THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 2, Issue of January 12, pp. 993–998, 2001
Printed in U.S.A.

The Tumor Suppressor PTEN Is Phosphorylated by the Protein Kinase CK2 at Its C Terminus

IMPLICATIONS FOR PTEN STABILITY TO PROTEASOME-MEDIATED DEGRADATION*

Received for publication, August 25, 2000
Published, JBC Papers in Press, October 16, 2000, DOI 10.1074/jbc.M009134200

Josema Torres‡ and Rafael Pulido§

From the Instituto de Investigaciones Citológicas, 46010 Valencia, Spain

The tumor suppressor phosphatase PTEN regulates cell migration, growth, and survival by dephosphorylating phosphatidylinositol second messengers and signaling phosphoproteins. PTEN possesses a C-terminal noncatalytic regulatory domain that contains multiple putative phosphorylation sites, which could play an important role in the control of its biological activity. The protein kinase CK2 phosphorylated, in a constitutive manner, a cluster of Ser/Thr residues located at the PTEN C terminus. PTEN-phosphorylated defective mutants showed decreased stability in comparison with wild type PTEN and were more rapidly degraded by the proteasome. Inhibition of PTEN phosphorylation by the CK2 inhibitor 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole also diminished the PTEN protein content. Our results support the notion that proper phosphorylation of PTEN by CK2 is important for PTEN protein stability to proteasome-mediated degradation.

The tumor suppressor gene *PTEN* (also named as *MMAC1* or *TEP-1*) (1–3) encodes a phosphatase with enzymatic activity toward both protein substrates and the lipid second messenger, phosphatidylinositol-3,4,5-triphosphate (4–6). PTEN regulates distinct signal transduction pathways, including the phosphatidylinositol 3-kinase/ protein kinase B cell survival- and integrin-triggered signaling pathways (for recent reviews, see Ref. 7–10). Structurally, PTEN protein is composed of an N-terminal dual specificity phosphatase-like enzyme domain and a C-terminal regulatory domain, which binds to phospholipid membranes (11). Mutations in the *PTEN* gene are present in a great number of tumors, as well as in the germ line cells of patients with several inherited cancer syndromes (reviewed in Refs. 12 and 13). The importance of PTEN catalytic activity in its tumor suppressor function is underscored by the fact that the majority of *PTEN* missense mutations detected in tumor specimens target the phosphatase domain and cause a loss in PTEN phosphatase activity. In addition, a large number of *PTEN* nonsense or frame-shift mutations found in tumors are targeted to the C-terminal domain of the protein, suggesting an important role for this domain in the regulation of the PTEN tumor suppressor activity. In this regard, the C-terminal region of PTEN has been shown to be important in the regulation of the stability and half-life of the molecule (14, 15). Also, the C-terminal PTEN amino acid sequence possesses a putative PDZ binding motif, which has been proposed to modulate PTEN functions by association to PDZ domain-containing proteins (16–19). Finally, the C-terminal PTEN domain is rich in putative phosphorylation sites, and phosphorylation of the PTEN C terminus has been recently reported to affect PTEN protein stability and function (20); however, the kinase responsible for such phosphorylation remains unidentified.

Protein kinase CK2[1] (formerly casein kinase II) is a highly conserved, ubiquitously expressed, messenger-independent serine/threonine-kinase that phosphorylates a wide variety of substrates involved in essential cell processes, including cell cycle and cell growth (21–23). In mammals, CK2 is a heterotetramer *in vivo*, composed of two catalytic (α and/or α') and two regulatory (β) subunits. Alterations in CK2 expression have been found in distinct types of tumors (24–26), and overexpression of the catalytic or regulatory CK2 subunits differentially affects cell growth and transformation (27–31). Also, antibody-mediated CK2 depletion inhibited cell cycle progression and growth of fibroblasts (32), and CK2 antisense oligonucleotide treatment blocked neurite outgrowth in neuroblastoma cells (33).

In this report, we demonstrate that the C terminus of the tumor suppressor PTEN is constitutively phosphorylated by CK2 at several residues in intact cells. An insight is provided on the putative role of phosphorylation as a regulatory mechanism of PTEN biological activity by modulating its stability to proteasome-mediated degradation.

MATERIALS AND METHODS

Plasmids, Antibodies, and Reagents—The cDNA encoding full-length human PTEN was obtained by reverse transcriptase-polymerase chain reaction amplification of poly(A)+ mRNA from MCF-7 cells using primers flanking the human PTEN coding region (1–3), and the PTEN nucleotide sequence was confirmed by DNA sequencing. pRK5 mammalian expression vectors were made by polymerase chain reaction amplification of PTEN or GST cDNAs and subcloning. For the construction of bacteria expression plasmids encoding GST-PTEN fusion proteins, PTEN cDNAs were cloned into the pGEX-4T1 or pGEX-5X1 expression vectors. PTEN C terminus truncation mutants and amino acid substitution mutants were made by polymerase chain reaction oligonucleotide site-directed mutagenesis, and mutations were confirmed by DNA sequencing. The 12CA5 anti-HA and the 4G10 anti-phosphotyrosine mAb have been described previously (34). The rabbit polyclonal anti-PTEN CS486 and BT166 antibodies were from R. Parsons and C. Eng, respectively (35, 36). The anti-GST mAb (clone 22.A)

* This work was supported by grants from the Ministerio de Educación y Cultura (PB96-0278) and the Generalitat Valenciana (GV-C-VS-20-047-96). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a fellowship from Generalitat Valenciana.

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

[1] The abbreviations used are: CK2, protein kinase CK2 or casein kinase II; DRB, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole; GST, glutathione *S*-transferase; HA, hemagglutinin; mAb, monoclonal antibody; EGF, epidermal growth factor; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis.

was obtained in our laboratory and is directed against *Schistosoma japonicum* GST. Horseradish peroxidase-conjugated goat anti-mouse secondary antibody was from Promega (Madison, WI). The following activating agents were used: phorbol 12-myristate 13-acetate (Sigma Chemical), 10 ng/ml, 30 min; EGF (Life Technologies, Inc.), 50 ng/ml, 10 min; insulin (Sigma), 1 μg/ml, 10 min; okadaic acid (Roche Molecular Biochemicals), 1 μM, 30 min; sodium pervanadate (Sigma), 10 μM, 30 min; A23187 calcium ionophore (Roche Molecular Biochemicals), 10 μM, 30 min. All incubations were made at 37 °C. Human recombinant CK2 holoenzime was from Roche Molecular Biochemicals. The following CK2 modulators were used: spermine (Sigma), 1 mM; heparine (Sigma), 1 μg/μl; 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA), 100 μM. The MEK1/2 inhibitor PD98059 (New England Biolabs Inc., Beverly, MA) was used at 10 μM, and dibutyryl cAMP (Roche Molecular Biochemicals) was used at 50 μM. The proteasome inhibitor MG132 (Peptide Institute Inc., Osaka, Japan) was used at 50 μM, leupeptin (Peptide Institute Inc.) was used at 0.1 mM, and NH_4Cl was used at 20 mM. For the experiments of inhibition of degradation, chases were performed in the continuous presence of the inhibitors.

