Mammalian TOR Controls One of Two Kinase Pathways Acting upon nPKCδ and nPKCε*

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There are three conserved phosphorylation sites in protein kinase C (PKC) isotypes that have been termed priming sites and play an important role in PKC function. The requirements and pathways involved in novel (nPKC) phosphorylation have been investigated here. The evidence presented for nPKCδ shows that there are two independent kinase pathways that act upon the activation loop (Thr-505) and a C-terminal hydrophobic site (Ser-662) and that the phosphorylation of the Ser-662 site is protected from dephosphorylation by the Thr-505 phosphorylation. Both phosphorylations require C1 domain-dependent allosteric activation of PKC. The third site (Ser-643) appears to be an autoprophosphorylation site. The serum-dependent phosphorylation of the Thr-505 and Ser-662 sites increases nPKCδ activity up to 80-fold. Phosphorylation at the Ser-662 site is independently controlled by a pathway involving mammalian TOR (mTOR) because the rapamycin-induced block of its phosphorylation is overcome by co-expression of a rapamycin-resistant mutant of mTOR. Consistent with this role of mTOR, amino acid deprivation selectively inhibits the serum-induced phosphorylation of the Ser-662 site in nPKCδ. It is established that nPKCε behaves in a manner similar to nPKCδ with respect to phosphorylation at its C-terminal hydrophobic site, Ser-729. The results define the regulatory inputs to nPKCδ and nPKCε and establish these PKC isotypes downstream of mTOR and on an amino acid sensing pathway. The multiple signals integrated in PKC are discussed.

The allosteric control of a number of protein kinases is an essential feature of their operation as signal transducers. This includes the various second messenger-dependent kinases such as cAMP-dependent protein kinase, PKG, the CAMkinases, and the lipid-dependent protein kinases C (PKC)1 (see Ref. 1 for classification). The ability of the relevant effectors to cause activation of these protein kinases has colored the view that they operate exclusively to relay changes in their particular effector concentrations to their respective target proteins. While this remains a key component of their action, it has become clear that for many of these protein kinases, there are additional controls. Broadly these fall into two categories, one relating to the subcellular compartmentalization of the protein kinases and the other to the phosphorylation of the kinases themselves. The subcellular distribution has profound effects upon the specificity of action of these proteins, by localizing the kinase close to substrate(s) and regulators (reviewed Refs. 2 and 3). The control through phosphorylation can serve a number of functions, including the regulation of catalytic potential (e.g. Refs. 4–6). PKC is an interesting example of this class of proteins, and there is ample evidence for both types of control acting, i.e. PKC-targeting proteins (7) and PKC phosphorylation (8); both are established as critical to the roles of PKC in controlling cell behavior (for example Refs. 9 and 10). Despite the critical nature of these controls, there is only limited information on the additional signaling pathways that input to PKC through these mechanisms.

Classical PKC (cPKC) isotypes (α, βI, βII, γ) are known to be phosphorylated in at least three sites (11–14). These are well conserved within the entire PKC subfamily, excluding a C-terminal hydrophobic site that is replaced with a glutamic acid residue in the two aPKC isotypes (ξ, δ) and with an aspartic acid in the two rPKC-related kinases, PRK1 and PRK2. The phosphorylation of the cPKC isotypes within these three sites acts cooperatively to maintain the kinase in an active conformation, although still requiring diacylglycerol (DAG) for activity (13, 14). The critical phosphorylations are within the activation loops of the kinase domains and mutations in these profoundly block activities (15–17). The other two sites are toward the C terminus and include a predicted autophosphorylation site followed by (19 residues C-terminal) a hydrophobic acid in the two PKC-related kinases, PRK1 and PRK2. The phosphorylation of the PKC isotypes downstream of these three sites acts cooperatively to maintain the kinase in an active conformation, although still requiring diacylglycerol (DAG) for activity (13, 14). The critical phosphorylations are within the activation loops of the kinase domains and mutations in these profoundly block activities (15–17). The other two sites are toward the C terminus and include a predicted autophosphorylation site followed by (19 residues C-terminal) a hydrophobic site in an FXXFS/TTF/Y motif. These phosphorylations play more subtle roles in maintaining a closed conformation that has optimum thermal stability and resistance to proteases and phosphatases (14). Regulatory domain contacts are also influenced by phosphorylation at the C terminus, as evidenced by a shift in Ca2+ dependence (18). For other PKC isotypes (novel/typical PKC (n/aPKC)), there is less information on the detailed behavior of phosphorylation site mutants although there is accumulating evidence that in mammalian cells these proteins are all phosphorylated to some degree in these conserved sites (19, 20).

