p73β Is Regulated by Protein Kinase Cδ Catalytic Fragment Generated in the Apoptotic Response to DNA Damage*

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Protein kinase C (PKC) δ is cleaved by caspase-3 to a kinase-active catalytic fragment (PKCδCF) in the apoptotic response of cells to DNA damage. Expression of PKCδCF contributes to the induction of apoptosis by mechanisms that are presently unknown. Here we demonstrate that PKCδCF associates with p73β, a structural and functional homologue of the p53 tumor suppressor. The results show that PKCδCF phosphorylates the p73β transactivation and DNA-binding domains. One PKCδCF-phosphorylation site has been mapped to Ser-289 in the p73β DNA-binding domain. PKCδCF-mediated phosphorylation of p73β is associated with accumulation of p73β and induction of p73β-mediated transactivation. By contrast, PKCδCF-induced activation of p73β is attenuated by mutating Ser-289 to Ala (S289A). The results also demonstrate that PKCδCF stimulates p73β-mediated apoptosis and that this response is attenuated with the p73β(S289A) mutant. These findings demonstrate that cleavage of PKCδ to PKCδCF induces apoptosis by a mechanism in part dependent on PKCδCF-mediated phosphorylation of the p73β Ser-289 site.

The p53 tumor suppressor regulates the transcription of genes involved in control of the cell cycle and apoptosis (1). Levels of p53 protein increase in the response of cells to DNA damage and certain other forms of stress. Activation of p53-mediated growth arrest or apoptosis prevents the replication of damaged DNA and thereby maintains integrity of the genome (2). Two p53 homologs, designated p73 and p63, have been identified that activate transcription from p53-responsive promoters and induce apoptosis (3–5). Both p73 and p63 share homology with the transactivation, DNA-binding and oligomerization domains of p53. In contrast to p53, p73 and p63 are expressed as multiple isoforms (3, 5). The p73 and p63 isoforms can fold into stable homotetramers through interactions of their oligomerization domains (6). The available findings further indicate that the oligomerization domain of wild-type p53 does not interact with those of p73 or p63 (6). These findings have suggested that p73 and p63 can activate p53-responsive genes by mechanisms independent of p53.

Several studies have indicated that p73 is involved in the cellular response to DNA damage. Initial reports showed that, unlike p53, p73 is not subject to accumulation in cells treated with genotoxic agents (3). Other work has shown that the α and β isoforms of p73 interact with the c-Abl tyrosine kinase in the genotoxic stress response. c-Abl is activated by DNA damaging agents and contributes to the induction of apoptosis by p53-dependent and -independent mechanisms (7, 8). The findings demonstrate that c-Abl also stimulates p73-mediated transactivation and that p73 participates in the apoptotic response to DNA damage (9–11). Moreover, studies have indicated that p73 is transcriptionally regulated by DNA damage and that a binding site in the p73 promoter is activated by p53 and p73 (12). These findings have provided support for involvement of p73 in response to genotoxic stress.

The protein kinase C (PKC) family of serine/threonine kinases consists of multiple isoforms with conserved catalytic domains (13). Differences in their regulatory domains have resulted in classification of the PKC isoforms into conventional, novel, and atypical subgroups. The ubiquitously expressed PKCδ isoform is a member of the novel PKC subgroup and is activated by diacylglycerol or phorbol esters in a calcium-independent manner (14–16). PKCδ is also activated by c-Abl in the cellular response to stress (17, 18). In this regard, treatment of cells with ionizing radiation (IR) is associated with c-Abl-dependent phosphorylation of PKCδ and translocation of PKCδ to the nucleus (17). Other studies have demonstrated that PKCδ is activated by caspase-3-mediated cleavage at the third variable region (V3) to a 38-kDa regulatory domain and a 40-kDa constitutively active catalytic fragment (CF) (19, 20). The finding that expression of PKCδCF results in DNA fragmentation has supported a role for PKCδ cleavage in the induction of apoptosis (21).

The present studies demonstrate that PKCδCF associates with p73β. The results show that PKCδCF phosphorylates p73β in part on Ser-289. The results also demonstrate that PKCδCF-mediated phosphorylation of Ser-289 contributes to p73β-dependent activation and apoptosis.

MATERIALS AND METHODS

Cell Culture—HCT 116-3 (22) and 293T cells were grown in Dulbecco’s modified Eagle’s minimum essential medium F-12 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 2 mM l-glutamine, and 400 μg/ml genetin sulfate. SAOS-2 cells and HeLa cells were grown as described earlier (23, 24). Cells were treated with 40 μM cisplatin (Sigma), 20 gray IR using a Gammacell 1000 (2.98 Gray/min; Atomic Energy of Canada) or 20 ng/ml tumor necrosis factor-α (TNF-α; Promega, Madison, WI) and 10 μg/ml cycloheximide (Sigma).

