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cGMP-dependent protein kinase I promotes cell apoptosis through hyperactivation of death-associated protein kinase 2

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ABSTRACT

cGMP-dependent protein kinase-I (cGK-I) induces apoptosis in various cancer cell lines. However, the signaling mechanisms involved remain unknown. Using protein microarray technology, we identified a novel cGK substrate, death-associated protein kinase 2 (DAPK2), which is a Ca^{2+}/calmodulin-regulated serine/threonine kinase. cGK-I phosphorylated DAPK2 at Ser^{299}, Ser^{367} and Ser^{368}. Interestingly, a phospho-mimic mutant, DAPK2 S299D, significantly enhanced its kinase activity in the absence of Ca^{2+}/calmodulin, while a S367D/S368D mutant did not. Overexpression of DAPK2 S299D also resulted in a twofold increase in apoptosis of human breast cancer MCF-7 cells as compared with wild-type DAPK2. These results suggest that DAPK2 is one of the targets of cGK-I in apoptosis induction.

1. Introduction

The intracellular second messenger cGMP is generated by guanylate cyclases in response to natriuretic peptides and nitric oxide (NO), and primarily activates cGMP-dependent protein kinase (cGK, PKG). Two genes encoding for cGK-I and cGK-II have been identified, of which cGK-I has two splicing isoforms: cGK-Iα and cGK-Iβ [1]. cGMP/cGK signaling has been shown to be associated with anti-tumor activities, including induction of apoptosis and inhibition of metastasis and angiogenesis in many cell types [2–4]. Treatment of human breast cancer cell lines, MCF-7 and MDA-MB-468, with a cell-membrane permeable cGMP analog resulted in cell growth inhibition and apoptosis induction [5,6]. On the other hand, the expression of cGK-I isoforms is reduced in many tumors compared to normal tissues, and ectopic expression of cGK-Iβ results in decreased tumor growth and invasiveness in nude mouse xenografts [6]. In addition, ectopic expression of cGK-I isoforms in the human colon carcinoma lines SW480 and SW620, which do not express endogenous cGK-I, promoted anoikis (apoptosis resulting from a loss of cell–matrix interactions) [7]. A pro-apoptotic drug exisulind, an inhibitor of cGMP phosphodiesterase PDE5, increased intracellular cGMP levels in SW480 cells [8]. Furthermore, exisulind treatment resulted in induction of cGK-Iβ protein expression in addition to enzyme activation. Thus, it is highly likely that cGK activation is correlated with tumor cell apoptosis. Although recent studies suggested the involvement of the oncogene β-catenin and c-jun N-terminal kinase in cGMP/cGK-induced apoptosis [9–11], the detailed mechanism remains unknown.

The death-associated protein kinase (DAPK) family is a group of highly related serine/threonine kinases that are associated with a wide spectrum of apoptotic signals including interferon γ, tumor necrosis factor-α and anoikis [12]. The DAPK family consists of five members: DAPK1, DAPK2/DRP-1, DAPK3/ZIPK/DLK, DRAK1 and DRAK2. DAPK family members show high sequence homology in their N-terminal catalytic domains, while the structures of their C-terminal regions are different [13]. Both DAPK1 and DAPK2 possess a Ca^{2+}/calmodulin (CaM)-binding domain, while DAPK3 lacks this domain but has a leucine zipper domain and two nuclear localization signals. Although both DAPK1 and DAPK2 are activated by Ca^{2+}/CaM, autophosphorylation in their CaM-binding domains reduces their binding affinity for CaM and their activities [12,14]. In contrast, DAPK3 activity is enhanced by autophosphorylation [15]. Additionally, some members of the DAPK family are transcriptionally regulated by tumor suppressors and are frequently silenced by hypermethylation of their promoter regions in various cancers [13]. Recent studies have shown DAPK2 to be down-regulated in malignant epithelial cells by β-catenin and involved in anoikis [16].

