Mitotic and Stress-induced Phosphorylation of HsPI3K-C2α Targets the Protein for Degradation*

Received for publication, February 17, 2003, and in revised form, April 24, 2003
Published, JBC Papers in Press, April 28, 2003, DOI 10.1074/jbc.M301657200

Svetlana A. Didichenko, Cristina M. Fragoso, and Marcus Thelen‡

From the Institute for Research in Biomedicine, Via Vincenzo Vela 6, Bellinzona CH 6500, Switzerland

Phosphoinositide 3-kinases (PI 3-kinases)¹ regulate diverse cellular processes, which include cell signaling, intracellular protein sorting, cell cycle progression, cell survival, and apoptosis (2). PI 3-kinases phosphorylate the D3 hydroxyl group on the inositol ring leading to 3-phosphoinositides which act as membrane-embedded second messengers mediating the activation of downstream effectors (3). Class I PI 3-kinases are heterodimeric enzymes consisting of a catalytic and a regulatory subunit. They are involved primarily in growth factor and chemotactic agonist-mediated signal transduction (4, 5). In vitro, these PI 3-kinases are able to utilize phosphatidylinositol (PtdIns), PtdIns(4)P, and PtdIns(4,5)P2 as substrates, but most likely produce PtdIns(3,4,5)P3 in vivo (6). Following activation of resting cells, these kinases are recruited rapidly from the cytosol to the plasma membrane where they generate PtdIns(3,4,5)P3. Class II PI 3-kinases are monomeric proteins. Three human isoforms, HsPI3K-C2α, HsPI3K-C2β, and HsPI3K-C2γ (7–10), and their homologs in rodents have been characterized (11–13). PI 3-kinases of this class have been found also in Drosophila melanogaster (14) and in Caenorhabditis elegans (15), but not in yeast. Members of class II are distinguished from other PI 3-kinases by the presence of two tandem domains at their carboxyl terminus, a phox homology domain and a C2 domain, a module that is known to confer Ca2+-dependent phospholipid binding (16). However, the C2 domains of class II PI 3-kinases lack a critical Asp residue in the calcium binding loop (17), which is consistent with the finding that they do not bind to membranes in a calcium-dependent manner (14, 18). In vitro, all class II PI 3-kinases phosphorylate PtdIns and PtdIns(4)P, but their in vivo substrate remains to be determined (2). Both HsPI3K-C2α and HsPI3K-C2β are implicated in signaling downstream of epidermal growth factor and platelet-derived growth factor receptors (18). HsPI3K-C2α was shown to concentrate in the trans-Golgi network and in clathrin-coated pits (19), whereas P3K-C2β was found in the nuclei of rat liver cells (20). In general, the role of class II PI 3-kinases in signal transduction and mode of activation is poorly understood, and specific downstream targets have not been characterized.

Class I PI 3-kinases activities were shown to be regulated by phosphorylation (21–23). Phosphorylation of the regulatory subunit p85 by the catalytic subunit p110α of class I PI 3-kinase (p110α/p85 heterodimer) down-regulates lipid kinase activity of the complex (21, 22). Phosphorylation of class II PI 3-kinases was demonstrated; however, the physiological role of this phosphorylation remained unclear. Increased phosphorylation of class II PI 3-kinase C2α was found to correlate with a moderately elevated enzyme activity in insulin-stimulated cells (24). In contrast, our data demonstrated that the phosphorylation status neither changes the lipid kinase activity of P3K-C2α nor affects the substrate specificity, but influences the intranuclear localization (1).

In this study we investigated the phosphorylation of HsPI3K-C2α induced by genotoxic stress and during the cell cycle. We show that the kinase becomes phosphorylated upon exposure of cells to UV irradiation and in proliferating cells at the G2/M transition of cell cycle. Stress-dependent and mitotic phosphorylation of HsPI3K-C2α occurs on the same serine residue (Ser259) within a recognition motif for proline-directed kinases, such as mitogen-activated protein kinase (MAPK), MEK, mitogen-activated protein kinase kinase (MAPKK), and mitogen-activated protein kinase kinase kinase (MAPKKK).

*This work was supported by the Swiss Cancer League and the Helmut Horten Foundation. The costs of publication of this article were therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed. E-mail: marcus.thelen@irb.unisi.ch.

¹The abbreviations used are: PI 3-kinases, phosphoinositide 3-kinases; Cdk, cyclin-dependent protein kinase; CMV, cytomegalovirus; DMEM, Dulbecco’s modified Eagle’s medium; EGFP, enhanced green fluorescent protein; ERK, extracellular signal regulated kinase; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; HEK, human embryonic kidney; JNK, c-Jun NH2-terminal kinase; JNKK, JNK kinase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MAP, mitogen-activated protein; MAPKK, MAP kinase kinase; MEK, mitogen-activated protein kinase; PI3K, PI 3-kinase; PtdIns, phosphatidylinositol; SAPK, stress-activated protein kinase.
Phosphorylation of HsPI3K-C2α

protein (MAP) kinases and cyclin-dependent protein kinases (Cdk). By using different selective inhibitors of MAP kinases and Cdk5 in vitro and in vivo assays, we found that Cdc2 mediates mitotic phosphorylation, whereas JNK/SAPK is responsible for stress-induced phosphorylation of HsPI3K-C2α. In either case phosphorylation provides a signal for proteasome-dependent degradation of the protein.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies against PI3K-C2α (AXIX and AXXIII) were described previously (1). Anti-GFP rabbit polyclonal antibody was purchased from Clontech, anti-Cdc2 polyclonal rabbit antibody was from Oncogene, anti-cyclin B1 antibody was from Pharmingen, anti-phospho-Jun (pSer63) and anti-phospho-Jun (pSer73) rabbit antibodies were from Upstate Biotechnology. Secondary horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were obtained from Bio-Rad.