Cell Culture, Transfections, and Isotope Cell Labeling—Human breast carcinoma MCF-7 cells were grown in RPMI medium supplemented with 10% heat-inactivated FCS (Life Technologies, Inc.). COS-7 cells were grown in Dulbecco's minimal essential medium containing high glucose (4.5 g/l) supplemented with 5% heat-inactivated FCS. COS-7 cells were transfected with the DEAE-dextran method and processed 48–72 h after transfection. For [^{35}S]methionine labeling, cells were incubated for 1 h in methionine/cysteine-free Dulbecco's minimal essential medium containing 2% dialyzed FCS and then labeled with [^{35}S]methionine/cysteine (50 μCi/ml) for 2 h (pulse). Chases were performed by substituting the [^{35}S]methionine/cysteine-containing medium for Dulbecco's minimal essential medium, 2% FCS containing an excess of cold methionine (1 mM). For ^{32}P labeling, cells were incubated in phosphate-free medium with 2% FCS for 1 h and then labeled with [^{32}P]orthophosphate (50 μCi/ml) for 4 h. Cell lysis, immunoprecipitation, and immunoblot were performed as described (34). For protein turnover measurements, HA-PTEN radiolabeled bands, resolved by SDS-PAGE after immunoprecipitation with the anti-HA 12CA5 mAb, were quantitatively analyzed using a phosphorImager. Results were plotted, and linear fits were performed ($r > 0.97$).

GST Fusion Proteins and in Vitro Kinase Assays—GST-PTEN fusion proteins overexpressed in bacteria were purified with glutathione-Sepharose using standard procedures. For *in vitro* CK2 kinase assays, GST-PTEN fusion proteins (0.5–1 μg) were incubated with 0.2 milliunits/μl CK2 for 30 min at 30 °C in buffer A (20 mM Tris-HCl, pH 7.5, 0.3 μM ATP, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 150 mM KCl; 30 μl final volume) containing 2 μCi of γ-[^{32}P]ATP/sample. For kinase assays using COS-7 cell extracts, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 100 mM NaF, 2 mM Na_3VO_4, and 20 mM $Na_4P_20_7$). Lysates were centrifuged for 10 min at 14,000 r.p.m., and supernatant aliquots (5 μg) were left untreated or incubated with the distinct agents for 5 min at 30 °C in buffer B (20 mM HEPES, pH 7.5, 50 μM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM Na_3VO_4; 20 μl/sample). Then, 20 μl of buffer B containing the GST fusion proteins used as substrates (1 μg/sample) and [γ-^{32}P]ATP (0.25 μCi/μl) were added, and samples were incubated further for 30 min at 30 °C. The reactions were stopped by adding SDS-PAGE sample buffer and boiling followed by SDS-PAGE and autoradiography, and radiolabeled bands were quantified in a PhosphorImager. For quantitative analysis of phosphate incorporation into GST-PTEN 202–403, 1 μg of the fusion protein was phosphorylated by CK2 (0.2 milliunits/μl) for 16 h at 30 °C in buffer B containing 0.5 mM [γ-^{32}P]ATP (70 cpm/pmol) and 1 mM spermine (40 μl final volume). Gel segments containing the labeled bands were dissolved in 0.2 ml of 30% H_20_2 for 3 h at 95 °C, diluted in scintillation liquid, and counted.

RESULTS

PTEN Is a Phosphoprotein under Standard Cell Growth Conditions—To study the putative role of post-translational modifications in the regulation of PTEN biological functions, the possibility that PTEN could be phosphorylated in intact cells was investigated. MCF-7 cells, known to carry a wild type *PTEN* gene (1), were labeled with ^{32}P, and cell lysates were subjected to immunoprecipitation using two distinct anti-PTEN antibodies (Fig. 1*A*). As shown, both anti-PTEN antibodies immunoprecipitated a radiolabeled protein that migrated at about 55 kDa (Fig. 1*A*, *lanes 2* and *3*), as expected for PTEN (36), whereas no signal was detected using a control serum (Fig. 1*A*, *lane 1*). Next, the region of PTEN that is phoshorylated in intact cells was analyzed using recombinant forms of this molecule. ^{32}P labeling was carried out in COS-7 cells transfected with HA-tagged PTEN wild type (residues 1–403) or C-terminal truncated forms (residues 1–343, 1–369, or 1–386), followed by immunoprecipitation with the anti-HA 12CA5 mAb. As observed for the endogenous PTEN in MCF-7 cells, the recombinant HA-PTEN-(1–403) was also phosphorylated in COS-7 cells (Fig. 1*B, upper panel*, *lane 2*). A similar extent of phosphorylation was detected on the truncated HA-PTEN-(1–386) molecule (Fig. 1*B, upper panel, lane 5*), whereas the truncated HA-PTEN-(1–343) and –(1–369) forms were not phosphorylated under these conditions (Fig. 1*B, upper panel*, *lanes 3* and *4*). The *lower panel* in Fig. 1*B* shows the expression of the distinct HA-PTEN molecules. The difference in the intensity of the endogenous and the recombinant PTEN-phosphorylated bands is explained by the difference in the amount of PTEN protein immunoprecipitated in our assays from MCF-7 or transfected COS-7 cells, as indicated by [^{35}S]methionine labeling experiments (data not shown). These results indicate that PTEN is a phosphoprotein under normal conditions of cell growth, suggesting that PTEN phosphorylation takes place between residues 369 and 386.