In this study, we identified DAPK2 as a novel substrate for cGK-I using a protein microarray. cGK-I phosphorylated DAPK2 at Ser^{299}, Ser^{367} and Ser^{368}. Phosphorylation of DAPK2 at Ser^{299} enhanced its kinase activity. Furthermore, overexpression of a phospho-mimic DAPK2 mutant, DAPK2 S299D, strongly induced apoptosis in human breast cancer MCF-7 cells compared with wild-type DAPK2.

Abbreviations: cGK, cGMP-dependent protein kinase; DAPK, death-associated protein kinase; NO, nitric oxide; CaM, calmodulin; MLC, myosin light chain; GST, glutathione S-transferase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; APC, adenomatous polyposis coli.

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These findings highlight the importance of the cGK-I/DAPK2 signaling pathway in regulating apoptosis.

2. Materials and methods

2.1. Plasmid construction

cDNAs encoding mouse myosin light chain 2 (MLC2) and mouse DAPK2 were cloned by PCR using the respective specific primers. PCR products were cloned into TA-cloning vector pGEM-T Easy (Promega), and the inserted DNA sequences were confirmed by DNA sequencing. A cDNA encoding for mouse DAPK2 was subcloned into the mammalian expression vector pFLAG-CMV-2 (Sigma). A cDNA encoding for mouse MLC2 was subcloned into a glutathione S-transferase (GST) expression vector, pGEX (GE Healthcare). Site-directed mutagenesis was performed using PrimeSTAR Mutagenesis Basal Kit (Takara Bio) according to the manufacturer's instructions. The mutation was confirmed by DNA sequencing analysis.

2.2. Cell culture and transfection

COS-7 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

2.3. Protein microarray

ProtoArray Human Protein Microarray Kinase Substrate Identification (KSI) Complete Kit (Invitrogen) was used according to the manufacturer's instructions. After equilibration at 4 °C for 15 min, the arrays were blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 2 h at 4 °C. 120 μl of kinase buffer [100 mM MOPS, pH 7.2, 1% Nonidet P40, 100 mM NaCl, 10 mg/ml BSA, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM dithiothreitol and 10 μCi/μl of [γ-^{33}P]ATP (33 nM final concentration) (Perkin Elmer)] containing 50 nM purified bovine cGK-Iα (Promega) and 5 μM cGMP was overlaid onto the array and incubated for 1 h at 30 °C. As a negative control, buffer was overlaid onto the array. The arrays were washed twice with 0.5% SDS and twice with H_2O at room temperature, dried and exposed to X-ray films. Spots were identified using GenePix Pro (Molecular Devices).

2.4. In vitro kinase assay

In vitro phosphorylation by cGK-I was performed as previously described [17]. MLC2 was used as a substrate for a DAPK2 activity assay. GST–MLC2 fusion protein was expressed in *Escherichia coli* and purified as previously described [17]. COS-7 cells transfected with pFLAG-DAPK2 wild type or mutant, were harvested with TNE buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40 and 1 mM EDTA) supplemented with protease inhibitors (10 μg/ml leupeptin and 10 μg/ml aprotinin). Cell lysates were incubated with an anti-FLAG M2 antibody (Sigma) and protein G Sepharose (GE Healthcare) overnight at 4 °C. The beads were washed three times with TNE buffer and twice with 50 mM Tris–HCl, pH7.5. The kinase reaction was carried out by resuspending the complexes in 100 μl of kinase buffer [50 mM Tris–HCl pH 7.5, 20 mM magnesium acetate, 100 μM or 50 μM ATP, 2 μCi [γ-^{32}P]ATP, phosphatase inhibitor cocktail (Nacalai Tesque) and 30 μg/ml purified GST–MLC2] including either 100 mM $CaCl_2$ and 10 nM CaM, or 5 mM EGTA, and incubating for 30 min at 30 °C. Phosphorylated GST–MLC2 was separated by SDS–PAGE and visualized with a BAS-1500 Bioimaging Analyzer (Fuji Film). Quantitative densitometric analysis was performed using Image J.

