Protein kinase C delta is phosphorylated on five novel Ser/Thr sites following inducible overexpression in human colorectal cancer cells

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Abstract

Phosphorylation plays an important role in regulation of protein kinase C delta (PKC δ). To date, three Ser/Thr residues (Thr 505, Ser 643, and Ser 662) and nine tyrosine residues (Tyr 52, Tyr 64, Tyr 155, Tyr 187, Tyr 311, Tyr 332, Tyr 512, Tyr 523, and Tyr 565) have been defined as regulatory phosphorylation sites for this protein (rat PKC δ numbering). We combined doxycycline-regulated inducible gene expression technology with a hypothesis-driven mass spectrometry approach to study PKC δ phosphorylation pattern in colorectal cancer cells. We report identification of five novel Ser/Thr phosphorylation sites: Thr 50, Thr 141, Ser 304, Thr 451, and Ser 506 (human PKC δ numbering) following overexpression of PKC δ in HCT116 human colon carcinoma cells grown in standard tissue culture conditions. Identification of potential novel phosphorylation sites will affect further functional studies of this protein, and may introduce additional complexity to PKC δ signaling.

Keywords: PKC δ ; phosphorylation; colon cancer; HCT116; Tet on; MIDAS; mass spectrometry

In mammalian cells the protein kinase C (PKC) family of serine/threonine kinases consists of at least 11 members (Mellor and Parker 1998). They play important roles in a broad spectrum of cellular processes ranging from proliferation to programmed cell death. Abnormalities in PKC signaling have been associated with several human diseases including cancer (Koivunen et al. 2006; Teicher 2006).

Based on their structure and activation properties, the PKC isoenzymes are commonly divided into three major classes: (1) conventional or classical (α , β , and γ), (2) novel (δ , ϵ , η , θ), and (3) atypical (μ , ξ , ι). Activation of

classical enzymes (cPKCs) can be triggered by calcium (Ca^{2+}) and diacylglycerol (DAG), novel enzymes (nPKCs) are activated by DAG but not by Ca^{2+} , and atypical enzymes (aPKCs) are activated independently of Ca^{2+} or DAG (Corbalan-Garcia and Gomez-Fernandez 2006). Also, the phosphorylation pattern is a very important factor in determining the activity status and protein stability of PKC isoforms (Parekh et al. 2000; Newton 2001).

Protein kinase C delta (PKC δ) is the oldest member of the nPKCs class. It is a ubiquitously expressed protein with a calculated molecular weight of 77.5 kDa. The available data suggests its role in growth inhibition, differentiation, apoptosis, and tumor suppression (Gschwendt 1999; Kikkawa et al. 2002; Brodie and Blumberg 2003; Jackson and Foster 2004). It seems that the functions of PKC δ may differ in diverse cell types. Even in the same cell type, depending on the physiological state and external stimuli, PKC δ may perform multiple different functions.

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This flexibility in PKC δ signaling is possible due to the complex structure and regulation mechanisms of the protein (Steinberg 2004; Corbalan-Garcia and Gomez-Fernandez 2006).

PKC δ consists of the N-terminal regulatory domain and the C-terminal catalytic domain that are connected by a short “hinge” region (Fig. 1C). The regulatory domain

contains the C2-like region and the C1 region. There is a pseudosubstrate motif between the C2-like and C1 regions. The C1 region includes two cysteine-rich sequences (C1A and C1B, respectively) that are able to bind DAG and phorbol esters triggering the activation of PKC δ . Activation of PKC δ by DAG or phorbol ester is usually associated with protein translocation to the membranes. The C2-like region structurally resembles the calcium binding C2 motifs of cPKC isoforms; however, it lacks the amino acids necessary for Ca²⁺ coordination, and thus is unable to trigger PKC δ activation in response to increases in Ca²⁺ concentration. The pseudosubstrate motif is believed to occupy the substrate recognition site in the catalytic domain, and thus helps to maintain the protein in an inactive conformation. The catalytic domain of PKC δ contains two conserved regions, C3 and C4, that are essential for substrate binding and kinase activity. The catalytic domain terminates with the turn motif and the hydrophobic motif that are important for full catalytic competence and interactions with PKC δ binding partners. The “hinge” region harbors the caspase cleavage site. Cleavage by caspase 3 results in the generation of a catalytically active C-terminal fragment (Brodie and Blumberg 2003; Steinberg 2004; Corbalan-Garcia and Gomez-Fernandez 2006).