Recent studies have led to the conclusion that phosphorylation of PKC isotypes in their activation loop sites is under the control of PDK1 or a closely related kinase (19, 20). This input to PKC provides an explanation for the controlling influence of PI 3-kinase that has been observed (21, 22), i.e. PI 3-kinase activation of PKC is channeled through PDK1 via PtdIns-3,4,5-P3 production. The pathway(s) to the phosphorylation of the hydrophobic C-terminal site in PKC is less well understood. Previous studies have suggested that this may be an autophosphorylation site for cPKC isotypes (23, 24). This does not appear to be the case for nPKC isotypes. For these latter proteins, there is evidence that an aPKC may be responsible for this...
phosphorylation (25). However, the particular upstream kinase involved physiologically remains to be identified unequivocally.

In assessing the role of upstream kinases in controlling the phosphorylation of the C-terminal hydrophobic sites in nPKC<sub>d</sub> in vivo, it has been noted that their phosphorylation is inhibited by the treatment of cells with the potent immunosuppressant drug rapamycin (25). This parallels the effect of rapamycin on the equivalent phosphorylation in p70<sub>60k</sub> (26) and suggests that PKC may lie on a similar signaling pathway with respect to the phosphorylation of this site. Here we define the requirements for serum-induced phosphorylation of nPKC<sub>d</sub> and nPKCe and show that mTOR plays a selective role in controlling phosphorylation in the hydrophobic C-terminal site.

Consistent with the defined role for TOR in nutrient sensing (reviewed in Ref. 27), amino acid deprivation is shown to influence nPKC phosphorylation specifically at this C-terminal site.

**EXPERIMENTAL PROCEDURES**

**Phosphorylation-site Specific Antisera and Western Analysis**—The Ser-phosphate/657, site-specific polyclonal antiserum was raised against peptides FEGFSPPYVPN, based upon the region flanking the FSY-motif in cPKCα. Activation loop phospho-site polyclonal antiserum for nPKC<sub>d</sub> and nPKCe (Thr-phosphate/505 and Thr-phosphate/566 respectively) were raised, immunizing with the peptides RAST(P)FCGT and TTTT(P)FCGT, respectively. All the sera were tested against phosphorylated and dephosphorylated forms of the immunogenic peptides. The sera show some cross-reactivity for the unphosphorylated peptide, therefore all Western analyses were performed in the presence of the cognate dephosphorylated peptide (dephosphopeptide) at 1 μg/ml; this was sufficient to compete immunoreaction with the dephosphopeptides (data not shown). Transfected cells were lysed in SDS-sample buffer, and protein samples were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) and analyzed for phosphorylation at the FSFY and activation loop sites. Western blots were developed using horseradish peroxidase–coupled donkey anti-rabbit IgG secondary antiserum (Amersham Pharmacia Biotech) (1:5000) and ECL<sup>TM</sup> (Amersham Pharmacia Biotech).

**Transfection and Cell Culture**—HEK293 cells were transfected with nPKC<sub>d</sub> or nPKCe alone or either with wild-type mTOR or rapamycin-insensitive mTOR using calcium phosphate, as described previously (28). Cells were starved for 24 h while maintained in suspension before stimulation with 10% FCS for the times indicated in the text or figure legends. Where indicated, LY294002 (10 μM) or rapamycin (20 nM) were added to cells for 30 min prior to stimulation.