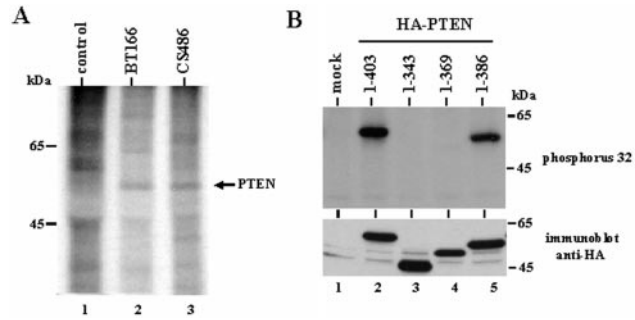

FIG. 1. **PTEN is a phosphoprotein under normal cell growth conditions.** *A,* MCF-7 cells were labeled with ^{32}P, and cell lysates were immunoprecipitated with a control antibody (*lane 1*) or anti-PTEN BT166 or CS486 sera (*lanes 2* and *3*, respectively). The *arrowhead* indicates the migration of phosphorylated PTEN. *B,* COS-7 cells were mock-transfected (pRK5 vector alone) (*lane 1*) or transfected with pRK5 HA-PTEN–(1–403) (*lane 2*), pRK5 HA-PTEN-(1–343) (*lane 3*), pRK5 HA-PTEN-(1–369) (*lane 4*), or pRK5 HA-PTEN-(1–386) (*lane 5*). In the *upper panel*, cells were labeled with ^{32}P, and cell lysates were immunoprecipitated with the anti-HA 12CA5 mAb. In the *lower panel*, 10 μg of total cell lysates were loaded and subjected to immunoblot with the anti-HA 12CA5 mAb. All samples were resolved by 10% SDS-PAGE under reducing conditions followed by autoradiography (*A* and *B*, *upper panel*) or immunoblot (*B*, *lower panel*).

To ascertain the identity of the kinase(s) involved in the phosphorylation of PTEN, ^{32}P-labeled COS-7 cells were transfected with HA-PTEN and incubated in the presence of stimulators of several signal transduction pathways, including okadaic acid, sodium pervanadate, A23187 calcium ionophore, insulin, phorbol 12-myristate 13-acetate, and EGF. As shown, none of these stimuli significantly modified the phosphorylation level of HA-PTEN (Fig. 2*A*, *lanes 1–7*). We also tested the possibility that PTEN could be phosphorylated on tyrosine residues. For these experiments, a GST-PTEN fusion protein was overexpressed in COS-7 cells, precipitated in one step using glutathione-Sepharose, and subjected to immunoblot with the anti-phosphotyrosine 4G10 mAb (Fig. 2*B*). No reactivity of the 4G10 mAb toward GST-PTEN was detected (Fig. 2*B, upper panel, lanes 1* and *2*), whereas a strong signal was observed toward GST-ERK2 (included as a positive control of tyrosine phosphorylation) upon EGF cell stimulation (Fig. 2*B,*

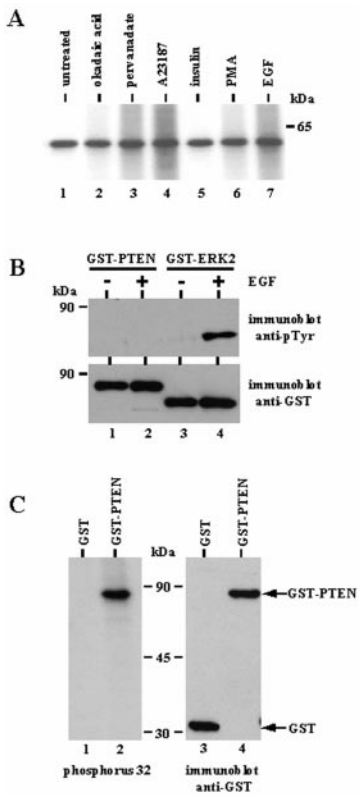

FIG. 2. **Constitutive phosphorylation of PTEN in intact cells.** *A,* COS-7 cells were transfected with pRK5 HA-PTEN-(1–403) and labeled with ^{32}P. Cells were left untreated (*lane 1*) or were treated for 30 min (okadaic acid, sodium pervanadate, calcium ionophore A23187, and phorbol 12-myristate 13-acetate) or 10 min (insulin and EGF) with the distinct stimuli, as indicated (*lanes 2–7*), and cell lysates were immunoprecipitated with the anti-HA 12CA5 mAb. *B,* COS-7 cells were transfected with pRK5 GST-PTEN-(1–403) (*lanes 1* and *2*) or pRK5 GST-ERK2 (*lanes 3* and *4*). Cells were left untreated (*lanes 1* and *3*) or were treated with EGF (50 ng/ml, 10 min) (*lanes 2* and *4*), and cell lysates were precipitated with glutathione-Sepharose followed by immunoblot analysis with the anti-phosphotyrosine 4G10 (*upper panel*) or an anti-GST mAb (*lower panel*). *C.*,COS-7 cells were transfected with pRK5 GST (*lanes 1* and *3*) or pRK5 GST-PTEN-(1–403) (*lanes 2* and *4*). In the *left panel*, cells were labeled with ^{32}P, and cell lysates were precipitated with glutathione-Sepharose. In the *right panel*, 10 μg of total cell lysates were loaded and subjected to immunoblot with an anti-GST mAb. All samples were resolved by 10% SDS-PAGE under reducing conditions followed by autoradiography (*A* and *C*, *left panel*) or immunoblot (*B* and *C*, *right panel*).

