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Phosphorylation-regulated Cleavage of the Tumor Suppressor PTEN by Caspase-3

IMPLICATIONS FOR THE CONTROL OF PROTEIN STABILITY AND PTEN-PROTEIN INTERACTIONS*

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Josema Torres‡§, Joe Rodriguez¶, Michael P. Myers¶, Miguel Valiente‡ ‡‡, Jonathan D. Graves‖, Nicholas K. Tonks¶, and Rafael Pulido‡**

From the ‡Instituto de Investigaciones Citológicas, Amadeo de Saboya 4, Valencia 46010, Spain, ¶Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, and ‖Department of Immunology, University of Washington Medical Center, Seattle, Washington 98195

PTEN phosphatase is one of the most commonly targeted tumor suppressors in human cancers and a key regulator of cell growth and apoptosis. We have found that PTEN is cleaved by caspase-3 at several target sites, located in unstructured regions within the C terminus of the molecule. Cleavage of PTEN was increased upon TNFα-cell treatment and was negatively regulated by phosphorylation of the C-terminal tail of PTEN by the protein kinase CK2. The proteolytic PTEN fragments displayed reduced protein stability, and their capability to interact with the PTEN interacting scaffolding protein S-SCAM/MAGI-2 was lost. Interestingly, S-SCAM/MAGI-2 was also cleaved by caspase-3. Our findings suggest the existence of a regulatory mechanism of protein stability and PTEN-protein interactions during apoptosis, executed by caspase-3 in a PTEN phosphorylation-regulated manner.

The *PTEN* gene, one of the tumor suppressor genes most frequently mutated in human cancers, encodes a protein of 403 amino acids with phosphatase activity (1–4). PTEN contains tyrosine phosphatase activity toward phosphorylated peptides and proteins *in vitro* (5, 6). It has been proposed that PTEN controls cell motility and invasiveness by tyrosine dephosphorylation of the focal adhesion kinase, Fak, and the adapter protein Shc (7). On the other hand, PTEN possesses lipid phosphatase activity toward 3-phosphoinositides, counteracting the action of the oncogenic phosphatidylinositol 3-kinases and blocking the activation of the proto-oncogene protein kinase B/Akt (8, 9). Structurally, PTEN is composed of two domains, an N-terminal phosphatase catalytic domain and the C-terminal C2 domain, which binds phospholipids *in vitro* and is thought to mediate the binding of the molecule to membranes (10). In addition, PTEN contains three unstructured regions: first, the first seven residues form part of a phosphatidylinositol (4,5)-diphosphate binding motif; second, an internal loop within the C2 domain (residues 286–310) of unknown function; third, a C-terminal tail (residues 354–403) that contains a region phosphorylated by the protein kinase CK2, as well as a PDZ binding motif located at the far C terminus (1–4). We and others have reported that the phosphorylation of PTEN by CK2 regulates its function and protein stability to proteasome-mediated degradation (11–13). The C-terminal PDZ binding motif of PTEN has been shown to interact with the second PDZ domain of the scaffolding proteins S-SCAM/MAGI-2 and MAGI-3. This interaction has been shown to enhance the inhibitory effect of PTEN on protein kinase B activation (14, 15).

Caspases (cysteinyl-directed aspartate-specific proteases) are a family of highly specific proteases that play a key role during the apoptotic cell death. Caspases, constitutively expressed as inactive zymogens, are activated in a proteolytic cascade, in which initiator caspases, such as caspase-8, are activated by autoproteolysis in response to apoptotic cell stimulation. Initiator caspases then proteolytically activate downstream executioner caspases, such as caspase-3, which target a restricted set of structural and signaling proteins. Cleavage of these substrates accounts for cell death, as well as the phenotypic alterations characteristic of apoptosis (16). Thus, the identification of caspase targets is crucial to understand how these caspases control the programmed cell death. In this regard, caspase-3 is the major apoptotic executioner protease and directly cleaves most of the proteins that are proteolyzed during apoptosis (17, 18).

PTEN plays a critical role in regulating cell survival; however, the regulation of its biological activity remains poorly understood. In this report, we describe that both the tumor suppressor PTEN and the scaffolding protein S-SCAM/MAGI-2 are targets of caspase-3. Interestingly, our results indicate that the proteolysis of PTEN by caspase-3 is negatively regulated by CK2-mediated phosphorylation of the phosphatase. The implications of the caspase-3-mediated proteolysis of PTEN, as a potential regulatory mechanism of protein stability and PTEN-protein interactions, are discussed.

EXPERIMENTAL PROCEDURES

Plasmids, Reagents, and Antibodies—pGEX-4T PTEN, pRK5 PTEN, and pRK5 HA[1]-PTEN wild type (w.t.) or the phosphorylation mutations

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§ Recipient of a fellowship from Generalitat Valenciana, Spain and a Journal of Cell Science traveling fellowship. Present address: Keratinocyte Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom.

** To whom correspondence should be addressed: Instituto de Investigaciones Citológicas, Amadeo de Saboya 4, Valencia 46010, Spain. Tel.: 34-96-3391256; Fax: 34-96-3601453; E-mail: rpulido@ochoa.fib.es.

‡‡ Recipient of a postdoctoral fellowship from Bancaja, Spain.

[1] The abbreviations used are: HA, hemagglutinin; w.t., wild type; TNFα, tumor necrosis factor α; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; HEK, human embryonic kidney; GST, glutathione *S*-transferase; PARP, poly(ADP)ribose polymerase.