Immunoprecipitation and Immunoblot Analysis—Cell lysates were prepared as described (25). Soluble proteins were incubated with anti-

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p73 is Regulated by PKCα

p73 (Neomarkers Inc., Fremont, CA), anti-PKCα (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-c-Abl (Santa Cruz) for 1 h and precipitated with protein A-Sepharose for an additional 1 h. The resulting immune complexes were washed in lysis buffer, separated by electrophoresis in SDS-PAGE, and transferred to nitrocellulose filters. The residual binding sites were blocked by incubating the filters with 5% dry milk in PBS/T (phosphate-buffered saline, 0.05% Tween 20) for 1 h at room temperature. Immunoblot analysis was performed with anti-p73, anti-PKCα, anti-FLAG (Sigma), anti-c-Abl (Calbiochem), or anti-p21 (Oncogene Research Products, Boston, MA).

Fusion Protein-binding Assays—Plasmids expressing glutathione S-transferase (GST)-p73β transactivation domain (TAD; amino acids 1–135), DNA-binding domain (DBD; amino acids 128–313), and oligomerization domain (OD; amino acids 311–499) were prepared by cloning the appropriate PCR product of human p73β into pGEX-2T (Promega). GST-PKCαCF and GST-PKCαCF(K-R) were prepared as described (17). Fusion proteins were purified by affinity chromatography using glutathione-Sepharose beads. Plasmids expressing histidine-(His)-PKCαCF and His-PKCαCF(K-R) were prepared by cloning PCR products obtained from pKVK-PKCα (21) into pET-28a+ (Novagen, Madison, WI). For fusion protein-binding assays, purified His proteins were incubated with immobilized GST fusion proteins for 1 h at 4°C. The resulting protein complexes were washed 4 times. The proteins were then separated by SDS-PAGE and subjected to immuno blot analysis with anti-p73 or anti-PKCα. Gels were also analyzed after staining with Coomassie Blue (Sigma).

In Vitro Phosphorylation Assays—Purified GST, GST-p73βTAD, GST-p73βDBD, GST-p73βOD, and myelin basic protein (Invitrogen) were incubated in kinase buffer (20 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, and 4 mM dithiothreitol) containing [γ-32P]ATP or cold ATP. Kinase-active recombinant PKCαFL (Panvera Corp., Madison, WI), His-PKCαCF, or kinase-inactive His-PKCαCF(K-R) was added for 30 min at 30°C. The reaction products were analyzed by SDS-PAGE and autoradiography.

Identification of In Vitro Phosphorylation Sites—Purified GST-p73βTAD, GST-p73βDBD, and GST-p73βOD was incubated with GST-PKCαCF(K-R) or PKCαCF. The reaction products were subjected to SDSPAGE. The p73β band was identified by Coomassie Blue staining and excised from the gel. In-gel digestion with trypsin was performed as described (26, 27). For 32P-labeled p73β, the trypsin-digested peptides were fractionated by reverse transcriptase-high performance liquid chromatography. Aliquots of the fractions were assayed for [32P]. Positive fractions were subjected to Edman sequencing. For unlabeled p73β, masses of the trypsin-digested peptides were analyzed by matrix-assisted laser desorption/ionization-mass spectroscopy using a Voyager DE-PRO (Perceptive Biosystem Inc., Framingham, MA).

Site-directed Mutagenesis—p73β(S289A) was generated using the site-directed mutagenesis kit (Stratagene, La Jolla, CA) to change Ser-289 to Ala. In Vitro Transfections—Cells were transfected with FLAG-p73β, GFP-p73β, pκV-PKCαCF, pκV-PKCαCF(K-R), pGFP-PKCαCF, pGFP-PKCαCF, or pGFP-PKCαCF(K-R) (21, 25, 28). These findings demonstrate that p73β binds to both PKCαFL and PKCαCF.