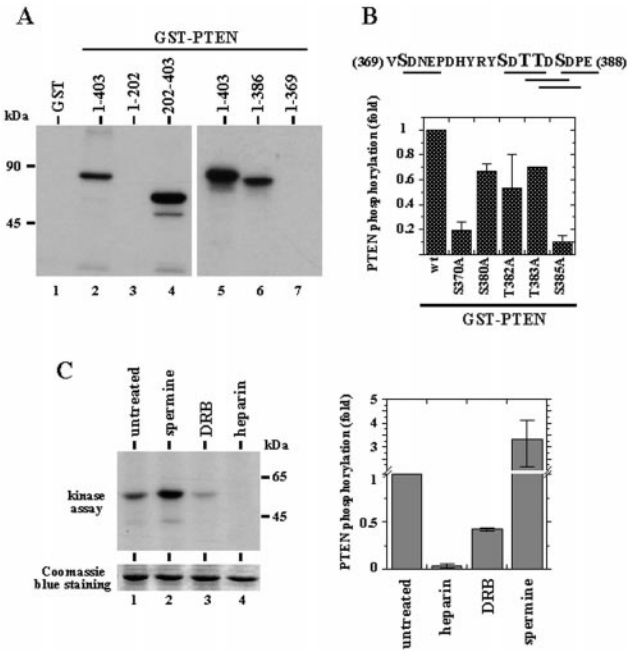

FIG. 3. **CK2 phosphorylates the C terminus of PTEN.** *A,* recombinant CK2 holoenzime was mixed with purified GST (*lane 1*), GST-PTEN-(1–403) (*lanes 2* and 5), or truncated GST-PTEN-(1–202) (*lane 3*), -(202–403) (*lane 4*), -(1–386) (*lane 6*), or –(1–369) (*lane 7*) fusion proteins (1 μg), and *in vitro* kinase assays were carried out in the presence of [γ-^{32}P]ATP. Samples were resolved by 10% SDS-PAGE followed by autoradiography. *B,* recombinant CK2 holoenzyme was mixed with purified GST-PTEN wild type or mutant fusion proteins (0.5 μg), as indicated, and kinase assays were performed as described in *A*. Data are presented as the incorporated radioactivity with respect to GST-PTEN wild type. Values represent the mean ± S.D. of two separate experiments. The *top* of the figure shows the PTEN amino acid sequence (residues 369–388) containing the CK2 phosphorylation sites (shown in *uppercase letters*). The distinct CK2 phosphorylation consensus motifs are *underlined*. Amino acids are indicated using the *single-letter code*. *C, left panel*, crude extracts from COS-7 cell lysates (5 μg) were left untreated (*lane 1*) or were preincubated separately with 1 mM spermine (*lane 2*), 100 μM DRB (*lane 3*), or 1 μg/μl heparin (*lane 4*) in buffer B and were then mixed with purified GST-PTEN-(202–403) (1 μg) in the presence of [γ-^{32}P]ATP and subjected to *in vitro* kinase assay (*upper panel*). The *lower panel* shows the identical amount of GST-PTEN-(202–403) substrate after all incubations, as detected by Coomassie blue staining. The *right panel* shows the quantification of GST-PTEN-(202–403) phospholabeling obtained after phosphorylation in the presence of the distinct CK2 modulators. Data are presented as the incorporated radioactivity with respect to control conditions. Values represent the mean ± S.D. of two separate experiments. For quantification, radiolabeled substrate bands were excised from the gel and counted in a scintillation counter or were analyzed using a PhosphorImager.

upper panel, *lane 4*). The *lower panel* in Fig. 2*B* shows the expression of the GST fusion proteins. The phosphorylation of GST-PTEN, precipitated from transfected COS-7 cells labeled with ^{32}P, is also shown as a control (Fig. 2*C*, *lane 2*). Together, these results indicate that the C-terminal region of PTEN is constitutively phosphorylated on serine and/or threonine residues.

PTEN Is Phosphorylated by CK2 Both in Vitro and in Vivo—The results described above suggest that a messenger-independent serine/threonine kinase may phosphorylate PTEN in intact cells. Residues 369–386 of the PTEN amino acid sequence include several consensus phosphorylation sites for protein kinase CK2 (S/TXXD/E/S(P)/T(P)) (residues Ser-370, Ser-380, Thr-382, Thr-383, and Ser-385; see Fig. 3*B*), which is known to phosphorylate *in vivo*, in a constitutive manner, a wide array of substrates (23). Thus, the possibility was tested that PTEN could be phosphorylated by CK2. First, *in vitro* CK2 kinase assays were performed, using bacteria-purified GST-PTEN molecules (wild type or truncated forms) as substrates and purified CK2 as the kinase. Both, GST-PTEN-(1–403) and GST-PTEN-(202–403) (full-length and C-terminal domain of PTEN, respectively) were strongly phosphorylated by CK2 (Fig. 3*A*, *lanes 2* and *4*, respectively), whereas this kinase did not phosphorylate GST alone or GST-PTEN-(1–202) (N-terminal domain of PTEN) (Fig. 3*A*, *lanes 1* and *3*, respectively). Furthermore, the C-terminal truncated GST-PTEN-(1–386), but not GST-PTEN-(1–369), was also phosphorylated by CK2 (Fig. 3*A*, *lanes 6* and *7*, respectively). Next, amino acid substitution mutants were generated that replaced to Ala the CK2 putative phosphorylation sites within the 369–386 PTEN region, and the mutant GST-PTEN-(1–403) fusion proteins were also tested for *in vitro* phosphorylation by CK2. As shown in Fig. 3*B*, CK2 phosphorylation of S370A and S385A mutants was greatly reduced, suggesting that Ser-370 and Ser-385 are major determinants for *in vitro* phosphorylation of PTEN by CK2. A partial contribution of the Ser-380, Thr-382, and Thr-383 PTEN residues to the *in vitro* phosphorylation by CK2 was also observed (Fig. 3*B*). In addition, stoichiometric analysis revealed that GST-PTEN-(202–403) incorporated about 4 mol

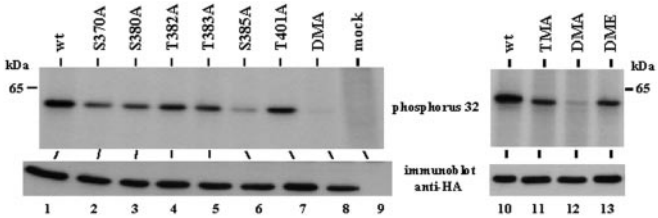

FIG. 4. **Identification of the PTEN-phosphorylated residues in intact cells.** COS-7 cells were mock-transfected (*lane 9*) or transfected with pRK5 HA-PTEN wild type (*lanes 1* and *10*) or mutants (*lanes 2–8* and *11–13*), as indicated. *DMA*, double mutant S370A/S385A; *DME*, double mutant S370E/S385E; *TMA*, triple mutant S380A/T382A/T383A. In the *upper panel*, cells were labeled with ^{32}P, and cell lysates were immunoprecipitated with the anti-HA 12CA5 mAb. In the *lower panel*, 10 μg of total cell lysates were loaded and subjected to immunoblot with the anti-HA 12CA5 mAb. Samples were resolved by 10% SDS-PAGE under reducing conditions followed by autoradiography (*upper panel*) or immunoblot with the anti-HA 12CA5 mAb (*lower panel*).

of phosphate/mol of protein after extensive *in vitro* phosphorylation by CK2.