In some experiments, cells were deprived of amino acids as follows. Transfected HEK293 cells were serum starved for 24 h, and then the cells were washed once in phosphate-buffered saline followed by incubation in serum-free media for 24 h. Amino acid-deprived cells were then stimulated with dialyzed 10% FCS either in the presence or absence of amino acids or leucine alone as indicated.

**Immunoprecipitation and nPKC Activity Determination**—Myc-immunocomplexes were prepared by lysing the cells on ice with 600 μl of ice-cold lysis buffer (20 μM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 15 μg/ml leupeptin, 100 μg/ml aprotinin, 100 μg/ml okadaic acid, and 1% Triton X-100). Insoluble material was removed after incubation with protein A-Sepharose for 10 min at 4 °C and centrifugation for 5 min (12,000 × g). The supernatants were incubated with 4 μg of anti-Myc antibody at 4 °C for 20 min, followed by 40 μl of protein G-Sepharose beads at 4 °C for 60 min. The beads were washed twice with lysis buffer and then once with lysis buffer containing 0.1% Triton X-100. Myc-immunoprecipitated nPKC<sub>d</sub> (10 μl) was incubated with 25 μl of a reaction mixture containing: 0.2 mg/ml myelin basic protein, 10 mM MgCl<sub>2</sub>, 20 mM Tris-HCl, pH 7.5, and 2.5 mM CaCl<sub>2</sub>, 20 μM ATP, 10 μCi/ml of [γ-<sup>32</sup>P]ATP) for the time period indicated. The reaction was stopped with 4 μl of 4× sample buffer (29), and the proteins were separated by 12.5% SDS-PAGE. Following this, an autoradiograph was taken, and the bands were quantitated by scanning stained PAGE gels to quantify nPKC<sub>d</sub> and expressing kinase activity as a function of this in arbitrary units.

**RESULTS**

**Serum-induced nPKC<sub>d</sub> and nPKCe Phosphorylation Requires Allosteric Activation**—It has been demonstrated previously that nPKC<sub>d</sub> from serum-starved cells accumulates in a dephosphorylated form (19). This phenomenon is further exaggerated when cells are maintained in suspension during the serum deprivation. As illustrated in Fig. 1, there is little nPKC<sub>d</sub> phosphorylation at either Thr-505 or Ser-662 in suspension cultures of 24 h serum-starved cells. On serum stimulation, both of these sites become phosphorylated (Fig. 1A). To assess the requirements for this serum-induced phosphorylation, we determined whether nPKC<sub>d</sub> activation at membranes was necessary, by employing the C1 domain DAG binding antagonist calphostin C (30). In the absence of serum, there is a very low basal level of phosphorylation and no perceptible effect of calphostin C. However, following serum addition, calphostin C blocked phosphorylation at the highest concentrations with partial effects at 50 nM. Thus, C1 domain-dependent activation of nPKC<sub>d</sub> is required for effective serum-induced phosphorylation.

Previously, the behavior of nPKCe has not been investigated with respect to the predicted activation loop site (Thr-566) and C-terminal hydrophobic site (Ser-729) phosphorylation. Antisera specific to the phosphorylated forms of these sites were derived to determine whether nPKCe was also subject to acute serum-induced phosphorylation. As observed for nPKC<sub>d</sub>, nPKCe was phosphorylated in serum-starved suspension cultures and became phosphorylated on serum addition (Fig. 1B). This response was inhibited by calphostin C with complete inhibition being observed at or above 100 nM. Hence both of these nPKC isotypes display acute serum-induced phosphorylation, dependent upon C1 domain-dependent membrane activation.

