolate anion represent the initiating events of a large-scale unfolding scheme underlying PKCδ activation (13, 14).

Why would the presence of retinol be of such importance to oxidative phosphorylation that its absence leads to diminished capacity for oxygen consumption and ATP synthesis in cells, and in vitamin A-deprived animals? In the latter example involving genetically modified, vitamin A storage-deficient mice maintained on a vitamin A-deficient diet, markedly reduced liver mitochondrial function can be restored to normal within hours of vitamin A

repletion (9). We propose that the key to understanding retinol function lies in the regulation of electron transfer between proteins. To permit interprotein electron transfer, the donor must be approaching Van der Waals contact with the acceptor; otherwise, an electron shuttle must be employed. In the electron transfer chain (ETC), cytochrome *b* uses ubiquinol as a shuttle. We suggest that retinol plays an analogous role in the PKCδ signalosome. Several observations support this paradigm: *1*) without retinol, the PKCδ signal circuit breaks down; *2*) retinol binds PKCδ by its β-ionone ring, leaving the polyene and hydroxyl group free to contact cytochrome *c*; *3*) eliminating the retinol binding pocket on PKCδ attenuates signaling (9); *4*) interrupting the conjugated double-bond structure, as observed with DH-Rol, disrupts PKCδ signaling.

In summary, we defined the function of 4 components, PKCδ, p66Shc, cytochrome *c*, and retinol in mitochondria. Based on data collected from chemical, genetic, and physiological approaches, we have assigned these components to a novel signaling module, the PKCδ signalosome. This pathway senses the redox state of mitochondria and sends a forward signal to adjust the flux of acetyl-CoA entering the Krebs cycle **(Fig. 6)**. This is the first demonstration of a direct functional link among cytochrome *c*, retinol, and redox signaling. Of major importance, the PKCδ signalosome clarifies how energy homeostasis is maintained within cells. FJ

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