2.5. Analysis of apoptosis

MCF-7 cells were transiently transfected with either FLAG-tagged DAPK2 wild type or mutants together with a green fluorescent protein (GFP) plasmid. Twenty-four hours after transfection, cells were stained with Hoechst 33342 (Invitrogen) for 10 min at 37 °C in 5% CO_2. They were washed with PBS and fixed with 3.7% formaldehyde in PBS for 30 min at room temperature. After washing with PBS, GFP-expressing cells were observed with a fluorescence microscope (IX71, Olympus). The number of apoptotic cells displaying both membrane blebbing and nuclear condensation was counted and expressed as a percentage of the total cell number; a minimum of 50 randomly chosen cells were counted for each sample.

3. Results

3.1. Identification of putative substrates for cGK-I by protein microarray analysis

To identify novel cGK substrates implicated in inducing apoptosis, we used a human protein microarray spotted with 1700

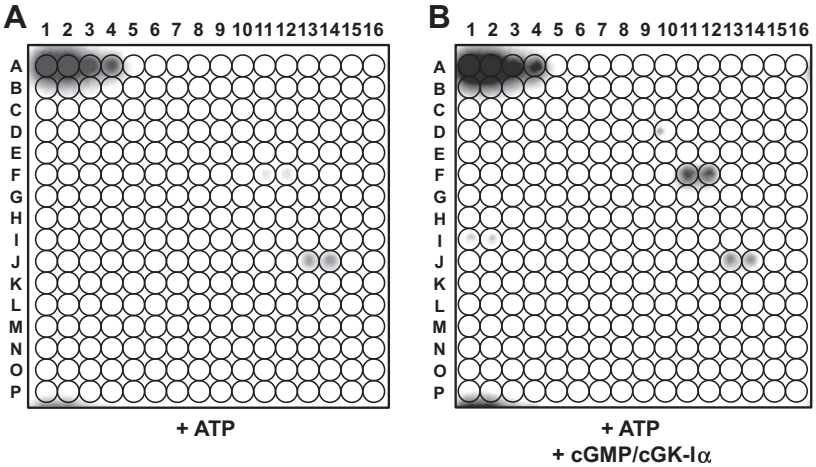

Fig. 1. Identification of DAPK2 as a putative substrate for cGK-I by protein microarray analysis. Human protein microarrays were incubated with [γ-^{33}P]ATP in the absence (A) or presence (B) of cGMP/cGK-Iα. After 30 min, the arrays were dried and exposed to X-ray films. At positions A1–4 and J13–14, protein kinases were autophosphorylated in the presence of ATP. Positions F11–12 were spotted with GST–DAPK2.

GST-tagged proteins in duplicate. The microarrays were incubated with [γ-^{33}P]ATP in the presence or absence of cGMP/cGK-Iα. Spots A1–4 and J13–14 on both arrays were protein kinases that were autophosphorylated in the presence of ATP (Fig. 1). Some spots with strong signals were found on the array incubated with cGMP and cGK-Iα (Fig. 1B) as compared with the control array (Fig. 1A). Spots F11–12, which were spotted with GST–DAPK2, showed greater than 20-fold differences in their signal intensities. DAPK2 is a Ca^{2+}/CaM-dependent protein kinase belonging to the DAPK family [18]. This family consists of five members and acts as a positive regulator of apoptosis [13,18–21]. Amino acid sequence analysis of human DAPK2 identified three potential phosphorylation sites, RRES^{299}, RRRS^{367} and RRSS^{368} for cGK (RR/KXS/T). These sequences are also conserved in mouse and rat DAPK2. These results suggested that DAPK2 was a putative substrate for cGK-I.

Amino acid sequence analysis of human DAPK1 indicated that DAPK1 also has some potential phosphorylation sites for cGK, suggesting that DAPK1 is also phosphorylated by cGK. However, we missed to identify DAPK1 as a novel substrate for cGK-I using protein microarray, because only small amount of GST–DAPK1 protein (nearly one-tenth of GST–DAPK2 protein) was spotted (data not shown).