To corroborate the requirement for an allosteric input, we monitored the effect of the direct activator TPA on the serum-induced phosphorylation of nPKC<sub>d</sub>. TPA alone had a modest effect on phosphorylation in the absence of serum (Fig. 1C). Serum alone induced a characteristic time-dependent phosphorylation of both the Thr-505 and Ser-662 sites in nPKC<sub>d</sub> that did not reach a maximum until 30 min. By comparison, serum + TPA induced a rapid phosphorylation that was optimum within 5 (Thr-505) or 10 (Ser-662) min. These results are consistent with observations in vitro where it has been shown that TPA acts cooperatively with PtdIns-3,4,5-P<sub>3</sub> to support PKD1 phosphorylation of nPKC<sub>d</sub> (Thr-505 site) (19).

**Two Kinase Pathways Act upon nPKC<sub>d</sub> to Control Activity**—The sensitivity of serum-induced nPKC<sub>d</sub> phosphorylation to other antagonists has provided circumstantial evidence for the operation of two independent pathways (19, 25). However the requirement for activation of nPKC<sub>d</sub> suggests that nPKC<sub>d</sub> catalytic activity may be involved in these responses; for nPKCe it has been proposed that in vitro it will autophosphorylate on its C-terminal hydrophobic site (Ser-660) (23, 24). To assess the requirement for activity, the effect of the PKC inhibitor bisindolylmaleimide I (BIM I) was investigated. The serum-induced phosphorylations of the nPKC<sub>d</sub> Ser-662 and Thr-505 sites were unaffected by BIM I (Fig. 2A). The same lack of effect was observed with a second inhibitor, G68693 (data not shown).

To demonstrate that BIM I inhibited nPKC<sub>d</sub> under these conditions, we also monitored the phosphorylation state of Ser-643 which has been reported to be an autophosphorylation site (19, 31). This site in nPKC<sub>d</sub> remains occupied in serum-starved cells and is influenced by neither LY294002 nor rapamycin (data not shown). By contrast, treatment of cells with BIM I led to a relatively rapid decrease in phosphorylation at Ser-643 which has been reported to be an autophosphorylation site (19, 31). This site in nPKC<sub>d</sub> remains occupied in serum-starved cells and is influenced by neither LY294002 nor rapamycin (data not shown).

 supplementary.

2 D. B. Parekh, and P. J. Parker, unpublished data.
to dephosphorylation of this autophosphorylation site (Fig. 2A). In these serum-deprived cultures, BIM I induced loss of phosphorylation of Ser-643 with a t₁/₂ of 5–10 min; for G6983, the t₁/₂ of loss was <5 min (data not shown). It is of interest that in serum-maintained cells where the Thr-505 and Ser-662 sites remain occupied, neither BIM I nor G6983 induced loss of phosphorylation of Ser-643 (data not shown), indicating that as documented for cPKCs there is a phosphatase protective effect on occupation of these sites (13, 14). The conclusion that can be drawn from these observations is that, under conditions in which nPKCs catalytic activity is inhibited, serum induces the phosphorylation of the Thr-505 and Ser-662 sites, consistent with two upstream pathways.

The serum-induced phosphorylation of nPKCe was also not dependent upon catalytic activity since BIM I concentrations over the range 100 nM to 30 μM had no significant effect upon either the Ser-729 or the Thr-566 phosphorylations (Fig. 2B). By contrast even at the lowest concentration employed (100 nM), BIM I suppressed phosphorylation at the predicted nPKCe autophosphorylation site (Thr-710, see Fig. 2C). It can be concluded that for both nPKCs and nPKCe, there are two upstream kinase inputs responsible for phosphorylation of their activation loop sites and their hydrophobic C-terminal sites.