In addition to activation by binding of DAG or phorbol ester and by caspase-mediated proteolysis, PKC δ can be also activated by phosphorylation. Regulation of PKC δ activity by phosphorylation is very complex (reviewed in Steinberg 2004). There are three conserved serine/threonine phosphorylation motifs in all PKC isoforms: a threonine within the activation loop, and two serine residues within the turn motif and hydrophobic motif, respectively. In many cell types PKC δ is stably phosphorylated at Ser 643 (turn motif) and Ser 662 (hydrophobic motif), but retains little phosphorylation at the activation loop (Thr 505). Phosphorylation of Thr 505 is associated with increased kinase activity. Nine tyrosine residues have been defined in addition to the known Ser/Thr residues as PKC δ phosphorylation sites. Tyrosine phosphorylation of PKC δ can be triggered by different stimuli. In some cases it results in activation of PKC δ ; however, the consequences of tyrosine phosphorylation vary, and seem to be associated with a cell type and particular phosphorylation pattern triggered by a given stimulus (Jackson and Foster 2004; Steinberg 2004).

Considering the complexity of PKC δ signaling in diverse tissues and the fact that human PKC δ contains multiple Ser, Thr, and Tyr residues (37, 30, and 20, respectively) (see Fig. 1B), it is possible, even likely, that additional, previously unidentified phosphorylation sites involved in the control of the functions of this enzyme in

Figure 1. Principles of the experimental approach and summary of the results obtained. (A) Schematic of the experimental protocol used (see text for details). (B) Amino acid sequence of human PKC δ . Ser, Thr, and Tyr residues are in green, dark blue, and red, respectively. The phosphorylation sites detected in this study are framed in red; other phosphorylation sites reported in the literature but not detected in our study are framed in black. Novel phosphorylation sites reported here are highlighted with a yellow background. Previously described Ser/Thr and Tyr phosphorylation sites are highlighted by the use of light blue and gray backgrounds, respectively. (C) Schematic illustrating localization of the detected phosphorylation sites within the functional modules of PKC δ .

vivo do exist (Gschwendt 1999). Identification of all possible phosphorylation sites on PKC δ may be essential for full understanding of the regulation of this protein in health and disease.

Results and Discussion

We decided to search for potential novel phosphorylation sites on PKC δ in colorectal carcinoma cells using a recently described hypothesis-driven mass spectrometry

Figure 2. (Legend on next page)

strategy termed MIDAS (MRM-initiated detection and sequencing) (Unwin et al. 2005). In colorectal cancer, PKC δ is believed to act as a tumor suppressor, and its overexpression in colon cancer cells may lead to growth inhibition (Perletti et al. 2005; Cerda et al. 2006). To avoid possible complications associated with negative effects of prolonged PKC δ overexpression and to be able to obtain a sufficient amount of the protein for mass spectrometric analysis, we employed the previously developed HCT116 human colorectal carcinoma cells engineered for doxycycline (Dox)-regulated inducible gene expression (HCT116 SMV Luc-only B20 and HCT116 AWE17 cells) (Welman et al. 2005, 2006b). Dox-regulated systems provide a high degree of control over the timing and the expression levels of a protein of interest. They are becoming increasingly popular as a tool to study protein regulation and functions in eukaryotic cells (Berens and Hillen 2003). The cells were grown in the standard tissue culture conditions and transfected with the pBidsRed2–FlagPKC δ plasmid as described previously (Fig. 1A) (Welman et al. 2005, 2006a). The pBidsRed2–FlagPKC δ plasmid enables simultaneous Dox-inducible expression of the Flag-tagged version of the wild-type human PKC δ (GeneBank accession number NM_006254) and dsRed2 reporter protein (Welman et al. 2005). Flag-tag does not interfere with the functions of PKC δ , and is routinely used to facilitate studies of PKC family members (Konishi et al. 2001). The cells were induced with 2 μ g/mL Dox 24 h after transfection. After a subsequent 24 h the cells were washed twice with ice-cold PBS and harvested directly into 1 $\times$ Cell Lysis Buffer (cat no. 9803, Cell Signaling) containing protease inhibitor cocktail (cat no. P8340, Sigma-Aldrich). The Flag-tagged PKC δ was immunoprecipitated using anti-Flag-M2 agarose (cat no. A2220, Sigma-Aldrich). Subsequently, 40 μ L of Laemmli Sample Buffer (cat. no. 161–0737, Bio-Rad) was added and the samples were incubated for 5 min at 100°C to dissociate the resin–PKC δ complexes.