RESULTS

p73 Associates with PKCα in Cells—To define proteins that associate with p73, HCT116 cell lysates were subjected to immunoprecipitation with anti-p73. Analysis of the precipitates by SDS-PAGE and staining demonstrated a coprecipitating protein of 78 kDa. Further analysis of the protein by matrix-assisted laser desorption/ionization-mass spectroscopy demonstrated identity with PKCα (data not shown). To extend these findings, anti-p73 immunoprecipitates from HCT116 cells were subjected to immunoblotting with anti-PKCα. The results confirmed the association of p73 and full-length PKCα (PKCαFL) (Fig. 1). PKCαFL is cleaved by caspase-3 to a constitutively active catalytic fragment (PKCαCF) in the apoptotic response of cells to genotoxic stress (19, 20). In concert with these findings, treatment of HCT116 cells with cisplatin was associated with cleavage of PKCαFL to PKCαCF (Fig. 1, second lane). Moreover, analysis of anti-p73 immunoprecipitates from cisplatin-treated HCT116 cells demonstrated coprecipitation of p73 with both PKCαFL and PKCαCF (Fig. 1, third and fourth lanes).

Binding of p73 and PKCα in Vitro—To assess regions of p73 involved in the association with PKCα, GST-p73β fusion proteins (Fig. 2A) containing the TAD (amino acids 1–135), DBD (amino acids 128–313), and OD (amino acids 311–499) were incubated with His-PKCαFL or His-PKCαCF. Immunoblot analysis of the adsorbents with anti-PKCα demonstrated binding of PKCαFL to each of the three domains (Fig. 2B). By contrast, binding of PKCαCF was detectable with p73β TAD and DBD, but not the OD (Fig. 2C). These findings demonstrate that p73β binds to both PKCαFL and PKCαCF.

PKCα Phosphorylates p73—To determine whether p73 is a substrate for PKCα, the GST-p73β fusion proteins were incubated with PKCαFL and [γ-32P]ATP. Analysis of the reaction products demonstrated a low level of p73β TAD and DBD phosphorylation (Fig. 3A). As a control, PKCαFL-mediated phosphorylation of myelin basic protein was readily detectable (Fig. 3A). In addition, PKCαFL autophosphorylation was detectable in each of the reactions (Fig. 3A). Similar studies performed with PKCαCF demonstrated clearly detectable phosphorylation of p73β TAD and DBD, but not OD (Fig. 3B). By contrast, there was no detectable phosphorylation of p73β in reactions containing the kinase-inactive PKCαCF(K-R) mutant (Fig. 3B). To define sites of phosphorylation, p73β was incubated with PKCαCF and [γ-32P]ATP, purified by high-performance liquid chromatography, and analyzed by mass spectrometry. The results showed that p73β is phosphorylated, at least in part, on Ser-289 in the DBD (data not shown). To confirm these findings, Ser-289 was mutated to Ala. Incubation of the p73β(DBD/S289A) mutant with PKCαCF showed decreased phosphorylation compared with that obtained with wild-type p73βDBD, but not complete abrogation of the signal (Fig. 3C).
In concert with these findings, PKCδ-mediated phosphorylation of p73β (S289A) was decreased compared with that found with wild-type p73β (Fig. 3D). These results demonstrate that PKCδ phosphorylates the p73β DBD on Ser-289 and that there are additional sites for PKCδ phosphorylation in the DBD and TAD.

PKCδ Regulates p73 Expression in Vivo—To extend the finding that endogenous PKCδ and PKCδ associate with p73β in HCT116 cells, we expressed GFP-p73β and PKCδ in HeLa cells (Fig. 4A, first to fourth lanes). Immunoblot analysis of anti-GFP immunoprecipitates with anti-PKCδ demonstrated binding of GFP-p73β to endogenous PKCδ and that the formation of GFP-p73β–PKCδ complexes is increased by overexpression of PKCδ (Fig. 4A, fifth to seventh lanes). The results also demonstrate binding of GFP-p73β and PKCδ (Fig. 4A, eighth lane). Similar results were obtained whenFLAG-tagged p73β was expressed with PKCδ or PKCδ (data not shown). To determine whether PKCδ affects p73β expression, cells were transfected withFLAG-p73β and GFP-PKCδ or GFP-PKCδ. Immunoblot analysis of...
were transfected with GFP-p73 and analyzed by immunoblotting with anti-PKC and anti-GFP.

To further assess the role of PKCζCF in p73β-mediated transactivation, we assayed transfectants for induction of p21. As shown previously (11), transfection of p73ζ alone (Fig. 7A). Notably, cotransfection of p73β and PKCζCF, and not PKCζFL or PKCζCF(K-R), increased p73β expression in cell treatment with TNF-α, which lacks the c-Ab- phosphorylation site for PKCζCF in the response of cells to DNA damage and not by pro-apoptotic signaling through the TNF-α death receptor.