We further investigated the relationship between the phosphorylations of the PKCδ Thr-505 and Ser-662 sites, employing an alanine 505 substitution mutant (T505A) (32). In cells expressing nPKCδ T505A, no serum-induced phosphorylation of Ser-662 was observed in contrast to the wild-type PKCδ (Fig. 3). This suggested that Thr-505 might need to be phosphorylated prior to Ser-662. However, previous studies with cPKCs have shown that there is a mutually protective effect of these phosphorylations that reduces their susceptibility to dephosphorylation (13, 14). Thus it was possible that in nPKCδ there was no obligatory order of phosphorylation but that there was active dephosphorylation. To test this, we serum stimulated cells in the presence of the phosphatase inhibitor okadaic acid (Fig. 3). Under these circumstances, the nPKCδ T505A mutant became phosphorylated at the Ser-662 site. This response of Ser-662 phosphorylation was not due to an independent, okadaic acid-induced pathway since there was no effect of okadaic acid alone even though, interestingly, there was an effect of okadaic acid on Thr-505 phosphorylation. It can be concluded that on serum stimulation, there is no ordered conditional phosphorylation of the Thr-505 and Ser-662 sites in nPKCδ, but that there is an interdependence that reflects the turnover of these sites.

mTOR Controls One Pathway of nPKCδ Phosphorylation—The accumulated evidence defines two distinguishable pathways acting upon nPKCδ. One of these pathways has been shown previously to involve PDK1 (19), the second is sensitive to rapamycin (25) and may thus be regulated by the mammalian target of rapamycin (mTOR, also denoted FRAP/RAFT-1; reviewed in Ref. 27). To determine the role of mTOR, we investigated whether a rapamycin-resistant mTOR (mTORrap-res) modified the rapamycin sensitivity of nPKCδ Ser-662 phosphorylation. nPKCδ was transfected without or with hemag-
glutinin-tagged mTORrap-res into HEK293 cells and cells subsequently placed into suspension under serum-deprived conditions. Expression of mTOR was confirmed by western using the hemagglutinin tag (Fig. 4A). On stimulation with serum, nPKCδ became phosphorylated at Ser-662 and Thr-505; both phosphorylations were sensitive to LY294002, while only the Ser-662 phosphorylation was blocked by rapamycin (Fig. 4B). Co-transfection with mTORrap-res completely suppressed the effect of rapamycin, and Ser-662 became phosphorylated on serum stimulation despite the rapamycin treatment. No effect of mTORrap-res was observed on the sensitivity of Ser-662 phosphorylation to LY294002. This is consistent with the ability of this inhibitor to target the catalytic function of mTOR (33), although the LY294002 sensitive input to Thr-505 phosphorylation may also be important (see above). The phosphorylation of the Thr-505 site was not affected by rapamycin under any condition and similarly not influenced by coexpression of mTORrap-res.

To investigate the contributions of these phosphorylations to PKCδ activity, use was made of the selective inhibition of Ser-662 phosphorylation by rapamycin. In serum-starved cells (Thr-505/Ser-662-dephosphorylated), nPKCδ became phosphorylated at Ser-662 and Thr-505; both phosphorylations were sensitive to LY294002, while only the Ser-662 phosphorylation was blocked by rapamycin (Fig. 4B). Co-transfection with mTORrap-res completely suppressed the effect of rapamycin, and Ser-662 became phosphorylated on serum stimulation despite the rapamycin treatment. No effect of mTORrap-res was observed on the sensitivity of Ser-662 phosphorylation to LY294002. This is consistent with the ability of this inhibitor to target the catalytic function of mTOR (33), although the LY294002 sensitive input to Thr-505 phosphorylation may also be important (see above). The phosphorylation of the Thr-505 site was not affected by rapamycin under any condition and similarly not influenced by coexpression of mTORrap-res.

To investigate the contributions of these phosphorylations to PKCδ activity, use was made of the selective inhibition of Ser-662 phosphorylation by rapamycin. In serum-starved cells (Thr-505/Ser-662-dephosphorylated), nPKCδ activity determined in immunocomplexes is very low (Fig. 5). Serum stimulation induces a more than 80-fold activation of nPKCδ. On serum stimulation in the presence of either rapamycin (no Ser-662 phosphorylation) or LY294002 (neither Thr-505 nor Ser-662 phosphorylation), there is a near complete inhibition of nPKCδ activation (>90%). Thus, phosphorylation of the Thr-505 and Ser-662 sites appears to be necessary for optimum activity and/or stability of the immunopurified nPKCδ. The strong inhibitory effect of rapamycin on recovered activity implies that the Ser-662 phosphorylation has a significant contribution to activity.