The resulting mixture was resolved by polyacrylamide gel electrophoresis (10% SDS-PAGE) (Laemmli 1970), the gel was fixed for 1 h (7% acetic acid:40% methanol:53% water), then stained with colloidal Coomassie Blue until protein bands became visible (typically 2 h), and the PKC δ band excised from the gel. The band was chopped into $\sim$ 1-mm² pieces, destained in 10% acetic acid:25% methanol:65% water, dehydrated under vacuum, and digested overnight at 37°C with trypsin as described (Unwin et al. 2003). After digestion, the supernatant was removed and kept and any remaining peptides were extracted from the gel pieces by 10-min sonication in 50 μ L 50:50 acetonitrile:5% formic acid. This was then combined with the supernatant removed previously, dried down to minimum volume, and reconstituted to $\sim$ 20 μ L with 2% acetonitrile:98% of 0.1% formic acid in water.

The resultant peptide mixture was analyzed using a 4000 QTRAP mass spectrometer (Applied Biosystems) online to a nanoflow liquid chromatograph. Sample integrity was first assessed by carrying out a standard information-dependent acquisition (IDA) analysis on $\sim$ 5% of the sample. The remaining sample ($\sim$ 95%) was used for a focused search of potential phosphorylation sites using the MIDAS strategy. For all experiments, 1–5 μ L of digested sample was loaded onto a 15 cm $\times$ 75 μ m i.d. PepMap, C18, 3 μ m column (LC-Packings), using a standard LC Packings UltiMate pump and FAMOS autosampler. HPLC buffers A and B consisted of 2% (v/v) acetonitrile:0.1% (v/v) formic acid, and 80% (v/v) acetonitrile:0.1% (v/v) formic acid, respectively. Samples were desalted online prior to separation using a micro precolumn (5 mm $\times$ 300 μ m i.d.) cartridge. The washing solvent was 0.1% formic acid delivered at a flow rate of 30 μ L/min for 4 min. Peptides were separated over a typical gradient of 8% Buffer B to 40% Buffer B over 40 min at a flow rate of 300 nL/min.

The MIDAS protocol takes full advantage of the combined functionality of the 4000 QTRAP, and is