To test the involvement of CK2 in the phosphorylation of PTEN observed in intact cells, crude extracts of COS-7 cells were incubated, in the presence of inhibitors or activators of CK2, with the GST-PTEN fusion proteins purified from bacteria, and *in vitro* kinase assays were performed. In keeping with the results obtained after *in vitro* phosphorylation by CK2, GST-PTEN-(1–403) and GST-PTEN-(202–403) were specifically phosphorylated by the kinase activity present in the cell extracts, whereas GST-PTEN-(1–202) or GST alone were not (Fig. 3*C*, *lane 1*, and data not shown). Remarkably, this phosphorylation was substantially increased in the presence of spermine, an activator of CK2 (Fig. 3*C*). On the other hand, in the presence of any of two CK2 inhibitors, DRB or heparin, the phosphorylation of GST-PTEN was inhibited (Fig. 3*C*). No effect was observed in the presence of other protein kinase effectors, such as the MEK1/2 inhibitor PD98059 or the protein kinase A activator dibutyryl cAMP (data not shown). These results sustain the notion that the kinase activity that phosphorylates PTEN *in vivo* is attributable to CK2. Interestingly, treatment of cells with DRB resulted in a decrease in both the *in vivo* phospholabeling and the protein amount of PTEN, suggesting that DRB cell treatment could diminish PTEN phosphorylation and/or PTEN protein content (data not shown; and see Fig. 5*C*).

PTEN phosphorylation was also analyzed on ^{32}P-labeled COS-7 cells overexpressing the distinct HA-PTEN CK2 phosphorylation mutants (Fig. 4). A significant reduction in the *in vivo* PTEN phosphorylation was observed on the S370A and S385A mutants (Fig. 4, *upper panel*, *lanes 2* and *6*, respectively). Also, a consistently reduced labeling was observed on the S380A mutant (Fig. 4, *upper panel*, *lane 3*), whereas the effect on the phosphorylation of the individual T382A and T383A mutants was less manifest (Fig. 4, *upper panel*, *lanes 4* and *5*, respectively). Because these results suggested the involvement of multiple residues on PTEN phosphorylation in intact cells, combined mutations were generated and tested for *in vivo* phosphorylation. The phosphorylation of the double mutant S370A/S385A (*DMA*) was nearly absent (Fig. 4, *upper panel*, *lanes 8* and *12*), and the phosphorylation of the triple mutant S380A/T382A/T383A (*TMA*) was markedly decreased (Fig. 4, *upper panel*, *lane 11*), indicating an additive contribution of all these residues to PTEN phosphorylation in intact cells. The phosphorylation of a double phosphomimetic S370E/S385E (*DME*) mutant was also analyzed. As shown, the S370E/S385E mutant was hyperphosphorylated in comparison with the S370A/S385A mutant (Fig. 4, *upper panel*, *lane 13*), suggesting that phosphorylation of these two PTEN residues might prime for additional phosphorylation of nearby residues. Finally, the putative *in vivo* phosphorylation of the Thr-401 residue, located within the PDZ binding consensus motif of PTEN, was also tested. As shown, the phosphorylation level of the T401A mutant was almost indistinguishable from the wild type PTEN (Fig. 4, *upper panel*, *lane 7*). Together, these results support the notion that CK2 constitutively phosphorylates in COS-7 cells a cluster of Ser/Thr residues at the C terminus of PTEN.

Phosphorylation of PTEN C Terminus Is Important for PTEN Protein Stability to Proteasome-mediated Degradation—To analyze the effect of PTEN phosphorylation on the stability of the molecule, pulse-chase experiments were performed on [^{35}S]methionine-labeled COS-7 cells transfected with HA-PTEN wild type or phosphorylation mutants. The kinetics of degradation of HA-PTEN wild type was two to three times slower than that showed by the distinct Ser/Thr to Ala phosphorylation mutants (Fig. 5*A*). Comparative plots of degradation of wild type PTEN with the S370A, T380A, T382A, T383A, S385A, and S370A/S385A (*DMA*) mutants are shown (Fig. 5*A*). These results suggested that the presence of the phosphorylated residues could be important for PTEN stability. To test this possibility, the degradation of the phosphomimetic S370E/S385E (*DME*) mutant was analyzed. Interestingly, the stability of the S370E/S385E mutant was almost identical to that obtained with the wild type PTEN (Fig. 5*A, right panel*), reinforcing the hypothesis of a role of phosphorylation of PTEN in protein stabilization.

To ascertain the degradative pathway of PTEN under these conditions, transfected COS-7 cells were pulsed with [^{35}S]methionine, and chases were performed in the presence of the proteasome inhibitor MG132 or the lysosome inhibitors leupeptin + NH_4Cl. As shown, the proteasome inhibitor MG132 inhibited the degradation of both PTEN wild type and S370A/S385A mutant (Fig. 5*B*, *lanes 3* and *7*), whereas no inhibition was observed in the presence of leupeptin + NH_4Cl (Fig. 5*B*, *lanes 4* and *8*). The effect of MG132 on the degradation of the S370A/S385A mutant was consistently less pronounced than that observed for the wild type PTEN. Finally, experiments were carried out to elucidate whether the effect of DRB cell treatment in the decrease of ^{32}P-radiolabeled PTEN detected in our studies was, in fact, because of the inhibition of CK2. Cells were left untreated or were preincubated with the proteasome inhibitor MG132 to prevent PTEN degradation, were then labeled with ^{32}P or [^{35}S]methionine in the presence of DRB, and the amount of radiolabeled PTEN was detected as above. Upon DRB cell treatment, both the ^{32}P phospholabeling and the amount of [^{35}S]methionine-labeled PTEN were decreased (Fig. 5*C*, *lane 2*). Remarkably, in the presence of DRB plus MG132 the phosphorylation of PTEN was decreased without affecting the amount of PTEN protein detected by [^{35}S]methionine labeling (Fig. 5*C*, *lane 3*). Together, these results indicate that defective PTEN phosphorylation by CK2 accelerates the proteasome-mediated degradation of PTEN.