PKCζCF Regulates p73-mediated Transactivation—To determine whether PKCζCF affects p73 function, we transfected SAOS2 cells, which are deficient in both p53 (31) and p73 (3), with a construct containing the luciferase gene driven by a p53 enhancer from the p21 promoter (p21-Luc) (29). Co-transfection of p21-Luc with vectors expressing FLAG-p73β and PKCζCF was associated with a 5.1-fold increase in p73 levels as compared with that obtained in the absence of PKCζCF (Fig. 6A). As a control, cotransfection of FLAG-p73β and kinase-inactive PKCζCF(K-R) had no effect on p73β expression (Fig. 6A). To confirm these findings, similar transfection studies were performed with the p73β(S289A) mutant. The results demonstrate that, whereas PKCζCF increases expression of p73β, this response was attenuated with p73ζ (S289A) (Fig. 6B). In concert with these results, PKCζCF, and not PKCζCF(K-R), stimulated p73β-mediated activation of the luciferase reporter (Fig. 6C). In addition, the effects of PKCζCF were attenuated in part when coexpressed with the p73β(S289A) mutant (Fig. 6C).

To further assess the role of PKCζCF in p73β-mediated transactivation, we assayed transfectants for induction of p21. As shown previously (11), transfection of p73β was associated with increased expression of p21 protein (Fig. 7A). Notably, cotransfection of p73β and PKCζCF, and not PKCζFL or PKCζCF(K-R), induced p21 compared with that in cells transfected with p73β alone (Fig. 7A). Analysis at different intervals after transfection demonstrated that induction of p21 corresponds with levels of

**Fig. 4. PKCζCF regulates p73β expression in vivo.** A, HeLa cells were transfected with GFP-p73β and pKV-PKCζFL or pKV-PKCζCF. Lysates were subjected to immunoprecipitation (IP) with anti-GFP and analyzed by immunoblotting with anti-PKCζ. B, HeLa cells were transfected with the indicated plasmids. Lysates were analyzed by immunoblotting with anti-FLAG, anti-PKCζ, or anti-actin. C, 293T cells were transfected with the indicated plasmids. Anti-c-Ab- immunoprecipitates were analyzed for phosphorylation of GST-Crk-(120–225) (upper panel) or GST-Crk-(120–212) (second panel). Intensity of the phospho-Crk bands was determined by densitometric scanning and compared with that of the control. Anti-c-Ab- immunoprecipitates were also subjected to immunoblotting with anti-c-Ab- (third panel). Lysates not subjected to immunoprecipitation were analyzed by immunoblotting with anti-PKCζ (fourth panel) and anti-actin (lower panel).
p73β and PKCδCF expression (Fig. 7B). These results collectively demonstrate that PKCδCF induces p73β-mediated transactivation by a kinase-dependent mechanism.

Fig. 5. PKCδCF regulates p73β expression in response to genotoxic stress. HCT116-3 cells were treated with 40 μM cisplatin (CDDP) (A and B), 20 gray IR (C), or 20 ng/ml TNF-α and 10 μg/ml cycloheximide (CHX) (D) for the indicated times. Immunoblot analysis of the lysates was performed with anti-p73, anti-PKCδ, or anti-actin.

Fig. 6. PKCδCF induces p73β transactivation in vivo. HeLa cells were transfected with p21-Luc, β-galactosidase, and the indicated plasmids. A and B, cell lysates prepared from transfected cells were subjected to immunoblot analysis with anti-FLAG or anti-PKCδ. C, luciferase and β-galactosidase assays were performed at 36 h after transfection. Relative luciferase activity was determined by normalizing the luciferase activity with β-galactosidase activity. The results are expressed as the mean ± S.D. for two experiments each performed in triplicate.
PKCδ/δCF Regulates p73-mediated Apoptosis—To extend the functional significance of the interaction between PKCδ/δCF and p73β, studies were performed to assess whether PKCδ/δCF affects p73β-induced apoptosis. As shown previously (32), expression of PKCδ/δCF induces an apoptotic response (Fig. 8). Notably, coexpression of GFP-p73β and PKCδ/δCF caused a greater increase in the number of apoptotic cells than that achieved collectively with either alone (Fig. 8). Co-transfection of GFP-p73β and PKCδFL was associated with an increase in apoptosis compared with that found with GFP-p73β alone, but not to the extent observed with PKCδ/δCF (Fig. 8). By contrast, cotransfection of GFP-p73β and PKCδ(K-R) had little effect compared with the percentage of apoptotic cells resulting from expression of GFP-p73β alone (Fig. 8).