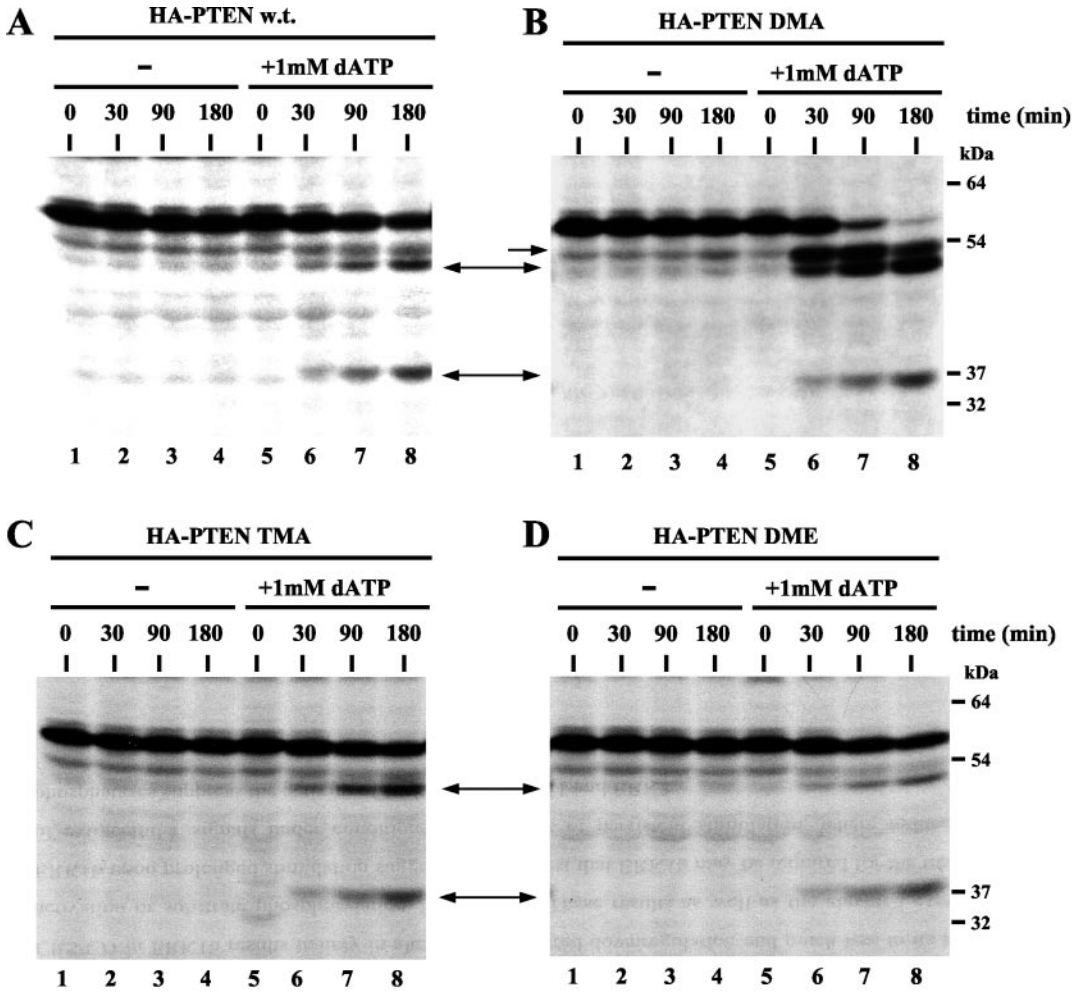

FIG. 1. **Proteolysis of PTEN by dATP-activated HEK293 cellular extracts.** ^{35}S-Labeled HA-PTEN w.t. (*panel A*) or the phosphorylation mutations HA-PTEN DMA (S370A/S385A) (*panel B*), HA-PTEN TMA (S380A//T382A/T383A) (*panel C*), or HA-PTEN DME (S370E/S385E) (*panel D*) were incubated at 30 °C with non-activated (*lanes 1–4*) or dATP-activated HEK293 cellular extracts (*lanes 5–8*). At the indicated times, aliquots were mixed with 15 μl of 2× SDS-PAGE sample buffer and boiled. Samples were resolved on 8% SDS-PAGE gels and analyzed by autoradiography. *Arrows* indicate the cleavage products of HA-PTEN common to all constructs (*double-head arrows*) or specific to HA-PTEN DMA mutation (*single-head arrow* in *B*).

S370A/S385A (DMA), S380A/T382A/T383A (TMA), and S370E/S385E (DME) have been described previously (12). PTEN catalytically inactive and caspase cleavage-site mutations were obtained by polymerase chain reaction oligonucleotide site-directed mutagenesis, and the mutations were confirmed by DNA sequencing. To obtain the construct pGEX-4T (S-SCAM)-PDZ 2, the second PDZ domain of rat S-SCAM (residues 591–732) was amplified by PCR from the plasmid pCMV Myc-S-SCAM (19) and subcloned into pGEX-4T. The broad-spectrum caspase inhibitor Boc-Asp(Ome)-fmk (Alexis Biochemicals) and the caspase-3 inhibitor z-DEVD-fmk (Calbiochem) were used at 50 and 4 μM, respectively. The caspase-3 colorimetric substrate, DEVD-*p*NA, was from MBL. Recombinant mouse TNFα (Sigma) was used at 100 ng/ml (for U87MG cells) or 20 ng/ml (for HeLa S3 cells). In some experiments, TNFα was used in combination with cycloheximide (10 ng/ml). Recombinant active His-tagged caspase-3 was purified from bacteria with nickel-nitrilotriacetic acid-agarose beads by standard procedures and used at 100 ng/μl for *in vitro* proteolysis assays. Recombinant active human caspase-9 was from MBL and was used at 20 milliunits/μl. Human recombinant CK2 holoenzyme was from Roche Applied Science and was used at 6 microunits/μl. The rabbit polyclonal antibody against the N terminus of PTEN was raised against residues 1–16 of human PTEN. The monoclonal antibodies against caspase-3, caspase-7, and caspase-9 have been described previously (20, 21). The rabbit polyclonal antibodies anti-PARP and anti-ERK1/2 were from Santa Cruz Biotechnology, Inc. The anti-HA and anti-Myc monoclonal antibodies were 12CA5 and 9E10, respectively.

Cell Lysis, Immunoblot, and Cell Fractionation in Triton X-100—Transfected COS-7 cells were rinsed in ice-chilled PBS and lysed in Buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 2 mM Na_3VO_4, 20 mM $Na_4P_2O_7$) and then the cell lysates were centrifuged (14000 rpm, 10 min at 4 °C), and the supernatants were used for immunoprecipitation or pull-down experiments, as indicated. For cell lysis of transfected U87MG cells (see Fig. 5*B* and Fig. 6*A*) and HeLa S3 cells (see Fig. 5*C*), all cells, attached and floating, were collected by centrifugation and washed once with ice-chilled PBS. Then, cells were lysed in SDS-PAGE sample buffer. For the fractionation of the cells in Triton X-100 (see Fig. 5*A*), transfected U87MG cells were rinsed in ice-chilled PBS and lysed in Buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA), followed by centrifugation (14000 rpm, 10 min at 4 °C), and the supernatant was considered as the Triton X-100-soluble fraction. The pellet was further extracted with SDS-PAGE sample buffer during 5 min at 95 °C, followed by centrifugation (14000 rpm, 5 min at room temperature), and the supernatant was considered as the Triton X-100-insoluble fraction. For the measurement of caspase-3 activity in cell extracts, transfected U87MG cells were lysed in NPM buffer (50 mM PIPES, pH 7.0, 50 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 μg/ml aprotinin) containing 0.1% Triton X-100.

Cell Culture, Transfections, and [^{35}S]Methionine Labeling of Proteins—COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 5% heat-inactivated fetal bovine serum. HEK293, HeLa S3, and U87MG cell lines were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated FBS. U87MG cells were transfected using FuGENE 6 (Roche Applied Science) following the recommendations of the manufacturer. COS-7 cells were transfected by the DEAE-dextran method. [^{35}S]Methionine labeling and purification of HA-tagged PTEN or Myc-tagged S-SCAM was carried out in transfected