DISCUSSION

On the basis that many CK2 substrates are oncogene products or tumor suppressor proteins as well as key signal transduction proteins, a role for CK2 in the regulation of cell growth- and cell cycle-related processes has been proposed (22, 23). In this report, we provide evidence that the tumor suppressor phosphatase PTEN is phosphorylated by CK2 both *in vitro* and in intact cells. CK2 phosphorylation sites in PTEN were located within a C-terminal cluster of Ser/Thr residues, which were found to be important for PTEN stability to proteasome-mediated degradation. The CK2 phosphorylation sites were identified as the PTEN residues Ser-370, Ser-380, Thr-382, Thr-383,

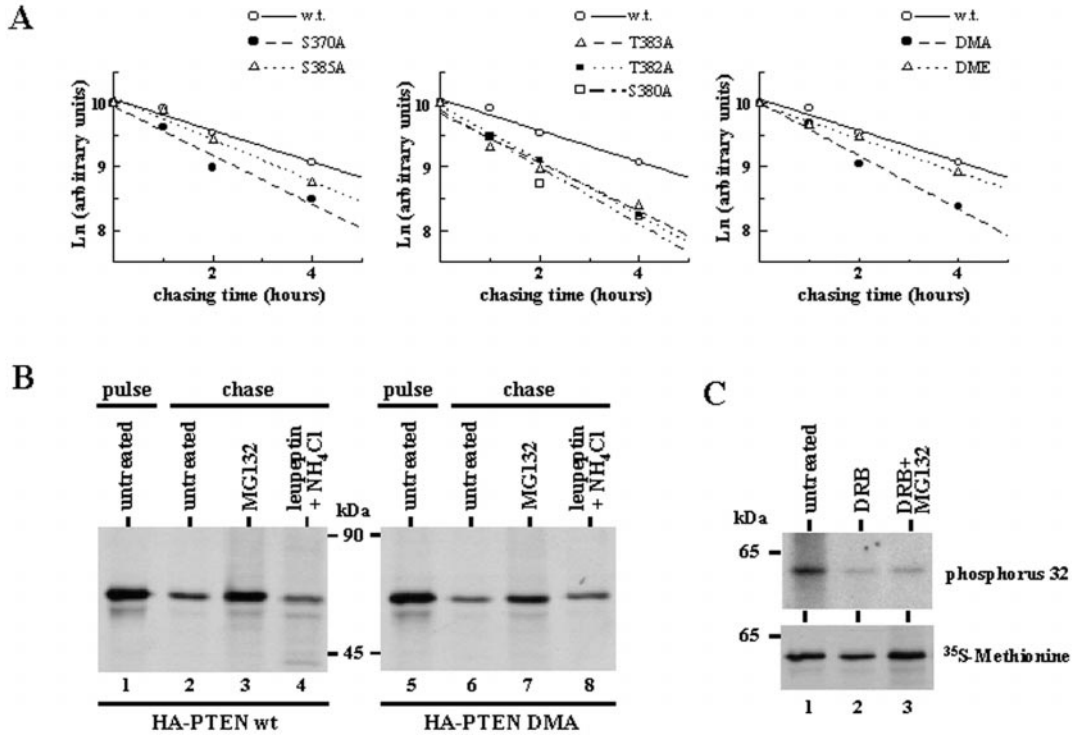

FIG. 5. **Stability of PTEN wild type and phosphorylation mutants.** *A,* COS-7 cells were transfected with pRK5 HA-PTEN wild type or mutants, as indicated. Cells were pulsed for 2 h in the presence of [^{35}S]methionine and then chased at different times, as indicated. Cell lysates were immunoprecipitated with the anti-HA 12CA5 mAb, and radiolabeled bands were resolved by 10% SDS-PAGE under reducing conditions and analyzed using a PhosphorImager. Kinetics degradation plots of PTEN wild type, single Ser/Thr to Ala mutants, and double S370A/S385A (*DMA*) and S370E/S385E (*DME*) mutants are shown. To facilitate comparison, results were grouped in sets of mutants; representative experiments are shown. Experiments were performed at least twice, and the difference between independent experiments was always less than 10%. *B,* COS-7 cells were transfected with pRK5 HA-PTEN wild type (*lanes 1–4*) or S370A/S385A (*DMA*) mutant (*lanes 5–8*). Cells were pulsed for 2 h in the presence of [^{35}S]methionine; then, cells were lysed (*lanes 1* and *5*) or were chased for 4 h in the absence (*lanes 2* and *6*) or presence (*lanes 3*, *4, 7, 8*) of inhibitors, as indicated, followed by cell lysis. Cell lysates were immunoprecipitated with the anti-HA 12CA5 mAb, and samples were analyzed by 10% SDS-PAGE under reducing conditions. *C*, COS-7 cells were transfected with pRK5 HA-PTEN wild type and labeled with ^{32}P (*upper panel*) or [^{35}S]methionine (*lower panel*) for 4 h in the absence (*lane 1*) or in the continuous presence of DRB (*lane 2*) or DRB plus MG132 (*lane 3*). Cells were lysed and processed as described in *B*.

and Ser-385. Because some of these sites can be generated after previous phosphorylation of nearby residues, and because the phosphomimetic S370E/S385E mutant is phosphorylated more efficiently than the S370A/S385A mutant, hierarchic CK2 multisite phosphorylation of this region of PTEN is likely to exist (37). Several findings support the notion that CK2 is the major kinase involved in the phosphorylation of PTEN in intact cells. First, cell treatment with a panel of different stimuli did not substantially affect the phosphorylation of PTEN, suggesting that such phosphorylation is constitutive and messenger-independent, as is known for most CK2-mediated phosphorylations. Second, tyrosine phosphorylation was not detected on PTEN, suggesting that tyrosine kinases do not directly phosphorylate PTEN. Third, phosphorylation of PTEN by the kinase activity present in COS-7 cell extracts was activated or inhibited by CK2 activators or inhibitors, respectively. Fourth, phosphorylation of PTEN in intact COS-7 cells was inhibited by cell treatment with the CK2 inhibitor DRB. Fifth, mutation to Ala of the PTEN CK2-phosphorylation sites, as well as deletion of the C-terminal portion of PTEN including these sites, resulted in an almost complete lack of PTEN phosphorylation in intact cells. However, the possibility cannot be ruled out that other kinase(s) not monitored in our assays, using overexpressed HA-PTEN in COS-7 cells, may contribute to the phosphorylation of endogenous PTEN *in vivo*. Remarkably, the CK2 phosphorylation sites in PTEN are conserved in species from mammals to *Xenopus laevis*, and clusters of putative CK2 phosphorylation sites are also present at the C terminus of PTEN from *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*.