The behavior of nPKCδ with respect to mTORrap-res was also
The control of PKC isotypes by phosphorylation has become a central feature of our understanding of PKC signaling. The results here elucidate three key aspects of these upstream controls acting upon nPKCs. First, there is a requirement for allosteric activation. Second, there are two distinguishable protein kinase inputs neither of which require activity of the nPKC itself. Third, one of these phosphorylations is shown to be under the control of mTOR. Coupled to previous studies on the activation loop phosphorylation of nPKCβ by PDK1, a summary of nPKC control can be made (Fig. 8) that serves as a working model (discussed below).

Elucidation of the phosphorylation of PKC by upstream protein kinases has been complicated by the behavior of the cPKC isotypes which, while best understood in respect of phosphorylation sites, have proven difficult to work with in dephosphorylation experiments either in vitro or in vivo (discussed in Ref. 14). Recent progress in this area has come from work on nPKC and aPKC isotypes (19, 20). These studies have provided compelling evidence that PDK1 phosphorylates nPKCδ and aPKCε in vitro and in vivo. In fact all PKC isotypes tested have been shown to form a complex with PDK1 (19), indicating that PDK1 has the potential to be a common upstream kinase for PKC isotypes. Evidence that this is indeed the case for cPKCs has been reported (24). The conservation of the PKC activation loop sequences at the predicted phosphorylation sites is consistent with this notion.

The two C-terminal phosphorylation sites identified in cPKC isotypes are also conserved in nPKC and aPKC isotypes although in aPKCs the serine/threonine at the more C-terminal hydrophobic site is replaced by a glutamic acid residue, with conservation of the surrounding FXXFEF/Y motif. It has been proposed that for cPKCs this C-terminal hydrophobic site is an autophosphorylation site (23, 24). While this has yet to be corroborated in vivo, the evidence for nPKC is that this phosphorylation involves an upstream kinase acting on this V5 domain. Thus nPKCδ expressed in bacteria is not phosphorylated on Ser-662 while it is phosphorylated on the adjacent autophosphorylation site Ser-664. More compelling evidence is presented here through use of the catalytic site inhibitors BIM I and Go6983, which under serum-starved conditions block Ser-643 phosphorylation of nPKCδ in vivo while having no such effect on the phosphorylation of Ser-662 (or Thr-505). The conclusion that there is a heterologous kinase required to phosphorylate PKC isotypes at these hydrophobic sites is consistent with the evidence that an aPKC complex may be responsible for this phosphorylation (25).

It is of note that both serum-induced phosphorylations (Thr-505 and Ser-662) are inhibited by the DAG competitive inhibitor calphostin C, indicating that activation at the membrane is important for effective phosphorylation. The PDK1 phosphorylation of PKC resembles that of PKB where co-recruitment to or allosteric activation at membranes is required for phosphorylation (35, 36). There is, however, a key difference for nPKC isotypes in that two distinct signaling pathways are involved,

Fig. 5. Activation of PKCδ by serum. PKCδ was immunoprecipitated from serum-starved cells (24 h) that had been left unstimulated (serum free), stimulated with 10% FCS for 30 min (FCS), or FCS stimulated in the presence of rapamycin (FCS+rap), or LY294002 (FCS+LY). Cells were pre-treated with inhibitors (rap, LY) 30 min prior to FCS stimulation. Extracts from these cells were subjected to immunoprecipitation employing the Myc-epitope tag on PKCδ. Immunocomplexes were then assayed for PKCδ activity using myelin basic protein. Relative PKC activity was based upon protein staining and scanning densitometry. Specific immunoprecipitation employing the Myc-epitope tag on PKCδ e