3.2. cGK-Iβ phosphorylates DAPK2 at Ser^{299}, Ser^{367} and Ser^{368}

To confirm the protein microarray results, we investigated if cGK-I could phosphorylate DAPK2 in an *in vitro* kinase assay. COS-7 cells were transfected with FLAG-tagged mouse DAPK2 along with FLAG-tagged cGK-Iβ. Cell lysates were immunoprecipitated with an anti-FLAG antibody, and the immunocomplex was incubated with [γ-^{32}P]ATP in the presence or absence of cGMP. As shown in Fig. 2A, wild-type DAPK2 was phosphorylated even without cGMP and cGK-Iβ because DAPK2 was autophosphorylated as reported previously [18,21]. To detect cGMP/cGK-mediated phosphorylation of DAPK2, we produced a kinase-dead mutant, DAPK2 K52A, and assessed it with an *in vitro* kinase assay. DAPK2 K52A was not autophosphorylated, but was efficiently phosphorylated by cGK-Iβ in a cGMP-dependent manner (Fig. 2A). This confirmed that DAPK2 was phosphorylated by cGK-I *in vitro*.

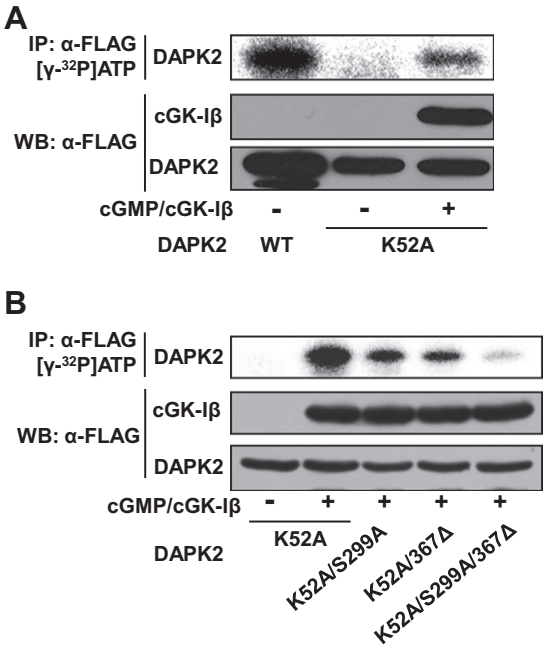

Fig. 2. cGK-I phosphorylates DAPK2 at Ser^{299}, Ser^{367} and Ser^{368}. (A and B) FLAG-tagged DAPK2 wild type or mutant was expressed in COS-7 cells along with FLAG-tagged cGK-Iβ. FLAG-tagged proteins were immunoprecipitated and incubated in a kinase buffer containing [γ-^{32}P]ATP with or without cGMP. To monitor the expression level of the FLAG-tagged proteins, cell lysates were blotted with an anti-FLAG antibody.

DAPK2 has three potential sites for phosphorylation by cGK: Ser^{299}, Ser^{367} and Ser^{368}. To identify DAPK2 sites phosphorylated by cGK-I, we created phospho-resistant mutants for these sites. Ser^{299} was replaced by non-phosphorylatable Ala (DAPK2 K52A/S299A). Because DAPK2 is a protein comprised of 370 amino acids and its Ser^{367} and Ser^{368} residues are close to the C-terminal end, Ser^{367} was replaced by a stop codon, DAPK2 K52A/367Δ. As shown in Fig. 2B, an *in vitro* kinase assay demonstrated that the phosphorylation of both DAPK2 K52A/S299A and K52A/367Δ by cGK-Iβ were reduced compared with that of DAPK2 K52A. Furthermore, cGK-Iβ failed to phosphorylate a triple mutant, DAPK2 K52A/S299A/367Δ. These results suggest that cGK-I phosphorylates DAPK2 at Ser^{299}, Ser^{367} and Ser^{368}.