We found that PTEN suffers a rapid degradation in COS-7 cells, which was inhibited by the proteasome inhibitor MG132 but not by lysosome inhibitors, indicating that the turnover of PTEN in cultured COS-7 cells depends mainly on proteasome-mediated degradation. The involvement of the proteasome in the degradation and regulation of the functions of short-lived proteins, including oncoproteins, tumor suppressors, and cell cycle proteins has been described extensively (for reviews, see Refs. 38–40). Thus, regulated degradation of PTEN via the proteasome could be envisaged as a major physiological mechanism that controls the amount of PTEN in specific cell types and tissues. In this context, it has been reported recently that C-terminal truncation mutants of PTEN have shorter half-lives than the wild type molecule (14, 15). In addition, we show that Ser/Thr to Ala amino acid mutations at the PTEN C terminus, leading to defective phosphorylation by CK2, accelerate the proteasome-mediated degradation of PTEN. However, a double Ser to Glu mutation (the S370E/S385E mutant), that mimicked constitutive CK2 phosphorylation of PTEN at these sites showed a degradation rate comparable with the wild type molecule. Furthermore, cell treatment with the CK2 inhibitor DRB, which inhibited PTEN constitutive phosphorylation, resulted in a diminution in PTEN protein content but only in the absence of the proteasome inhibitor MG132. While this manuscript was in preparation, Vazquez *et al.* (20) also showed that phosphorylation of some of the PTEN C-terminal residues an-

alyzed in our study regulate protein stability as well as PTEN-mediated G1 cell cycle arrest. Thus, constitutive phosphorylation of PTEN by CK2 may play a key role in the regulation of PTEN biological functions upon normal cell growth conditions.

Our findings suggest that phosphorylation of the PTEN C terminus itself does not provide enough PTEN protein stability (see below) but rather could be important to acquire the proper, stable conformation of the PTEN C-terminal tail. For instance, it is well documented that phosphorylation of the C terminus of IκBα by CK2 affects the stability and constitutive turnover of this NF-κB regulator (41–44). In addition, the possibility exists that phosphorylation of the C terminus of PTEN could modulate its subcellular location and function by affecting its association with regulatory and/or stabilizing molecules. In this regard, deletion of the C-terminal PDZ binding motif of PTEN results in the impairment of some PTEN cellular functions (16). Also, PTEN has been found to associate, through its PDZ binding motif, with PDZ domain-containing proteins, including hMAST205 and MAGUK scaffolding proteins (17–19). Interestingly, MAGUKs might regulate the functions of PTEN through the assembly and stabilization of multiprotein signaling complexes at specific subcellular compartments, as known for other related PDZ-containing proteins (45). It has been suggested that phosphorylation of the Thr-401 residue within the PDZ binding motif of PTEN could regulate its binding to PDZ-containing proteins (17). However, our results suggest that the PTEN Thr-401 residue is not phosphorylated *in vivo*. Mutations that did not abrogate PTEN phosphorylation, but are predicted to disrupt the association with PDZ-containing proteins (PTEN-(1–386) and T401A mutant), also diminished the stability of PTEN (data not shown), favoring the hypothesis of a putative role for phosphorylation by CK2 in PTEN stabilization through protein association. Frame-shift mutations at the end of the *PTEN* coding region have been found in tumors, indicating that such mutations could produce C-terminal truncated PTEN molecules with altered tumor suppressor function (13). Furthermore, by using anti-PTEN antibodies, variable expression levels of PTEN protein are detected in tumor specimens as compared with normal tissues (46–49), suggesting that mechanisms other than loss of the *PTEN* gene may account for alterations on PTEN protein expression in some tumors. The results presented here provide evidence that the CK2 phosphorylation sites contained within the C terminus of PTEN play an important role in its stabilization and turnover, in a process mediated by the proteasome. Our findings support a model in which changes in the phosphorylation status of the C terminus of PTEN might alter the basal expression levels of this tumor suppressor. In this regard, we have not detected dephosphorylation of PTEN after incubation with crude cell extracts, and PTEN phosphorylation status did not change upon cell incubation with the PP2A inhibitor, okadaic acid (Fig. 2).[2] The possibility exists that dephosphorylation of PTEN takes place only under particular conditions of activation of an unidentified phosphatase and/or under specific subcellular location of PTEN. Further work will be necessary to ascertain whether regulated dephosphorylation events could affect PTEN phosphorylation and function *in vivo*.

Acknowledgments—We thank C. Eng and R. Parsons for providing antibodies, E. Knecht for helpful discussions and critical reading of the manuscript, G. Fuertes for help with the degradation experiments, and I. Roglá for expert technical assistance.