Figure 2. MS/MS spectra for the PKC δ -derived peptides that contained detectable phosphorylation sites. The peptides were generated as illustrated in Figure 1A. “cps” stands for counts per scan and “amu” stands for atomic mass units. (A) MS/MS spectrum for the PKC δ tryptically derived peptide, KPTMYPEWK, containing a phosphorylation site at Thr50. Site of phosphorylation is confirmed by y-ions, y6, y7, and y8. In addition, y7* at m/z 936.5 is characteristic of the conversion of phosphothreonine to dehydroamino butyric acid under collisionally induced dissociation conditions. (B) MS/MS spectrum for the PKC δ tryptically derived peptide, SEDEAKFPTMNR, containing a phosphorylation site at Thr141. Site of phosphorylation is confirmed by y-ions, y4*–y8*, which are characteristic of the conversion of phosphothreonine to dehydroamino butyric acid under collisionally induced dissociation conditions. (C) MS/MS spectrum for the PKC δ tryptically derived peptide, RSDSASSEPVGIYQGFKEK, containing a phosphorylation site at Ser304. Site of phosphorylation is confirmed by b-ions, b3, b4*, and b5*. b* ions correspond to the characteristic conversion of a phosphoserine residue to dehydroalanine under collisionally induced dissociation conditions. (D) MS/MS spectrum for the PKC δ tryptically derived peptide, ATFYAAEIMCGLQLHASK, containing a phosphorylation site at Thr451. Site of phosphorylation is confirmed by b-ions, b3* and b5* characteristic of the conversion of phosphothreonine to dehydroamino butyric acid under collisionally induced dissociation conditions. (E) MS/MS spectrum for the PKC δ tryptically derived peptide, ASTFCGTPDYIAPEILQGLK, containing phosphorylation sites at Ser506 and Thr507. Sites of phosphorylation are confirmed by b-ions, b3*, b5*, and b7*–H₂O. b* ions correspond to the characteristic conversion of a phosphoserine residue to dehydroalanine and phosphothreonine to dehydroamino butyric acid under collisionally induced dissociation conditions. (F) MS/MS spectrum for the PKC δ tryptically derived peptide, NLIDSMDQSAFAGFSFVNPK, containing a phosphorylation site at Ser664. Site of phosphorylation is confirmed by y-ions, y6–y9 and y6*–y10*. y* ions correspond to the characteristic conversion of a phosphoserine residue to dehydroalanine under collisionally induced dissociation conditions.

described in detail elsewhere (Unwin et al. 2005). Briefly, in silico trypsin digestion of PKC δ generates a set of potential phosphopeptides. The mass spectrometer is instructed to sequentially search for the corresponding masses of these peptides, and, provided they generate certain phospho-indicative fragment ions, to then generate MS/MS spectra from which the sites of phosphorylation may be determined. Multiple injections were required due to the large number of potential phosphopeptides associated with this protein.

Following data acquisition and analysis we were able to identify seven Ser/Thr phosphorylation sites on PKC δ purified from HCT116 Luc-only B20 cells (Figs. 1B, 2). No P-Tyr residues could be detected. Two of the identified P-Ser/P-Thr sites represented previously reported phosphorylation sites in the activation loop (Thr 507; =Thr 505 in rat PKC δ) and hydrophobic motif (Ser 664; =Ser 662 in rat PKC δ), respectively, and five (Thr 50, Thr 141, Ser 304, Thr 451, and Ser 506) represented previously unreported phosphorylation sites. These novel phosphorylation sites could also be detected independently on PKC δ purified from HCT116 AWE17 cells (data not shown). Although the physiological significance and potential functions of these newly identified phosphorylation sites remain to be established, they are localized in functionally important regions of the protein (Fig. 1C). Thr 50 is located within the C2-like motif in close proximity of Tyr 52, which can serve as a docking site for the SH2 domain of Src family kinases when phosphorylated. Thr 141 lies within the pseudosubstrate motif. Ser 304 is positioned at the border between the regulatory domain and the “hinge” region. Thr 451 and Ser 506 are both situated within the C4 motif of the catalytic domain with Ser 506 being placed within the activation loop next to the regulatory Thr 507 (=Thr 505 in rat PKC δ). Thr 50, Thr 141, Thr 451, and Ser 506 represent evolutionary conserved residues, and homologous sites are present on PKC δ derived from diverse species including the house mouse (*Mus musculus*), Norway rat (*Rattus norvegicus*), cattle (*Bos taurus*), and dog (*Canis lupus familiaris*). Ser 304 is rather unique for human PKC δ sequence. Importantly, at least three of these newly discovered phosphorylation sites (Thr 50, Thr 141, and Ser 304) seem to result from phosphorylation by other kinases rather than from PKC δ autophosphorylation, as they could be readily detected following PKC δ overexpression in HCT116 cells grown in the presence of excessive amounts (10 μ M) of a cell-permeable PKC δ inhibitor, rottlerin (cat. no. 557370, Calbiochem) (data not shown).

Conclusion

Taken together, the data obtained in this study strongly suggests that the regulation of PKC δ in vivo by phosphorylation may be more complex than previously

thought. The identification of five novel Ser/Thr phosphorylation sites should have a significant impact on future functional studies of this protein.

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