Fig. 6. The C-terminal hydrophobic sites in both PKCδ and PKCε show rapamycin-sensitive phosphorylation mediated by mTOR. PKCδ or PKCε were transfected into HEK293 cells with or without mTOR or mTORrap-res, and cultures were serum starved for 24 h. Cells were then treated with 10% FCS + 100 nm TPA for 30 min in the presence or absence of rapamycin or LY294002 as indicated. PKC expression and hydrophobic site phosphorylation was followed by Western blotting.
Amino acid deprivation selectively inhibits serum-induced phosphorylation of the C-terminal hydrophobic site in PKCδ. A, cells were transfected with PKCδ and serum starved for 24 h. Cultures were then depleted of amino acids for the times shown. Following depletion, cells were stimulated with either FCS (10%) or with dialyzed FCS (10%) (FCS + amino acids). PKCδ expression and Ser-662 and Thr-505 phosphorylation were determined by Western blotting. B, PKCδ-transfected cells were serum deprived for 24 h and switched to media devoid of amino acids for 2.5 h. Cultures were then stimulated with dialyzed FCS (10%) in the presence or absence of added amino acids as shown. Extracts were prepared and PKCδ expression and Ser-662 and Thr-505 phosphorylation were determined by Western blotting. C, cells were transfected and then serum and amino acid deprived as in panel A. Cultures were then stimulated with dialyzed FCS (10%) in the presence or absence of added 0.8 mM l-leucine as indicated, and PKCδ phosphorylation and expression were determined.

i.e. PtdIns-phospholipase C (DAG) and PI 3-kinase (PtdIns-3,4,5-P3). PKC thus serves to integrate these two cellular inputs.

Once in an activated conformation, nPKCδ can be phosphorylated on either the Thr-505 or Ser-662 sites. However, the Ser-662 phosphorylation is subject to efficient dephosphorylation in the absence of Thr-505 phosphorylation. This is evidenced by the behavior of the nPKCδ T505A mutant, which only becomes phosphorylated at the Ser-662 site in the presence of the phosphatase inhibitor, okadaic acid. This property is partly reminiscent of that described for PKCα where lack of phosphates at one of the three priming sites can sensitize the other sites to TPA-induced dephosphorylation (13, 14). This relationship means that for PKCδ, while there is no conditional requirement, there would appear to be a preferred order of phosphorylations, with Thr-505 preceding Ser-662. This is also consistent with the response to rapamycin, which has a specific effect on the Ser-662 site without influencing the Thr-505 site, i.e. the occupation of the Ser-662 site has little influence on the Thr-505 site under these conditions.

With respect to the phosphorylation of the hydrophobic sites in nPKCδ/ε, the studies here demonstrate that mTOR has a dominant role in controlling phosphorylation; this is particular to the nPKCδ Ser-662 site (and nPKCe Ser-729 site), with no acute effect upon the activation loop site. In extending these observations to a physiological pathway, we have demonstrated that amino acid deprivation blocks nPKCδ-induced phosphorylation at the Ser-662 site. Under these conditions, no effect of amino acid depletion is observed for the Thr-505 site, showing that the PI 3-kinase/PDK1 pathway is not affected and illustrating the independent control of these phosphorylation events. Thus at least one further pathway acts upon nPKC, showing that the PI 3-kinase/PDK1 pathway is not affected and illustrating the independent control of these phosphorylation events. Thus at least one further pathway acts upon nPKC.

The various inputs to nPKC discussed above are summarized in Fig. 8, including the points at which inhibitors act. The overall picture is of agonist-dependent nPKC allostery activation at the membrane through the C1 domain. In this conformation, the nPKC is acted upon by two membrane-associated kinases, PDK1 and a hydrophobic site kinase, probably comprising an aPKC complex. This activated nPKC can also auto-