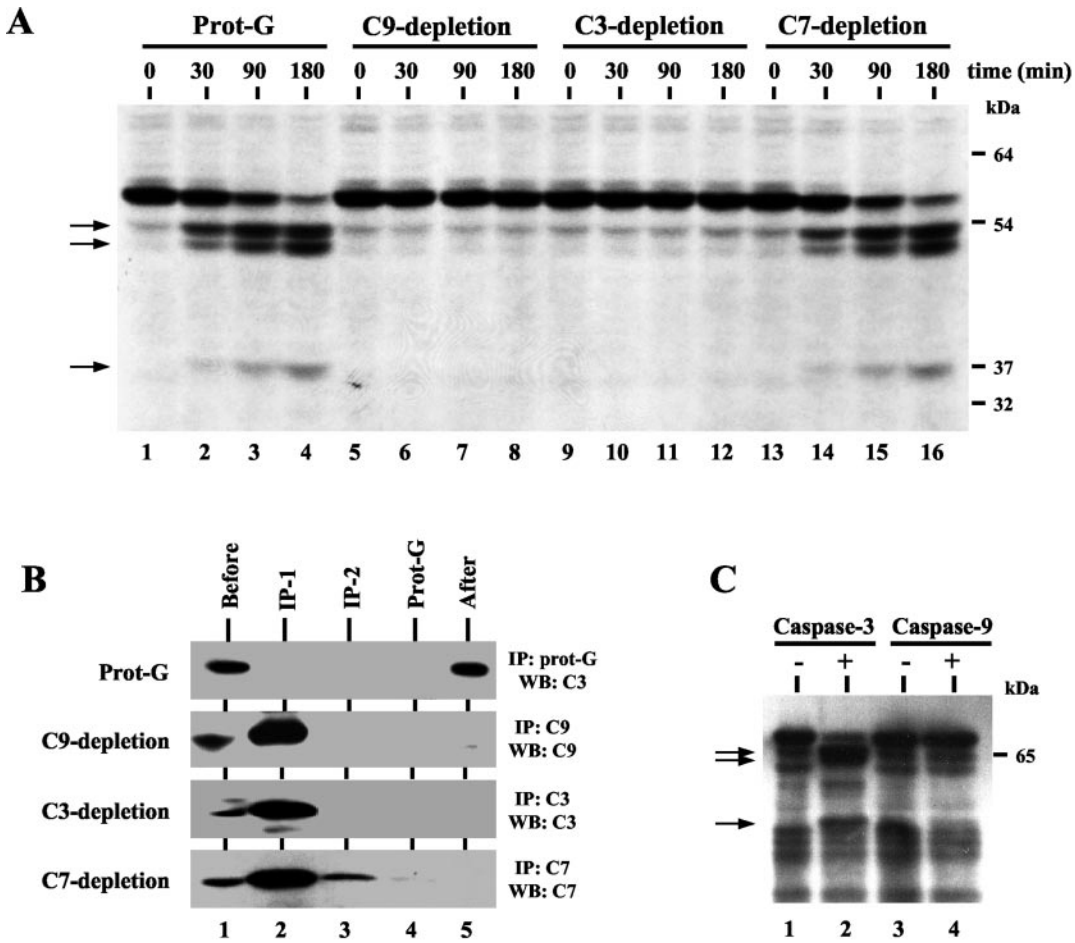

FIG. 2. **Cleavage of PTEN is caspase-3-dependent.** *A*, ^{35}S-labeled HA-PTEN DMA mutation was incubated with activated HEK293 cellular extracts that were not immunodepleted (*lanes 1–4*) or immunodepleted of caspase-9 (*lanes 5–8*), caspase-3 (*lanes 9–12*), or caspase-7 (*lanes 13–16*) prior to their activation with 1 mM dATP. At the indicated times, aliquots were processed and analyzed as in Fig. 1. *B*, immunoblot of the HEK293 cellular extracts before and after the immunodepletions (*lanes 1* and *5*, respectively) and of the precipitates corresponding to the first (*IP-1*; *lane 2*) and second (*IP-2*; *lane 3*) rounds of immunodepletion and after the incubation with protein G following the two immunodepletions steps (*Prot-G*; *lane 4*). *C9*, caspase-9; *C3*, caspase-3; *C7*, caspase-7; *IP*, immunoprecipitation; *WB*, immunoblot. *C*, GST-PTEN, purified from bacteria, was incubated in the absence (*lanes 1* and *3*) or in the presence of recombinant active caspase-3 (100 ng/μl) (*lane 2*) or caspase-9 (20 milliunits/μl) (*lane 4*) for 3 h and then samples were mixed with 15 μl of 2× SDS-PAGE sample buffer and boiled. Samples were resolved on 8% SDS-PAGE gels and analyzed by immunoblot with an anti-GST antibody. In *A* and *C*, the *arrows* indicate the cleavage products of HA-PTEN and GST-PTEN, respectively.

COS-7 cells as described (12). Briefly, transfected COS-7 cells were incubated for 1 h in methionine-free Dulbecco's modified Eagle's medium (Sigma) and then cells were labeled for 4 h with [^{35}S]methionine (100 μCi/μl), rinsed in PBS, and lysed in lysis Buffer B, as indicated above. The HA-tagged or Myc-tagged proteins were immunoprecipitated from the lysates with the anti-HA 12CA5 or the anti-Myc 9E10 monoclonal antibody, respectively, plus protein A-Sepharose beads, washed four times with HNTG (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1% glycerol), and used for the *in vitro* proteolysis experiments. Pulse-chase experiments of ^{35}S-labeled PTEN were performed as described (12).

In Vitro Proteolysis Experiments and Caspase-3 Activity Assays—Preparation of the HEK293 cellular extracts and activation of the caspase cascade with dATP were performed as described (21). For the *in vitro* proteolysis of PTEN by the HEK293 cellular extracts, purified ^{35}S-labeled HA-PTEN wild type or the indicated mutations, from COS-7 transfected cells, were incubated with 35 μl of the non-activated or the dATP-activated HEK293 cellular extracts at 30 °C. Immunodepletions were performed by sequential incubations of cellular extracts with caspase-specific antibodies and protein G-Sepharose. For the *in vitro* proteolysis by caspase-3 or caspase-9, Myc-S-SCAM, HA-PTEN, or GST-PTEN, wild type, or the indicated mutations were incubated with recombinant active caspase-3 (100 ng/μl) or caspase-9 (20 milliunits/μl) in 35 μl of NPM buffer at 30 °C. At the indicated times, equal aliquots of each sample were placed on 15 μl of 2× SDS-PAGE sample buffer, boiled, resolved on SDS-PAGE gels under reducing conditions, and analyzed by autoradiography or by immunoblot. Caspase-3 activity assays from U87MG cell extracts were performed using the caspase-3 colorimetric substrate DEVD-*p*NA. Briefly, U87MG cells were lysed as indicated above, and 0.2 mg of protein extract was diluted 1:1 in NPM buffer containing 20 μM DEVD-*p*NA. Samples were incubated at 30 °C for 30 min, and DEVD-*p*NA hydrolysis was measured by absorbance at 405 nm.

GST Fusion Proteins, in Vitro Kinase Assays, and GST Pull-down Experiments—The GST fusion proteins were expressed in bacteria and purified with glutathione-Sepharose using standard procedures. *In vitro* CK2 kinase assays of the GST fusion proteins were done as described (12). For GST pull-down experiments, COS-7 cells transfected with HA-PTEN wild type or mutations were lysed in lysis Buffer B as indicated above, and 0.5 mg of the cell lysates were incubated for 2 h on ice with 2 μg of the GST-(S-SCAM)-PDZ 2 fusion protein, followed by the addition of glutathione-Sepharose beads and further incubation for 2 h under constant shaking. Then, samples were washed four times with HNTG buffer, resolved on 10% SDS-PAGE gels, and analyzed by immunoblot with the anti-HA monoclonal antibody 12CA5.

RESULTS

PTEN Is Cleaved by Activated HEK293 Cell Extracts in a Phosphorylation-regulated Manner—We have described previously that PTEN is constitutively phosphorylated by the Ser/Thr protein kinase CK2 at a cluster of residues located at its C terminus. Analysis of the CK2 phosphorylated region of PTEN showed the existence of several putative caspase cleavage sites