DISCUSSION
Proteolytic Activation of PKCδ in Apoptotic Cells—Diverse substrates are subject to caspase-3-mediated cleavage in cells induced to undergo apoptosis. Whereas most substrates of caspase-3 are inactivated, certain proteins, such as PKCδ (19, 20), PKCγ (24), the p21-activated kinase 2 (33), cytosolic phospholipase A2 (34), and PITSLRE kinase a2-1 (35), are activated by caspase-3-mediated proteolysis. Cleavage of PKCδ at a DMQD/N site in the third variable region (V3) generates a 40-kDa fragment that contains the ATP-binding and kinase domains (19, 20). Loss of the N-terminal regulatory sequences results in a catalytic fragment that is constitutively active in the absence of diacylglycerol or phorbol esters (19, 20). The demonstration that overexpression of the PKCδ catalytic fragment (PKCδCF) is associated with chromatin condensation, nuclear fragmentation, appearance of sub-G₁ DNA, and lethality has supported a role for PKCδ cleavage in the induction of apoptosis (32). The mechanisms responsible for PKCδCF-induced apoptosis are, however, largely unknown.

Certain insights regarding the role of PKCδCF in apoptosis have been derived from the finding that PKCδCF phosphorlates the DNA-dependent protein kinase (DNA-PK) (25). Interaction of PKCδCF and DNA-PK inhibits the function of DNA-PK to associate with Ku-DNA complexes and to phosphorylate its downstream target, p53 (25). Notably, cells deficient in DNA-PK exhibit partial resistance to apoptosis induced by overexpression of PKCδCF (25). These findings have provided support for involvement of PKCδCF in the regulation of an effector of the DNA damage response. The present studies extend the functional role of PKCδCF by demonstrating an interaction with p73. As found previously for DNA-PK (25), p73 associates constitutively with both PKCδFL and PKCδCF. The significance of the association between p73 and PKCδFL is unclear, but conceivably represents a mechanism in which p73 is regulated by signals that activate PKCδFL in the absence of caspase-3-mediated cleavage.

Interaction of p73 and PKCδCF—Like other members of the p53 family, the p73α and p73β isoforms contain transactivation DNA-binding and oligomerization domains (3). The two isoforms differ at their C termini as a result of differential splicing of the p73 mRNA (3). Both isoforms activate p53-responsive promoters and induce apoptosis (4, 36). The homology between p53 and p73 suggested that p73 might function in the cellular stress response. Indeed, recent studies showed that p73 is activated by IR- and cisplatin-induced DNA damage and that this response is regulated in part by the c-Abl kinase (9–11).
The findings demonstrate that c-Ab1 stimulates p73-mediated transactivation (9–11). Moreover, p73-mediated apoptosis is regulated by a c-Ab1-dependent mechanism (9–11). Other studies have indicated that transcription of the p73 gene is activated by DNA damage (12). These findings have supported a role for p73 in the genotoxic stress response.

The present studies demonstrate that, in addition to c-Ab1, p73 is regulated by PKCθ. In this regard, it is noteworthy that c-Ab1 and PKCθ have been found to interact by cross-activating their kinase functions in the cellular responses to genotoxic and oxidative stress (17, 18). The present results show that both PKCθFL and PKCθCF associate with p73. The results also show that activation by cleavage to PKCθCF is necessary for the detection of p73 phosphorylation. These findings do not exclude the possibility that activation of PKCθ by other mechanisms, such as through interactions with c-Ab1, could similarly result in PKCθFL-mediated phosphorylation of p73. Our results further show that PKCθCF phosphorylates p73θ, at least in part, on Ser-289 in the DBD. Thus, mutation of Ser-289 to Ala was associated with a decrease in, but not complete, activation (7), TNF-α/H9252 also interacts with p73 and stimulates p73-mediated phosphorylation of Ser-289 and that a second signal mediated by c-Ab1 may be necessary to fully activate p73θ.

Previous work has shown that p73α and p73β can induce apoptosis (4) and that c-Ab1 contributes to p73-mediated apoptosis in response to genotoxic stress (9–11). Other studies have demonstrated that E2F-1 induces transcription of the p73 gene and that p73 is functional in mediating E2F-1-induced apoptosis (41). In concert with these findings and the demonstration that PKCθCF also induces apoptosis (32), the present results demonstrate that the interaction between PKCθCF and p73 contributes to the apoptotic response. As the generation of PKCθCF is conferred by activation of caspase-3, the interaction between PKCθCF and p73 would serve to amplify, rather than initiate, the induction of apoptosis. Thus, cleavage of PKCθCF to the constitutively activated PKCθCF would appear to function as a fail-safe mechanism to ensure that once a cell has committed to undergo apoptosis then pro-apoptotic effectors (i.e. p73) are subject to potentially irreversible induction by PKCθCF-dependent signaling.

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