3.3. Phosphorylation of DAPK2 at Ser^{299} by cGK-I increases its kinase activity

Next, we examined whether DAPK2 phosphorylation affects its kinase activity. Although the three putative phosphorylation sequences (RRES^{299}, RRRS^{367} and RRSS^{368}) were completely matched to the consensus motif for cGK (RR/KXS/T), RSST^{369} also partially corresponded to this phosphorylation motif. Thus, three phospho-mimic mutants were generated in which the putative cGK phosphorylation sites were replaced by Asp: DAPK2 S299D, DAPK2 S367D/S368D and DAPK2 S367D/S368D/T369D. Myosin light chain 2 (MLC2) was used as a substrate for DAPK activity as previously described [13]. As shown in Fig. 3A, wild-type DAPK2 phosphorylated GST–MLC2 in a Ca^{2+}/CaM-dependent manner (3.4-fold increase). Interestingly, the activity of the DAPK2 S299D mutant was significantly increased compared with that of the wild type in the presence of Ca^{2+}/CaM (twofold increase), whereas the activities of DAPK2 S367D/S368D and DAPK2 S367D/S368D/T369D mutants were not. In addition, the DAPK2 S299D mutant showed a significantly high activity even without Ca^{2+}/CaM. The activity of another phospho-mimic mutant, DAPK2 S299E, was also examined. As expected, the DAPK2 S299E mutant also exhibited enhanced kinase activity like that of the DAPK2 S299D mutant (Fig. 3B). These findings suggested that phosphorylation of DAPK2 at Ser^{299}, but not at Ser^{367} and Ser^{368}, stimulated its kinase activity independently of Ca^{2+}/CaM.

A previous study demonstrated that DAPK2 was autophosphorylated at Ser^{318} and inactivated, and that DAPK2 activation is required for dephosphorylation of Ser^{318} [12]. To examine whether phosphorylation of Ser^{299} affects autoinhibition by phosphorylation at Ser^{318}, we generated DAPK2 S318E and DAPK2 S299D/S318E mutants and examined their activities with an *in vitro* kinase assay. As shown in Fig. 3C, the DAPK2 S318E mutant was not activated by Ca^{2+}/CaM, consistent with a previous report [12]. On the other hand, the kinase activity of the DAPK2 S299D/S318E double mutant was similar to that of the DAPK2 S299D mutant and overcame the inhibitory effect of phosphorylation at Ser^{318}. These results suggested that DAPK2 phosphorylation at Ser^{299} increased its kinase activity by a Ca^{2+}/CaM-independent mechanism and also interfered with the autoinhibitory mechanism resulting from phosphorylation at Ser^{318}.

3.4. A phospho-mimic mutant DAPK2 S299D strongly induces apoptosis compared with wild-type DAPK2

A previous study showed that DAPK2 overexpression induced apoptosis in various cell lines, and that a dominant-negative