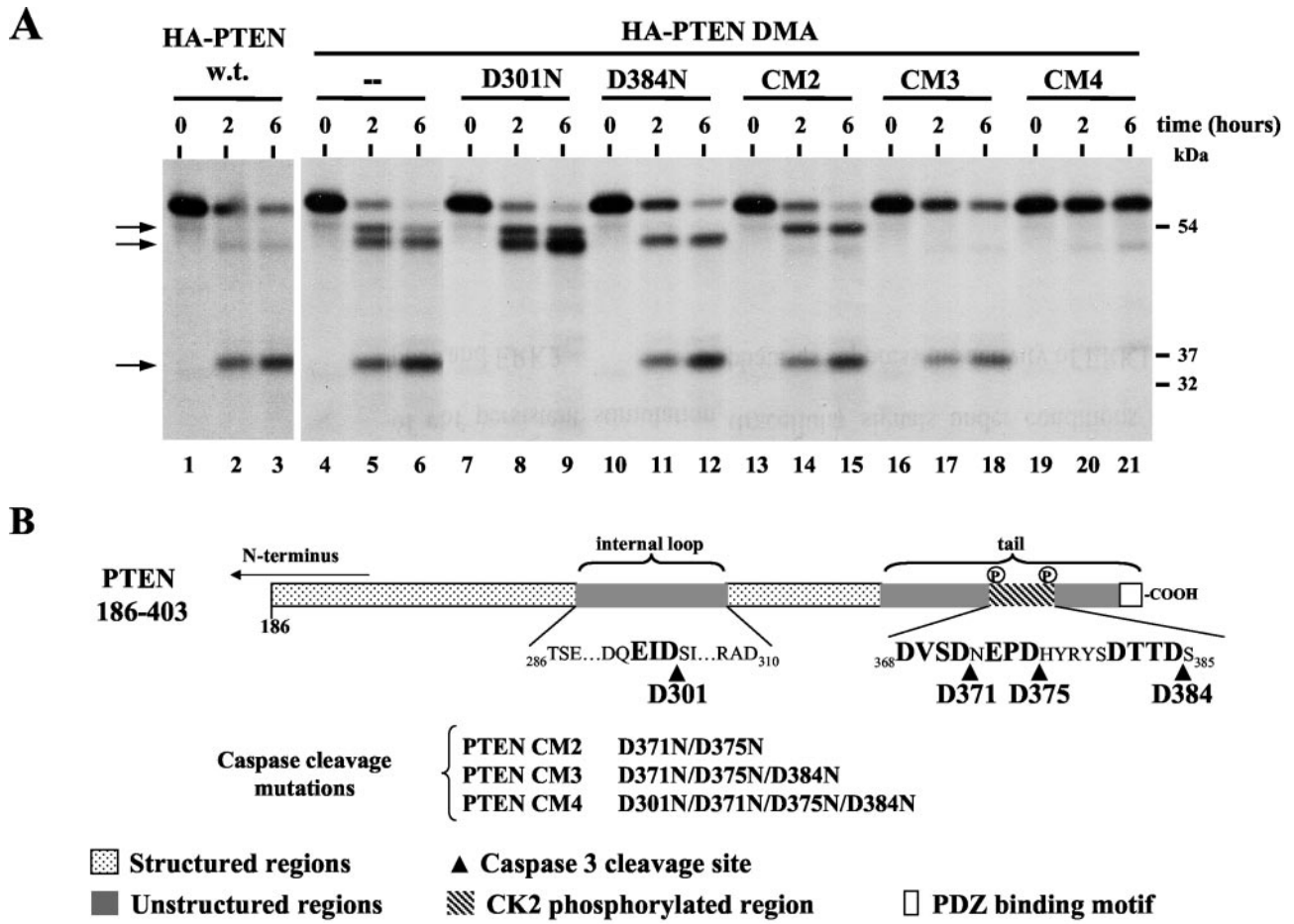

FIG. 3. **PTEN is cleaved by caspase-3 at four target sites.** *A*, ^{35}S-labeled HA-PTEN, w.t., or the indicated mutations, were incubated with recombinant-active caspase-3 (100 ng/μl). At the indicated times, aliquots were processed and analyzed as in Fig. 1. *Arrows* indicate the cleavage products of HA-PTEN. *DMA*, S370A/S385A; *CM2*, D371N/D375N; *CM3*, D371N/D375N/D384N; *CM4*, D301N/D371N/D375N/D384N. *B*, schematics of the C-terminal portion of PTEN (residues 186–403, corresponding to the C2 domain and the tail of the molecule) and the caspase-cleavage mutations used in our study. The structural features of the C-terminal portion of PTEN and the cleavage target sites for the caspase-3 (*arrowheads*) are indicated.

(D*XX*D and D*XX*D-like sequences) (12, 17), suggesting that PTEN could be cleaved by caspases within this region. To test this hypothesis, we performed *in vitro* proteolysis assays using ^{35}S-labeled HA-PTEN, immunoprecipitated from transfected COS-7 cells, as the substrate. As the source of inactive or active caspases, non-activated or dATP-activated HEK293 cellular extracts were used. No changes in HA-PTEN (58-kDa apparent molecular mass) were observed upon incubation with non-activated HEK293 cellular extracts (Fig. 1*A*, *lanes 1–4*), indicating that PTEN is not a target for proteases present in the non-activated extracts. However, upon incubation with dATP-activated cellular extracts, the intensity of the band corresponding to HA-PTEN diminished in a time-dependent manner, concomitant with the appearance of two new bands of lower apparent molecular mass (50 and 37 kDa) (Fig. 1*A*, *lanes 5–8*). Thus, activation of caspases in cell extracts triggers the cleavage of PTEN at several sites.

Next, the involvement of phosphorylation in PTEN cleavage was investigated. *In vitro* proteolysis assays were performed using the phosphorylation-defective HA-PTEN S370A/S385A mutation (DMA), which targets the two major residues of PTEN phosphorylated by CK2 (12, 13; see also Fig. 4*B*). Incubation of HA-PTEN DMA with activated extracts led to a prominent proteolysis of the PTEN molecule (Fig. 1*B*, *lanes 5–8*), generating three bands of lower molecular mass: two faster migrating bands (50 and 37 kDa) that comigrated with the two cleavage products from HA-PTEN wild type (*double-head arrows*) and an additional band of 53 kDa. The intensity of the 50-kDa band, but not the 37-kDa band, was increased in the HA-PTEN DMA mutant, compared with HA-PTEN wild type (see below). Moreover, the time course of the proteolysis showed that the HA-PTEN DMA mutant was cleaved faster than the HA-PTEN wild type. PTEN residues Ser^{380}, Thr^{382}, and Thr^{383} are also involved, to a minor extent, in the phosphorylation of PTEN by CK2 (12). Accordingly, a HA-PTEN S380A/T382A/T383A mutation (TMA) was also tested for proteolytic cleavage, as above. As shown, the proteolysis pattern of HA-PTEN TMA was indistinguishable with that of HA-PTEN wild type (Fig. 1*C*). The proteolysis of the HA-PTEN S370E/S385E mutation (DME), which mimics the phosphorylation of the residues Ser^{370} and Ser^{385} observed in PTEN wild type, was also analyzed. Upon incubation with activated extracts, HA-PTEN DME showed a proteolysis pattern analogous to that observed for HA-PTEN wild type (Fig. 1*D*). Taken together, these results demonstrate that HA-PTEN DMA mutation is cleaved more efficiently and contains more target sequences for proteolysis by activated cellular extracts than HA-PTEN wild type. This suggests that the phosphorylation of residues Ser^{370} and/or Ser^{385} negatively regulates the cleavage of PTEN by caspases.

PTEN Is Cleaved by the Caspase-3 at Several Target Sites—To identify the caspase(s) involved in the cleavage of PTEN, ^{35}S-labeled HA-PTEN DMA was incubated with HEK293 cellular extracts that were immunodepleted of