Fig. 7. Amino acid deprivation selectively inhibits serum-induced phosphorylation of the C-terminal hydrophobic site in PKCδ. A, cells were transfected with PKCδ and serum starved for 24 h. Cultures were then depleted of amino acids for the times shown. Following depletion, cells were stimulated with either FCS (10%) (FCS + amino acids) or with dialyzed FCS (10%) (FCS - amino acids). PKCδ expression and Ser-662 and Thr-505 phosphorylation were determined by Western blotting. B, PKCδ-transfected cells were serum deprived for 24 h and switched to media devoid of amino acids for 2.5 h. Cultures were then stimulated with dialyzed FCS (10%) in the presence or absence of added amino acids as shown. Extracts were prepared and PKCδ expression and Ser-662 and Thr-505 phosphorylation were determined by Western blotting. C, cells were transfected and then serum and amino acid deprived as in panel A. Cultures were then stimulated with dialyzed FCS (10%) in the presence or absence of added 0.8 mM l-leucine as indicated, and PKCδ phosphorylation and expression were determined.

Fig. 8. Multiple inputs required for serum-induced nPKC phosphorylation. Following serum deprivation, the phosphorylation of nPKC requires the membrane activation of the protein through its C1 domain as evidenced by sensitivity to calphostin C. In response to serum, this activation is effected through the DAG/phorbol binding site, presumably via agonist-dependent phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P2). In this active conformation at the membrane, the low specific activity (low) PKCδ can be phosphorylated by PDK1 at the activation loop site (505). This requires PtdIns-3,4,5-P3-dependent PDK1 recruitment to the membrane since it is inhibited by the PI 3-kinase inhibitor LY294002. The C-terminal hydrophobic site (662) within the V5 domain of PKCδ also becomes phosphorylated on the membrane by a membrane-associated kinase (probably an aPKC complex, Ref. 25). This phosphorylation is sensitive to the action of mTOR; inhibition of mTOR by the specific inhibitor rapamycin inhibits serum-induced phosphorylation at this site. The mTOR-dependent control is predicted to operate through a protein phosphatase as indicated (see “Discussion”). The agonist/effector-dependent inputs immediately upstream of nPKC are thus: (1) phospholipase C, (2) PI 3-kinase/PDK1, (3) nutrient/mTOR, and (4) an aPKC, or functionally related, kinase complex.
phosphorylate. A permissive input (mTOR) operates in parallel to this such that, under certain unfavorable conditions (e.g., amino acid deprivation), the hydrophobic C-terminal site remains dephosphorylated. Hence nPKC acts to integrate information from three defined inputs and a fourth yet to be fully defined. It is the combined effect of these inputs that leads to optimum nPKC function. Lack of phosphorylation at Ser-662 (rapamycin) or Thr-505/Ser-662 (LY294002) reduces the specific activity of immunopurified nPKC8 by ~10- and ~90-fold, respectively, while lack of DAG interaction would both block phosphorylation and prevent allosteric activation of otherwise phosphorylated nPKC. This integration of information and the ability to store it (PKC once phosphorylated can remain phosphorylated for minutes to hours) represent important features of cellular control.

It is established that in yeast, TOR1/2 controls amino acid sensing with consequent effects upon translation and cell cycle progression (27, 38). In mammals, a similar situation pertains with mTOR acting to control translation through p70S6k and progression (27, 38). In mammals, a similar situation pertains sensing with consequent effects upon translation and cell cycle of cellular control.

phosphorylation and prevent allosteric activation of otherwise specific kinases. The demonstration that PKC
is also subject to inhibition of these phosphorylation events. However, the studies here identify the nPKC isotypes as additional downstream targets of this pathway. The site-specific effect of amino acid starvation on PKC8 and the control of this phosphorylation by rapamycin, leads to the conclusion that mTOR couples this sensing pathway to PKC8. The recent studies on p70S6k (34, 40) suggests that mTOR may play a pleiotropic role in controlling the phosphorylation of hydrophobic sites in multiple AGC subfamily kinases. The demonstration that PKC8 is also subject to mTOR control supports assignment of such a broad role. The placement of nPKCs on an amino acid sensing pathway implies that this is part of the cells adaptive response. How nPKC function may be deployed to protect cells from amino acid deprivation (e.g. in reducing protein synthesis, enhancing protein degradation) remains to be determined.

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