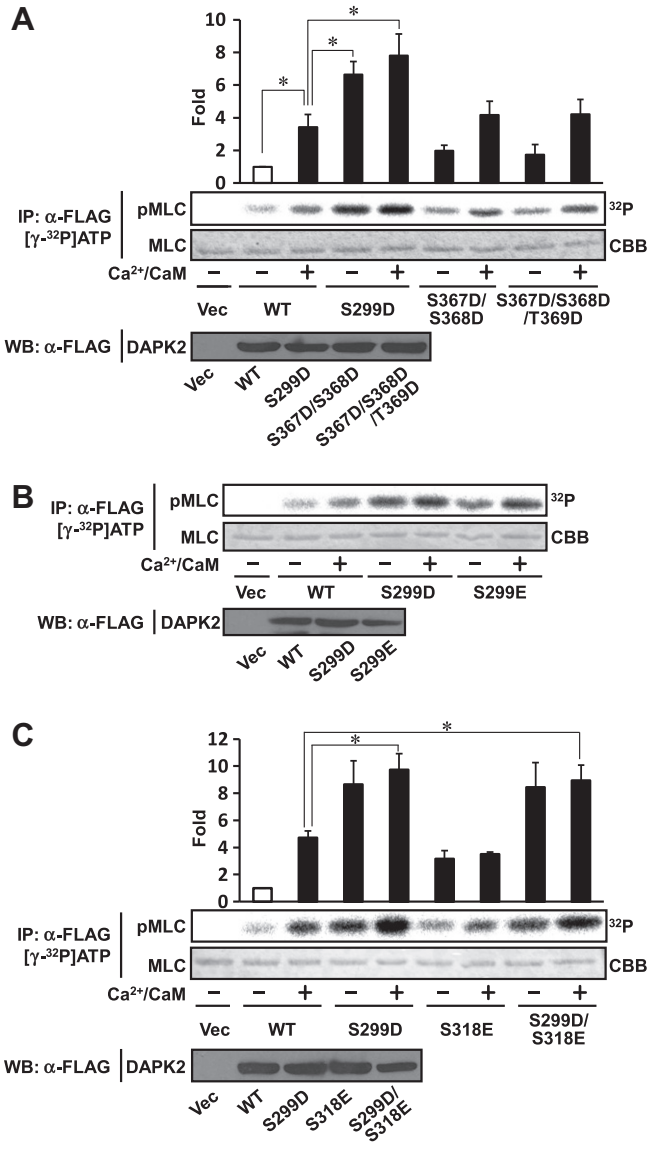

Fig. 3. Phosphorylation of DAPK2 at Ser^{299} enhances its kinase activity. (A, B and C) COS-7 cells were transiently transfected with FLAG-DAPK2 wild type or mutant. FLAG-DAPK2 proteins were immunoprecipitated and used in an *in vitro* kinase assay with recombinant GST–MLC2 as a substrate in the presence of either $CaCl_2$/CaM or EGTA. GST–MLC2 was separated on SDS–PAGE, after which the gel was analyzed by Coomassie blue staining and a bioimaging analyzer. The relative kinase activity of DAPK2 was quantified by densitometric analysis. The activity of wild-type DAPK2 without Ca^{2+}/CaM was taken as 1. All experiments were performed three times independently. Results are expressed as means ± S.E. Statistical significance was determined by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

DAPK2 mutant protected cells from TNF-α-induced apoptosis [18]. These results suggested a strong association between DAPK2 catalytic activity and apoptosis. Thus, we tested whether DAPK2 phosphorylation at Ser^{299} affected the induction of apoptosis in human breast cancer MCF-7 cells. In a previous study [22], apoptotic morphological changes, such as membrane blebbing and nuclear condensation, were clearly observed in MCF-7 cells that were transfected with DAPK2. We transiently transfected MCF-7 cells with either DAPK2 wild type or mutants along with GFP. In GFP-expressing cells, the number of apoptotic cells displaying both membrane blebbing and nuclear condensation was counted. As shown in Fig. 4, overexpression of wild-type DAPK2 significantly induced apoptosis, whereas a DAPK2 inactive mutant, S318E, induced fewer apoptotic changes than wild type consistent with a previous report [12]. In agreement with the data for kinase activity,

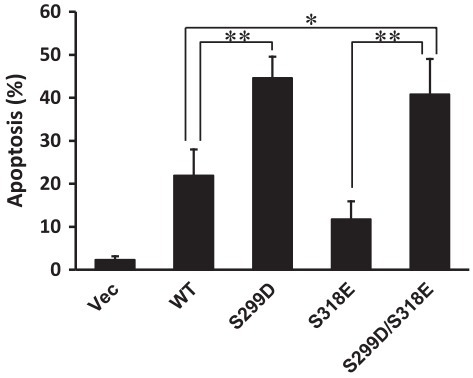

Fig. 4. Phospho-mimic mutant DAPK2 S299D enhances apoptosis induction in MCF-7 cells. pFLAG-DAPK2 wild type or mutant was transiently transfected into MCF-7 cells along with pEGFP. After 24 h, cells were stained with Hoechst 33342 and fixed with formaldehyde. Fixed cells were observed under a fluorescence microscope. The number of cells showing apoptotic morphologies was expressed as a percentage of the total number of GFP-expressing cells counted. Experiments were performed three times independently. Results are expressed as means ± S.E. Statistical significance was determined by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

forced expression of the phospho-mimic mutant DAPK2 S299D resulted in a twofold increase in apoptotic cells compared with wild-type DAPK2. In addition, the same result was observed for DAPK2 S299D/S318E-expresseing cells. These results suggested that cGK-I induced apoptosis through DAPK2 phosphorylation.