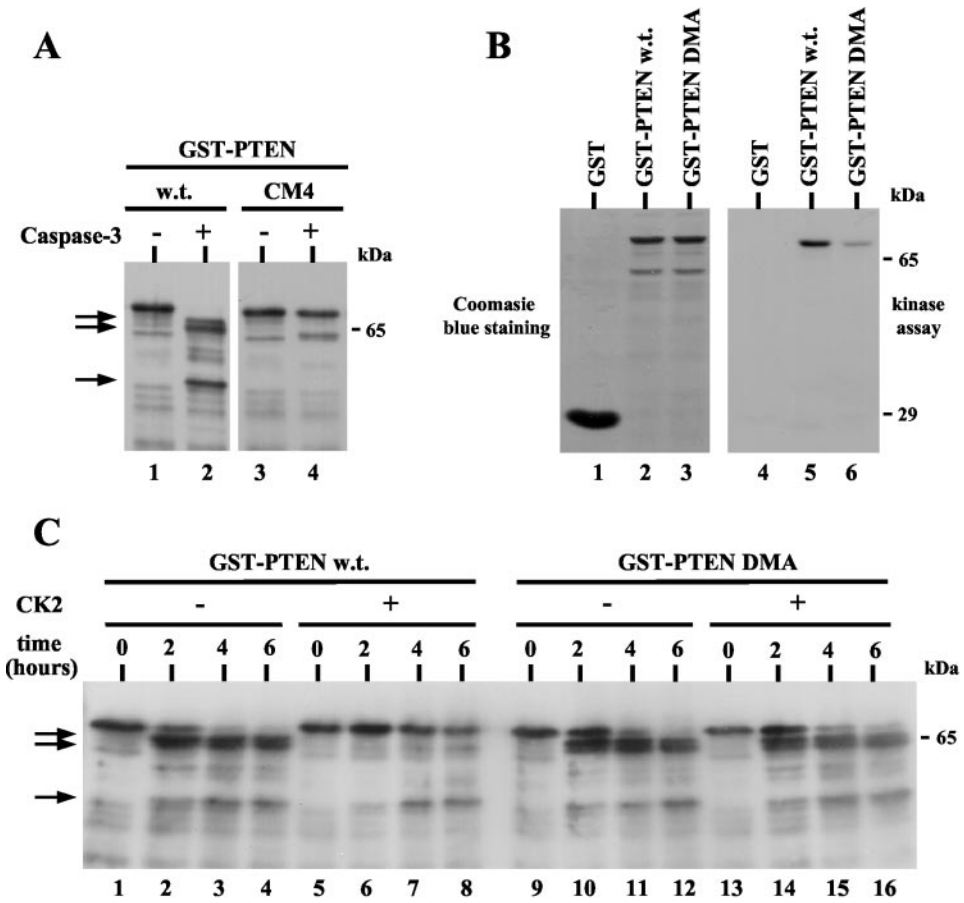

FIG. 4. **CK2 phosphorylation blocks PTEN cleavage by caspase-3.** *A*, GST-PTEN w.t. or CM4 mutation (D301N/D371N/D375N/D384N), purified from bacteria, were incubated in the absence (*lanes 1* and *3*) or in the presence of recombinant caspase-3 (100 ng/μl) (*lanes 2* and *4*) for 4 h. Samples were processed as in Fig. 2*C* and analyzed by immunoblot with an anti-GST antibody. *B*, GST or GST-PTEN w.t. or DMA mutation (S370A/S385A), purified from bacteria, were phosphorylated *in vitro* by recombinant CK2 holoenzyme in the presence of [γ-^{32}P]ATP. Samples were resolved by 10% SDS-PAGE, followed by Coomassie Blue staining (*lanes 1–3*) or autoradiography (*lanes 4–6*). *C*, GST-PTEN wild type or DMA mutation were left untreated (*lanes 1–4* and *9–12*) or were phosphorylated by CK2 (*lanes 5–8* and *13–16*), as in *panel B*, in the presence of cold ATP. Samples were incubated in the presence of recombinant caspase-3 (100 ng/μl) for the indicated times and were subjected to immunoblot with anti-GST antibody, as in *panel A*.

caspase-9, caspase-3, or caspase-7 by specific antibodies, prior to their activation with dATP. As shown, depletion of caspase-7 from the extracts had no effect in the cleavage of HA-PTEN DMA (Fig. 2*A*, *lanes 13–16*), when compared with the control set points (Fig. 2*A*, *lanes 1–4*). However, immunodepletion of caspase-9 or caspase-3 (Fig. 2*A*, *lanes 5–8* and *6-12*, respectively) abrogated the cleavage of HA-PTEN DMA by the activated extracts. As a control, the extent of depletion of the different caspases from the extracts is shown (Fig. 2*B*). These results indicate that HA-PTEN DMA is cleaved by the activated HEK293 cellular extracts in a caspase-9/caspase-3-dependent manner. The activation of caspase-9 in the dATP-activated extracts triggers the activation of caspase-3 (21), suggesting that PTEN could be directly proteolyzed by caspase-3. To test this possibility, a GST-PTEN fusion protein was incubated in the presence of recombinant active caspase-3 or caspase-9, followed by immunoblot with an anti-GST antibody. As shown in Fig. 2*C*, caspase-3, but not caspase-9, cleaved GST-PTEN, rendering products equivalent to that observed in the dATP-activated extracts. Furthermore, both ^{35}S-labeled HA-PTEN wild type and DMA mutation were also proteolyzed upon incubation with active caspase-3 (Fig. 3*A*, *lanes 1–3* and *4-6*, respectively). Under these conditions, caspase-9 proteolyzed its specific synthetic substrate, LEHD-*p*NA (data not shown). These results demonstrate that PTEN is specifically cleaved by caspase-3 *in vitro* and support the notion that, in the experiments using activated HEK293 cellular extracts, PTEN is directly proteolyzed by caspase-3.

To identify the caspase-3 cleavage sites on PTEN, experiments were performed using mutations that affect putative caspase-3-like sites at the C-terminal portion of PTEN (Asp^{301}, Asp^{371}, Asp^{375}, and Asp^{384}; see Fig. 3*B*). Mutation of Asp^{301} and Asp^{384} to Asn (D301N and D384N) abrogated the appearance of the 37- and 54-kDa bands, respectively, upon incubation with recombinant caspase-3 (Fig. 3*A*, *lanes 7–9* and *10–12*). This implicates these residues are sites for cleavage by caspase-3. Analogous mutation of the residues Asp^{371} or Asp^{375} alone had no effect in the proteolysis of PTEN by the caspase-3 (data not shown); however, the combined mutation of these two residues (D371N/D375N; CM2) abrogated the appearance of the 50-kDa band (Fig. 3*A*, *lanes 13–15*), indicating that the mutation of the two caspase putative target sequences at Asp^{371} and Asp^{375} is necessary to abolish the cleavage of PTEN in this region. The combined mutation of the critical Asp in the three target sequences found within the CK2 phosphorylated region (D371N/D375N/D384N; CM3) eliminated the appearance of the 50- and 54-kDa bands upon incubation with caspase-3 (Fig. 3*A*, *lanes 16–18*). Finally, the combined mutation of all the caspase target sequences found in PTEN (D301N/D371N/D375N/D384N; CM4) completely blocked the cleavage of PTEN by the caspase-3 *in vitro* (Fig. 3*A*, *lanes 19–21*). Altogether, these results demonstrate that caspase-3 cleaves the tumor suppres-