REFERENCES

1. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittman, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. (1997) *Science* **275,** 1943–1947
2. Steck, P. A., Pershouse, M. A., Jaser, S. A., Yung, W. K. A., Lin, H., Ligon, H. A., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H. F., and Tavtigian, S. V. (1997) *Nat. Genet.* **15,** 356–362
3. Li, D,-M., and Sun, H. (1997) *Cancer Res.* **57,** 2124–2129
4. Myers, M. P., Stolarov, J. P., Eng, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R., and Tonks, N. K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 9052–9057
5. Maehama, T., and Dixon, J. E. (1998) *J. Biol. Chem.* **273,** 13375–13378
6. Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K. (1998) *Science* **280,** 1614–1617
7. Cantley, L. C., and Neel, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4240–4245
8. Maehama, T., and Dixon, J. E. (1999) *Trends Cell Biol.* **9,** 125–128
9. Tamura, M., Gu, J., Tran, H., and Yamada, K. M. (1999*) J. Natl. Cancer Inst.* **91,** 1820–1828
10. Di Cristofano, A., and Pandolfi,. P. P. (2000) *Cell* **100,** 387–390
11. Lee, J.-O., Yang, H., Georgescu, M.-M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J. E., Pandolfi, P. P., and Pavletich, N. P. (1999) *Cell* **99,** 323–334
12. Myers, M. P., and Tonks, N. K. (1997) *Am. J. Hum. Genet.* **61,** 1234–1123
13. Ali, I. U., Schriml L. M., and Dean, M. (1999) *J. Natl. Cancer Inst.* **9,** 1922–1932
14. Georgescu, M.-M., Kirsch, K. H., Akagi, T., Shishido, T., and Hanafusa, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10182–10187
15. Tolkacheva, T., and Chan, A. M.-L. (2000) *Oncogene* **19,** 680–689
16. Morimoto, A. M., Berson, A. E., Fujii, G. H., Teng, D. H.-F., Tavtigian, S. V., Bookstein, R., Steck, P. A., and Bolen, J. B. (1999) *Oncogene* **18,** 1261–1266
17. Adey, N. B., Huang, L., Ormonde, P. A., Baumgard, M. L., Pero, R., Byreddy, D. V., Tavtigian S. V., and Bartel, P. L. (2000) *Cancer Res.* **60,** 35–37
18. Wu, X., Hepner, K., Castelino-Prabhu, S., Do, D., Kaye, M. B., Yuan X-Y., Wood, J., Ross, C., Sawyers, C. L., and Whang Y. E. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4233–4238
19. Wu, Y., Dowbenko, D., Spencer, S., Laura, R., Lee, J., Gu, Q., and Lasky, L. A. (2000) *J. Biol. Chem.* **275,** 21477–21485
20. Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W. R. (2000) *Mol. Cell Biol.* **20,** 5010–501820
21. Pinna, L. A. (1990) *Biochim. Biophys. Acta* **1054,** 267–284
22. Meisner, H., and Czech, M. P. (1991) *Curr. Opin. Cell Biol.* **3,** 474–483
23. Allende, J. E., and Allende, C. C. (1995) *FASEB J.* **9,** 313–323
24. Münstermann, U., Fritz, G., Seitz, G., Yiping, L., Schneider, H. R., and Issinger, O.-G. (1990) *Eur. J. Biochem.* **189,** 251–257
25. ole-MoiYoi, O. K., Brown, W. C., Iams, K. P., Nayar, A., Tsukamoto, T., and Macklin, M. D. (1993) *EMBO J.* **12,** 1621–1631
26. Stalter, G., Siemer, S., Becht, E., Ziegler, M., Remberger, K., and Issinger, O.-G. (1994) *Biochem. Bioph. Res. Commun.* **202,** 141–147
27. Seldin, D. C., and Leder, P. (1995) *Science* **267,** 894–896
28. Hériché, J-K., Lebrin, F., Rabilloud, T., Leroy, D., Chambaz, E. M., and Goldberg, Y. (1997) *Science* **276,** 952–955
29. Orlandini, M., Semplici, F., Ferruzzi, R., Meggio, F., Pinna, L. A., and Oliviero, S. (1998) *J. Biol. Chem.* **273,** 21291–21297
30. Li, D., Dobrowolska, G., Aicher, L. D., Chen, M., Wright, J. H., Drueckes, P., Dunphy, E. L., Munar, E. S., and Krebs, E. G. (1999) *J. Biol. Chem.* **274,** 32988–32996
31. Vilk, G., Saulnier, R. B., St. Pierre, R., and Litchfield, D. W. (1999) *J. Biol. Chem.* **274,** 14406–14414
32. Lorenz, P., Pepperkok, R., Ansorge, W., and Pyerin, W. (1993) *J. Biol. Chem.* **268,** 2733–2739
33. Ulloa, L., Díaz-Nido, J., and Ávila, J. (1993) *EMBO J.* **12,** 1633–1640
34. Pulido, R., Zúñiga, Á., and Ullrich, A. (1998) *EMBO J.* **17,** 7337–7350
35. Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M. P., Tonks, N., and Parsons, R. (1998) *Cancer Res.* **58,** 5667–5672
36. Dahia, P. L. M., Aguiar, R. C. T., Alberta, J., Kum, J. B., Caron, S., Sill, H., Marsh, D. J., Ritz, J., Freedman, A., Stiles, C., and Eng, C. (1999) *Hum. Mol. Genet.* **8,** 185–193
37. Roach, P. J. (1991) *J. Biol. Chem.* **266,** 14139–14142
38. Ciechanover, A. (1994) *Cell* **79,** 13–21
39. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) *Annu. Rev. Biochem.* **65,** 801–847
40. Lee, D. H. L., and Goldberg, A. L. (1998) *Trends Cell Biol.* **8,** 397–403
41. McElhinny, J. A., Trushin, S. A., Bren, G. D., Chester, N., and Paya, C. V. (1996) *Mol. Cell. Biol.* **16,** 899–906
42. Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. (1996) *Mol. Cell Biol.* **16,** 1401–1409
43. Schwarz, E. M., van Antwerp, D., and Verma, I. M. (1996) *Mol. Cell. Biol.* **16,** 3554–3559
44. Leslie, N. R., Gray, A., Pass, I., Orchiston, E. A., and Downes, C. P. (2000) *Biochem. J.* **346,** 827–833
45. Pawson, T., and Scott, J. D. (1997) *Science* **278,** 2075–2080
46. Perren, A., Weng, L.-P., Boag, A. H., Ziebold, U., Thakore, K., Dahia, P. L. M., Komminoth, P., Lees, J. A., Mulligan, L. M., Mutter, G. L., and Eng, C. (1999) *Am. J. Pathol.* **155,** 1253–1260
47. Zhang, P., and Steinberg, B. M. (2000) *Cancer Res.* **60,** 1457–1462
48. Gimm, O., Perren, A., Weng, L. P., Marsh, D. J., Yeh, J. J., Ziebold, U., Gil, E., Hinze, R., Delbridge, L., Lees, J. A., Mutter, G. L., Robinson, B. G., Komminoth, P., Dralle, H., and Eng, C. (2000) *Am. J. Pathol.* **156,** 1693–1700
49. Torres, J., Navarro, S., Roglá, I., Ripoll, F., Lluch, A., García-Conde, J., Llombart-Bosch, A., Cervera, J., and Pulido, R. (2001) *Eur. J. Cancer*, **37,** 114–121

[2] J. Torres and R. Pulido, unpublished observations.

PROTEIN SYNTHESIS
POST-TRANSLATION MODIFICATION
AND DEGRADATION:

The Tumor Suppressor PTEN Is Phosphorylated by the Protein Kinase CK2 at Its C Terminus: IMPLICATIONS FOR PTEN STABILITY TO PROTEASOME-MEDIATED DEGRADATION

Josema Torres and Rafael Pulido
J. Biol. Chem. 2001, 276:993-998.
doi: 10.1074/jbc.M009134200 originally published online October 16, 2000