4. Discussion

cGMP/cGK signaling can induce apoptosis in cancer cell lines and it has been suggested that this signaling pathway could be a target for anti-cancer agents [2–4]. However, the detailed signaling mechanisms remain unclear. In this study, we identified DAPK2 as a novel cGK substrate using protein microarray technology. DAPK2 belongs to the DAPK family comprised of five kinases that share a high sequence homology in their catalytic domains and are involved in apoptosis induction. DAPK2 is a CaM-dependent serine/threonine kinase that is activated by CaM in response to Ca^{2+} stimuli. In addition to its activation by Ca^{2+}/CaM, DAPK2 activity is also regulated by an autoinhibitory mechanism. By the mechanism called a "double-locking" mechanism, DAPK2 is kept in an inactive state [12]. DAPK2 activation is required for dephosphorylation of an autophosphorylated residue Ser^{318} within the CaM-binding domain and for Ca^{2+}/CaM binding. The negative charge of its phospho-Ser^{318} residue interacts with the positive charge of a Lys^{151} residue in its active site, which results in the inhibition of CaM binding. This autoinhibitory mechanism is necessary to prevent erroneous activation in response to random fluctuations in cellular Ca^{2+} levels. We found that DAPK2 phosphorylation at Ser^{299} increased its kinase activity in a Ca^{2+}/CaM-independent manner and overcame the autoinhibitory mechanism resulting from phosphorylation at Ser^{318}. Because the Ser^{299} residue phosphorylated by cGK-I is also present in the CaM-binding domain and is close to the autophosphorylation Ser^{318} site, we suggest that DAPK2 phosphorylation at Ser^{299} could disrupt the interaction between phospho-Ser^{318} and Lys^{151} and promote Ca^{2+}/CaM binding, thus triggering DAPK2 hyperactivation. However, a CaM overlay assay revealed that a phospho-mimic mutation at Ser^{299} did not influence the binding of CaM to DAPK2 (data not shown). DAPK2 activity has also been shown to be regulated by dimerization [12,23]. This suggests that other mechanisms may be involved in the hyperactivation of DAPK2 by Ser^{299} phosphorylation. DAPK1 and DAPK2 show high sequence homology in their CaM binding domains and have conserved autophosphorylation sites [12,14]. Sequence alignments indicate that DAPK1 has a Ser^{289} residue cor-

responding to Ser^{299} in DAPK2. Interestingly, a previous study showed that DAPK1 phosphorylation at Ser^{289} by p90 ribosomal S6 kinase inhibited its pro-apoptotic activity in a non-tumor cell line, HEK293E [24]. However, our findings may be supported by another study that showed that DAPK2 was activated via trans-phosphorylation of residue(s) other than Ser^{318} by unidentified kinase(s) [12]. Because the autoinhibitory mechanism of DAPK2 has been shown to be different from that of DAPK1 [23], phosphorylation at Ser^{289} in DAPK1 and Ser^{299} in DAPK2 may exert opposite effects.

In cancer cell lines, the expression of DAPK2 is generally silenced by hypermethylation of its promoter [13]. However, a previous report showed that knockdown of oncogenic β-catenin by RNA interference induced DAPK2 expression in a colon cancer cell line [16]. This cell line lacked a functional adenomatous polyposis coli (APC) tumor suppressor protein. Although APC induces β-catenin degradation in normal cells, which is dependent on phosphorylation by glycogen synthase kinase 3β, its degradation is often blocked by loss-of-function mutations of APC in carcinomas. On the other hand, cGMP/cGK signaling attenuates β-catenin-mediated transcription but not β-catenin degradation in colon and breast cancer cells, although the downstream target(s) directly regulated by cGK remain unclear [9,11]. Taken together, cGMP/cGK-I signaling may increase DAPK2 expression via suppression of β-catenin-mediated transcription and activate DAPK2 via phosphorylation at Ser^{299}, resulting in induction of apoptosis in cancer cells.

In summary, we identified a novel cGK-I substrate implicated in apoptosis induction. We determined that DAPK2 kinase activity was regulated by its trans-phosphorylation. Phosphorylation of DAPK2 at Ser^{299} enhanced its kinase activity, which resulted in inducing apoptosis in MCF-7 cells. Further research is required to unravel the cGMP/cGK-I signaling mechanisms involved in order to develop more efficacious anti-tumor drugs.

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