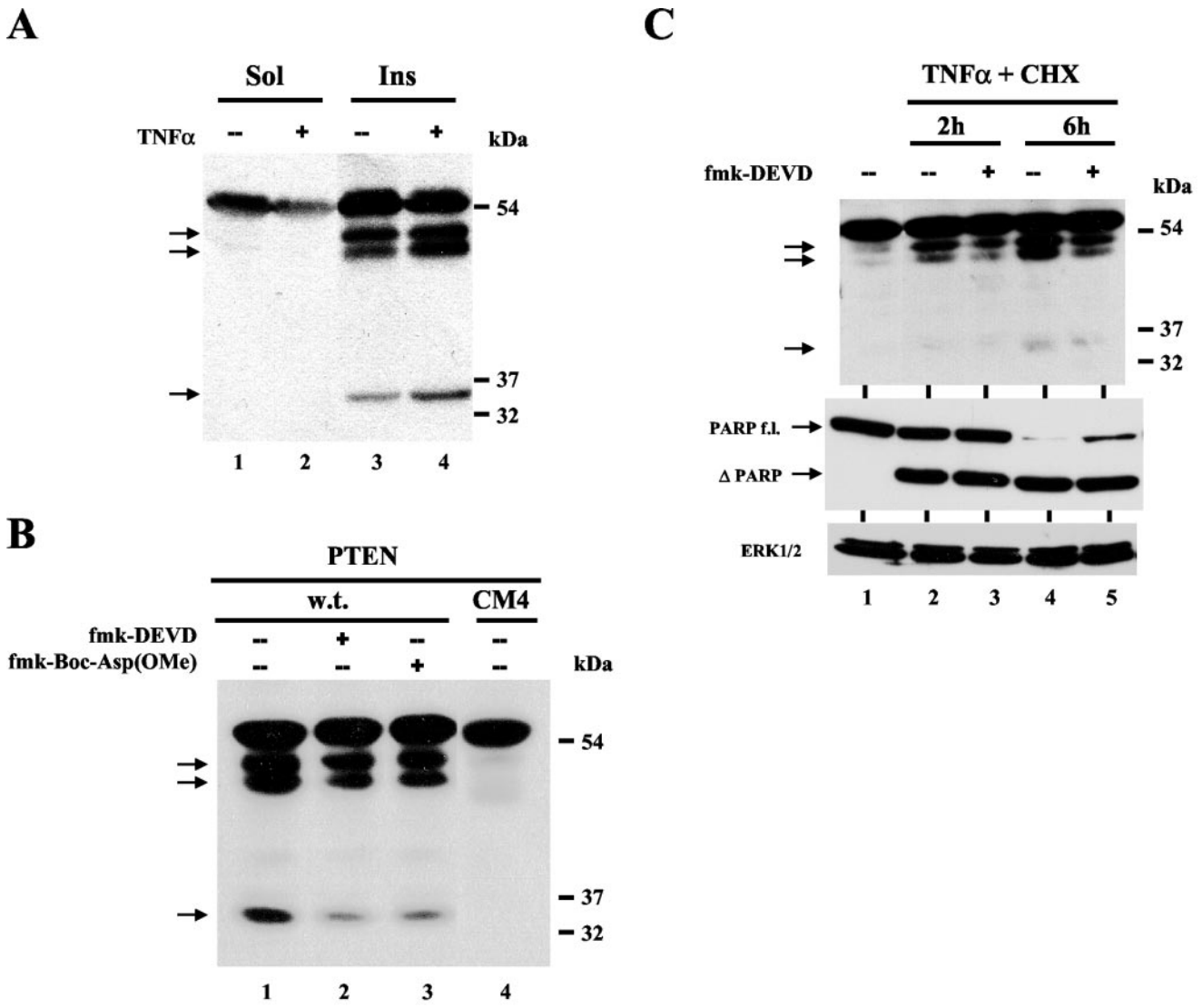

FIG. 5. **Partial proteolysis of PTEN *in vivo*.** *A*, U87MG cells, transfected with pRK5 PTEN wild type, were left untreated (*lanes 1* and *3*) or were treated for 6 h with TNFα (100 ng/ml) (*lanes 2* and *4*). Both floating and attached cells were collected by centrifugation and fractionated in Triton X-100 (see "Experimental Procedures"), and equal amounts of cellular fractions were analyzed by immunoblot using an anti-N-terminal PTEN antibody, which recognizes the N terminus of the molecule. *Sol*, Triton X-100 soluble fraction; *Ins*, Triton X-100 insoluble fraction. *B*, U87MG cells, transfected with pRK5 PTEN w.t. or pRK5 PTEN CM4 mutation (D301N/D371N/D375N/D384N), were left untreated (*lanes 1* and *4*) or incubated with 4 μM z-DEVD-fmk (*lane 2*) or 50 μM Boc-Asp(OMe)-fmk (*lane 3*) for 16 h. Then both floating and attached cells were collected by centrifugation and lysed in 1× SDS-PAGE sample buffer. Equal amounts of cellular lysates were analyzed by immunoblot as in *A*. *C*, HeLa S3 cells were left untreated (*lane 1*) or incubated with 4 μM z-DEVD-fmk (*lanes 3* and *5*) for 1 h. Then, cells were treated with TNFα (20 ng/ml) plus cycloheximide (*CHX*) (10 ng/ml) for 2 h (*lanes 2–3*) or 6 h (*lanes 4–5*). Cells were harvested as in *B*, and equal amounts of cellular lysates were analyzed by immunoblot using anti-N-terminal PTEN (*upper panel*), anti-PARP (*middle panel*), or anti-ERK1/2 (*lower panel*) antibodies. *Arrows* in *A* and *B* and in the upper *panel of C* indicate the cleavage products of PTEN; *arrows* in the *middle panel* of *C* indicate the full-length PARP protein (*PARP f.l.*) and the cleaved PARP product (Δ*PARP*).

sor PTEN at several target sites within the C-terminal domain.

CK2 Phosphorylation Blocks PTEN Cleavage by Caspase-3—To test the direct role of CK2 phosphorylation of the C-terminal tail of PTEN on its cleavage by caspase-3, experiments were performed using recombinant active caspase-3 and non-phosphorylated and CK2-phosphorylated GST-PTEN fusion proteins. As shown in Fig. 4*A* (see also Fig. 2*C*), GST-PTEN was cleaved by caspase-3 in an identical manner as HA-PTEN. Also, GST-PTEN wild type was efficiently phosphorylated by CK2 *in vitro*, whereas phosphorylation of the GST-PTEN DMA mutation was severely impaired (Fig. 4*B*; see also Ref. 12). Remarkably, after phosphorylation of GST-PTEN wild type by CK2, caspase-3 cleavage at the PTEN C-terminal tail was abolished, whereas no effect of CK2 was observed on the cleavage of GST-PTEN DMA (Fig. 4*C*). These results demonstrate that phosphorylation of PTEN by CK2 blocks PTEN cleavage at its C terminus.

Caspase-3-mediated Cleavage of PTEN in Vivo—To ascertain whether PTEN is cleaved by caspases *in vivo*, we examined by immunoblot the status of PTEN protein ectopically expressed in the PTEN-null cell line U87MG, using an antibody raised against the N terminus of PTEN. When expressed in U87MG cells, PTEN was detected in both Triton X-100-soluble and -insoluble fractions (Fig. 5*A*). Remarkably, a distinctive pattern of PTEN bands was observed in the insoluble fraction that included the PTEN full-length band (*upper band*) and three faster migrating bands, which were similar to that observed for the proteolysis of the PTEN DMA mutation by the caspase-3 *in vitro*. The appearance of these three faster migrating bands increased upon cell stimulation with the pro-apoptotic cytokine

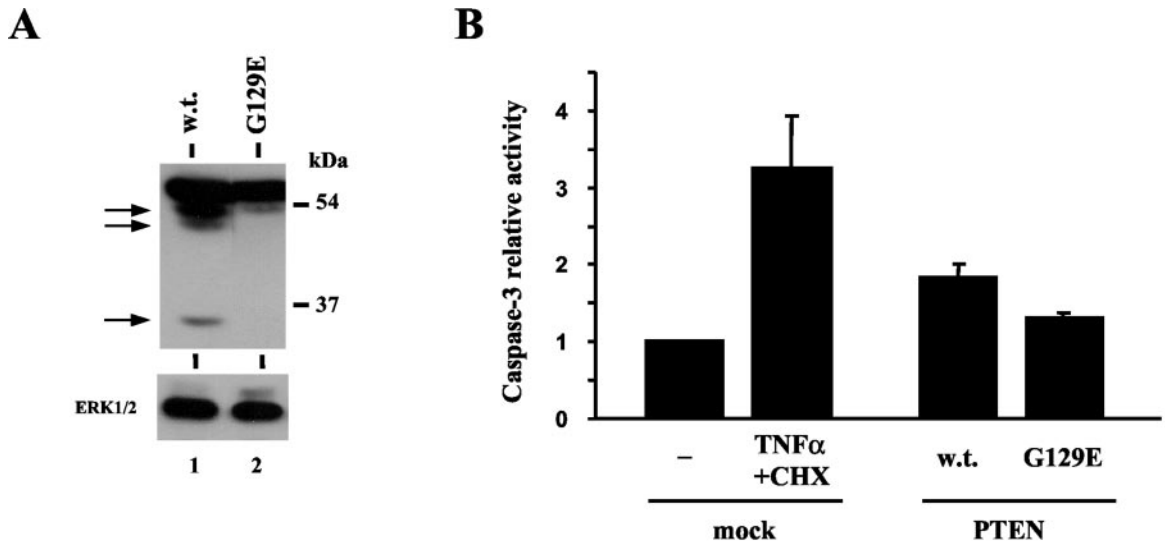

FIG. 6. **Caspase-3 activity is induced by ectopic expression of PTEN.** *A*, U87MG cells transfected with pRK5 PTEN w.t. or G129E mutation were harvested 24 h after transfection, lysed as in Fig. 5*B* and analyzed by immunoblot using an anti-N-terminal PTEN antibody (*upper panel*) or anti-ERK1/2 antibodies (as a control of protein-loading; *lower panel*). *B*, cell extracts from U87MG cells, transfected as in *A*, were processed for measurement of caspase-3 activity in the presence of the caspase-3-specific substrate DEVD-*p*NA. Mock-transfected cells, non-treated or treated with TNFα plus cycloheximide (*CHX*) for 12 h, were processed in parallel.

TNFα (Fig. 5*A*, *lane 4*). This cleavage was partially prevented by cell treatment with a specific caspase-3-selective inhibitor (z-DEVD-fmk) or with a broad spectrum caspase inhibitor (Boc-Asp(Ome)-fmk (Fig. 5*B*, *lanes 2* and *3*, respectively). Finally, only the PTEN full-length band was observed in the caspase-insensitive PTEN CM4 mutation (Fig. 5*B*, *lane 4*). We next tested whether endogenous PTEN is cleaved by caspases during TNFα-induced apoptosis, using HeLa S3 cells that harbor wild type alleles of PTEN (22). As observed, cleavage of endogenous PTEN was observed during TNFα-induced apoptosis in a time course-dependent manner and was partially inhibited by preincubation of the cells with the caspase-3 inhibitor (Fig. 5*C*, *upper panel*). The extent of caspase-3 activation upon the distinct conditions, as monitored by the cleavage of the caspase-3-specific protein substrate, PARP, is shown (Fig. 5*C*, *middle panel*). Note that only partial inhibition of PARP cleavage by z-DEVD-fmk was achieved under our experimental conditions. The expression of the mitogen-activated protein kinases ERK1/2 is also shown as a control of protein loading (Fig. 5*C*, *lower panel*). These results indicate that endogenous PTEN is cleaved in HeLa S3 cells during TNFα-induced apoptosis in a caspase-3-dependent manner and corroborate the results obtained both *in vitro* and *in vivo* with recombinant PTEN.

The cleavage pattern of the lipid-phosphatase inactive PTEN G129E mutation was also investigated in U87MG cells. Interestingly, the G129E PTEN mutation was less susceptible to cleavage than PTEN wild type (Fig. 6*A*, *upper panel*), suggesting that PTEN catalytic activity could affect caspase-3 activity in U87MG-transfected cells. To test this possibility, caspase-3 activity was measured in cell extracts from U87MG cells transfected with PTEN (Fig. 6*B*). As shown, caspase-3 activity was increased in extracts from wild type PTEN-transfected cells but not on those from cells transfected with G129E PTEN. Altogether, these results indicate that PTEN is cleaved by caspase-3 *in vivo* at the target sites identified in our *in vitro* studies and suggest a role for PTEN in the regulation of caspase-3 activity.

Caspase-3 Proteolysis May Affect PTEN Protein Stability and PTEN/S-SCAM Interactions—The cleavage of PTEN by caspase-3 removes the C-terminal portion of PTEN, which has been involved in the regulation of PTEN stability and half-life, as well as in the binding of PTEN to PDZ domain-containing proteins (1–4). To test the consequences of caspase-3 cleavage on PTEN stability, the degradation of PTEN deletion mutants mimicking the cleavage of the protein by caspase-3 (residues 1–384, 1–375, 1–371, and 1–301) was measured by pulse-chase experiments. COS-7 cells expressing HA-PTEN wild type or C-terminal deletions were ^{35}S-labeled, and degradation plots were obtained (Fig. 7*A*). As shown, PTEN C-terminal deletions were degraded faster than PTEN wild type.

Next, the association of HA-PTEN C-terminal deletion mutants with the second PDZ domain of the multi PDZ domain-containing protein S-SCAM/MAGI-2 (S-SCAM-PDZ 2) (14, 19) was tested by pull-down experiments, using a GST-(S-SCAM)-PDZ 2 fusion protein. PTEN wild type was efficiently precipitated with GST-(S-SCAM)-PDZ 2 (Fig. 7*B*, *lane 8*); however, the PTEN C-terminal deletion mutants lacked the capability to interact with this PDZ domain (Fig. 7*B*, *lanes 9–12*). The same results were obtained with S-SCAM/MAGI-2 full-length in co-immunoprecipitation experiments (data not shown). Remarkably, S-SCAM/MAGI-2 was also found to be partially proteolyzed by caspase-3 *in vitro* (Fig. 7*C*, *lanes 2* and *3*). Together, these results suggest a role for caspase-3 in the regulation of PTEN protein stability and in the disassembling of multimolecular complexes containing PTEN and S-SCAM/MAGI-2.

DISCUSSION

The role of the tumor suppressor PTEN in the control of cell survival and apoptosis is well documented (1–4). However, the regulatory mechanisms of PTEN biological activity remain poorly understood. In the present study, we have investigated the involvement of caspases in the partial proteolysis of PTEN during apoptosis and its potential role as a regulatory mechanism of PTEN protein stability and PTEN interaction with PDZ domain-containing proteins. We have found that PTEN is cleaved upon incubation with HEK293 cellular extracts in the presence of dATP, which activates the caspase cascade in these extracts (21). Partial proteolysis of PTEN was also observed in intact cells stimulated with the cytokine TNFα, a well known activator of the caspase cascade. Our results provide evidence that this proteolysis is because of the activity of the caspase-3 apoptotic protease. First, depletion of caspase-3, or its upstream activator caspase-9, from the dATP-activated HEK293 cellular extracts, impaired the cleavage of PTEN. Second, re-

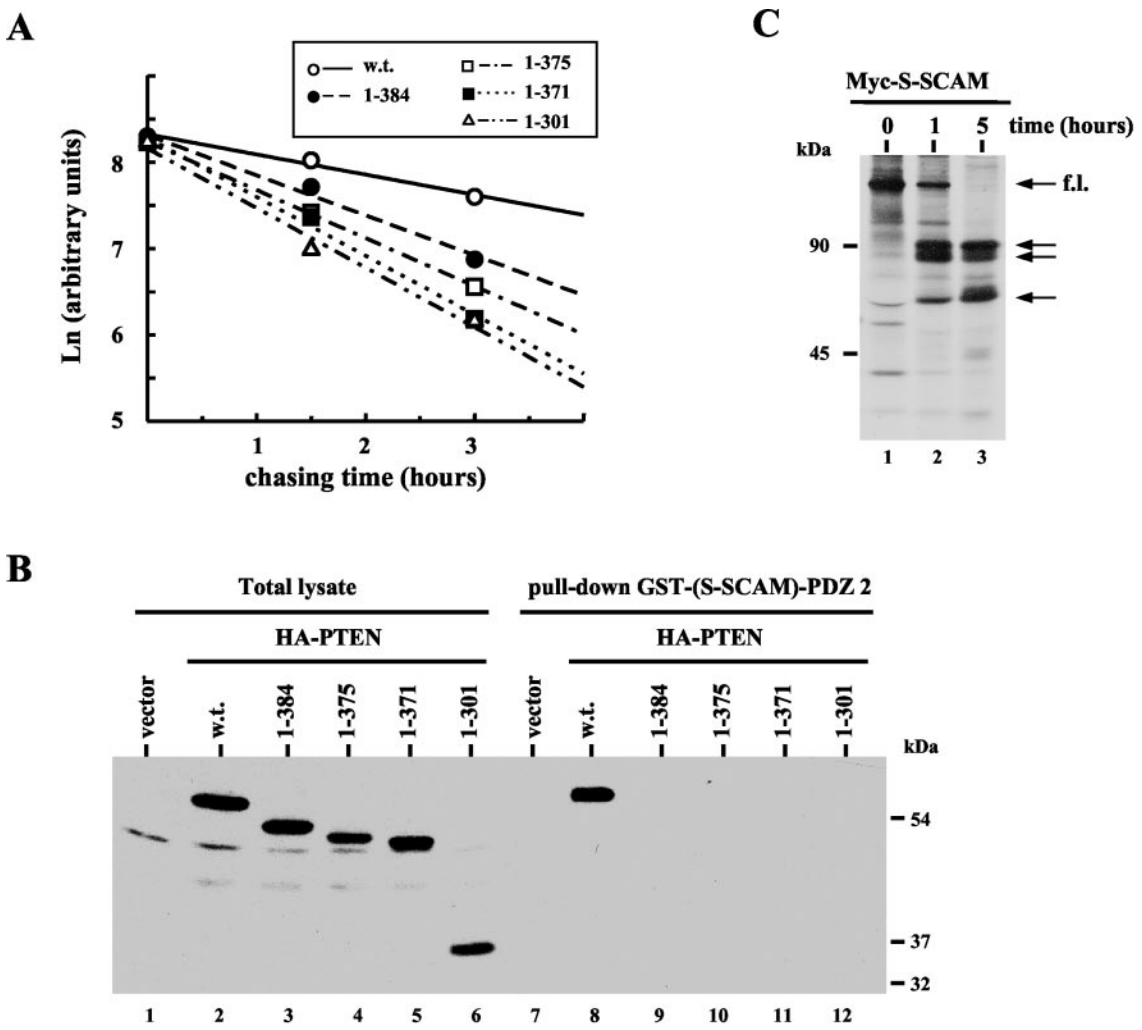

FIG. 7. **Caspase-3 proteolysis may affect protein stability and PTEN/S-SCAM interactions.** *A*, COS-7 cells were transfected with pRK5 HA-PTEN w.t. or deletion mutations, as indicated. Cells were pulse-labeled with [^{35}S]methionine for 2 h and chased at the indicated times. ^{35}S-Labeled HA-PTEN proteins were immunoprecipitated with the anti-HA monoclonal antibody, resolved on 10% SDS-PAGE gels, and analyzed using a PhosphorImager. Kinetic degradation plots of the mean from two separate experiments are shown. *B*, COS-7 cells were transfected with pRK5 alone (*vector*) or pRK5 HA-PTEN w.t. or deletion mutations, as indicated. Equal amounts of cellular lysates (0.5 mg) were subjected to pull-down with 2 μg of GST-(S-SCAM)-PDZ 2 (*lanes 7–12*). The presence of HA-PTEN proteins in total lysates (75 μg) (*lanes 1–6*) and in precipitates (*lanes 7–12*) was analyzed by immunoblot using anti-HA antibody. *C*, ^{35}S-labeled Myc-S-SCAM was incubated with recombinant-active caspase-3 (100 ng/μl). At the indicated times, aliquots were processed, loaded onto 10% SDS-PAGE, and analyzed as in Fig. 1. *Arrows* indicate the cleavage products of Myc-S-SCAM; *f.l.*, full-length Myc-S-SCAM.

combinant caspase-3 cleaved PTEN *in vitro* to generate proteolysis products identical to those observed upon incubation of PTEN with the dATP-activated 293 cellular extracts or upon cell stimulation with TNFα. Third, cleavage of PTEN during TNFα stimulation was diminished by preincubation of the cells with both the broad-spectrum caspase inhibitor Boc-Asp(OMe)-fmk or the caspase-3-selective inhibitor z-DEVD-fmk. Fourth, caspase-3 target sites were identified in PTEN amino acid sequence (see below). Mutation of these sites prevented PTEN cleavage in intact cells. Together, these results support the hypothesis that caspase-3, the major executioner caspase during apoptosis, directly cleaves the tumor suppressor PTEN in intact cells.

Our mutational analysis demonstrates that caspase-3 cleaves PTEN at several target sites, which are located at unstructured regions of the protein (see Fig. 3*B*). One of these sites, Asp^{301}, is located at an internal loop within the C2 domain of PTEN. This domain has been shown to facilitate the binding of PTEN to membranes; however, the precise function of its internal loop remains unknown. On the other hand, the residues Asp^{371}, Asp^{375}, and Asp^{384} are located at the C-terminal tail of PTEN, which regulates the stability and the function of PTEN (11–13, 23, 24). Interestingly, these three sites overlap with the CK2 phosphorylation sites found at the C terminus of PTEN (12) (see Fig. 3*B*), suggesting that the cleavage of the phosphatase in cells could be regulated by CK2-mediated phosphorylation. Indeed, our results using PTEN phosphorylation-defective mutations indicate that phosphorylation of the residues Ser^{370} and/or Ser^{385} inhibits the cleavage of PTEN by caspase-3. Furthermore, phosphorylation by CK2 abrogated caspase-3 cleavage of PTEN *in vitro*, reinforcing the hypothesis that CK2 could negatively regulate PTEN cleavage by caspase-3 during apoptosis. It is possible that the phosphorylation of Ser^{370} and Ser^{385} PTEN residues could mask the neighboring caspase-3 cleavage sites and/or decrease the accessibility of the protease to its recognition sites. In this regard, it has been shown that the phosphorylation by CK2 of several cell survival-regulatory proteins inhibits their cleavage by caspases (25). On the other hand, it has been reported that defective phosphorylation of PTEN at other C-terminal residues (Ser^{380}, Thr^{382}, and Thr^{383}) enhances its function and its binding to the PDZ domain-containing protein S-SCAM/MAGI-2 (11, 26, 27;

see also below), suggesting that specific phosphorylation of distinct residues at the C terminus of PTEN could differentially affect PTEN regulation. In general, CK2 is a constitutively active kinase, making possible that most of the CK2 sites within PTEN would always be phosphorylated. However, our findings also suggest that the phosphorylation of PTEN could be modulated in cells, because both recombinant and endogenous wild type PTEN show a proteolysis pattern similar to that observed for the PTEN DMA mutation. Furthermore, the fact that the cleavage of PTEN in cells was only detected in the Triton X-100-insoluble fraction suggests that pools of PTEN with distinct levels of phosphorylation may exist at different subcellular locations. Thus, it is possible that a pool of non-phosphorylated PTEN is localized at Triton X-100 insoluble fractions, such as components of nucleus, lipid rafts, and/or cytoskeleton, where it could be proteolyzed by caspase-3 in response to apoptotic stimuli. Indeed, PTEN localization at some of these subcellular compartments has been reported (28, 29). The possibility cannot be excluded that a protein phosphatase, located at Triton X-100-insoluble fractions, could target PTEN in response to caspase activation.

During apoptosis, caspases cleave a restricted set of signaling proteins involved in cell growth and survival, thereby turning off survival signals in addition to activating death signals (30). We have not observed significant differences in the inhibition of protein kinase B activity by transient overexpression of the C-terminal tail PTEN deletion mutations mimicking PTEN cleavage by caspase-3, when compared with PTEN wild type (data not shown), suggesting that PTEN catalytic activity is not directly affected by the caspase-3-mediated cleavage. However, the possibility cannot be ruled out that cleavage of PTEN modulates its phosphatase activity. On the other hand, the observation that the cleavage of the catalytically inactive PTEN G129E mutation is diminished in intact cells suggests that PTEN activity could favor its cleavage by caspases. In this regard, caspase-3 activity was increased in extracts from cells transfected with PTEN wild type but not on those transfected with PTEN G129E. These findings, and the observations on the reduced stability of C-terminal PTEN deletions (see Refs. 23 and 24 and this study), suggest that caspase-3-mediated cleavage of PTEN could be part of a physiological feedback mechanism whereby PTEN protein levels could be down-regulated during the commitment of cells to undergo apoptosis.

We have found that PTEN deletion mutations mimicking caspase-3 cleavage fail to bind to the second PDZ domain of the scaffolding protein S-SCAM/MAGI-2. Furthermore, S-SCAM/MAGI-2 was also proteolyzed by caspase-3 *in vitro*, suggesting that the interaction of the C-terminal tail of PTEN with PDZ domain-containing proteins could be controlled by caspase cleavage. The C2 domain of PTEN is also targeted by caspase-3 (see above), and it has been reported that this domain mediates the interaction of PTEN with the major vault protein (31). Thus, it is conceivable that, during apoptotic cell death, cleavage of PTEN by caspase-3 would result in changes in the interaction of PTEN with structural, scaffolding, and/or regulatory molecules at particular subcellular locations (see above). Altogether, the results presented here describe a potential regulatory mechanism of PTEN protein stability and PTEN-protein interactions, controlled by CK2 and mediated by caspase-3, that could take place under physiological conditions.

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Mechanisms of Signal Transduction:

Phosphorylation-regulated Cleavage of the Tumor Suppressor PTEN by Caspase-3: IMPLICATIONS FOR THE CONTROL OF PROTEIN STABILITY AND PTEN-PROTEIN INTERACTIONS

Josema Torres, Joe Rodriguez, Michael P. Myers, Miguel Valiente, Jonathan D. Graves, Nicholas K. Tonks and Rafael Pulido

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