Plasmids—The cDNA encoding human HsPI3K-C2α (8) was kindly provided by Dr. J. Domin (London). Various HsPI3K-C2α cDNAs were amplified by the PCR using gene-specific primers with incorporated restriction sites to facilitate their cloning into appropriate vectors. For expression in bacterial cells, GST-tagged fusion constructs were generated by cloning wild-type and mutant HsPI3K-C2α cDNAs into the BamHI site of pGEX-2T (Amersham Biosciences). For expression in mammalian cells, cDNAs were cloned into the eukaryotic expression vector pEGFP-C1 or pEGFP-N1 (Clontech). To generate pEGFP-HsPI3K-C2α expressing GFP-HsPI3K-C2α, a PCR product corresponding to the amino acids 240–275 of HsPI3K-C2α was inserted into Xho1-BamHI sites of pEGFP-C1. HsPI3K-C2α point mutations, S254A, S259A, S259D, S259E, S262A, and S264A were created by PCR amplification from pEGFP-C1-HsPI3K-C2α, using mutant sequence oligonucleotides. pBK-CMV-myc-HsPI3K-C2α, which encodes full-length HsPI3K-C2α tagged at the NH2 terminus with myc epitope was constructed as follows. The SacI-BspEI fragment from pBK-CMV-HsPI3K-C2α (8) containing the 5’ untranslated region and the first 52 nucleotides of the HsPI3K-C2α coding sequence was replaced by the SacI-BspEI fragment carrying a Kozak consensus sequence, an ATG start codon, and the sequence of myc tag joined in-frame to the HsPI3K-C2α coding sequence (4–52 bp). pBK-CMV-HA-HsPI3K-C2α, which encodes full-length HsPI3K-C2α tagged at the NH2 terminus with HA epitope, was constructed using a similar approach. To generate pEGFP-C1-GFP-HsPI3K-C2α, which encodes complete HsPI3K-C2α domain tagged at the NH2 terminus with GFP and HA epitope (GFP-HsPI3K-C2α), the SacI-EcoRI fragment from pBK-CMV-HA-HsPI3K-C2α, which encodes full-length HsPI3K-C2α tagged at the NH2 terminus with myc epitope was amplified by PCR amplification from pEGFP-N1:myc-HsPI3K-C2α, using the myc tagging primer (GFP-5’)-Irradiation—Cells were exposed to genotoxic agents and analyzed 1.5 h later. An UV dose of 300 J/m² was delivered in a single pulse using a Stratalinker (Stratagene). Prior to pulsing, the medium was removed, being replaced immediately after the treatment. 1 Gy of γ-irradiation was delivered using a Gammacell 1000 apparatus.

Cell Electrophoresis, Immunoprecipitation, and Western Blot Analysis—Proteins were separated on 8 or 6% SDS-polyacrylamide gels prepared from the stock (33.5% acrylamide, 0.3% bisacrylamide) and blotted onto Immobilon-P (Millipore). Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Triton X-100 and probed with specific antibodies. Immunoreactive bands were detected with horseradish peroxidase-labeled secondary antibodies and visualized by enhanced chemiluminescence (Pierce).

For immunoprecipitation, cells were washed twice in phosphate-buffered saline and lysed in buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1.5 mM MgCl2, 1 mM EDTA), supplemented with protease inhibitors (40 mM NaF, 0.5 mM sodium orthovanadate, 40 μM β-egrophosphate, 5 mM sodium pyrophosphate) and protease inhibitors (Complete, Roche). Cell homogenates were centrifuged at 13,000 × g for 10 min, and supernatants were precleared with Gammabind Plus-Sepharose (Amersham Biosciences) for 15 min. Immunoprecipitation of HsPI3K-C2α with antibody AXIX was carried out at 4 °C for 1–2 h. Immunocomplexes were bound to Gammabind Plus-Sepharose for 30 min, collected by centrifugation, and washed twice in lysis buffer, once in 10 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.05% SDS; then in 10 mM Tris-HCl (pH 8.0), once in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.05% SDS, and finally in 10 mM Tris-HCl (pH 8.0), 0.05% SDS. For α-phosphatase treatment immunoprecipitates were additionally washed twice in phosphate buffer (50 mM Tris-HCl (pH 7.5), 2 mM MnCl2, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35) and resuspended in 50 μl of the same buffer. After warming up at 30 °C for 5 min, 50 units of λ-phosphatase (New England Biolabs) was added, and samples were incubated at 30 °C for 40 min.

Pulse- chased Experiments—Subconfluent cultures of HeLa cells were labeled overnight with 50 μCi of [3H]methionine/cysteine (Amersham Biosciences)/ml in methionine-free DMEM (Invitrogen) supplemented with 10% dialyzed fetal calf serum. After labeling cells were washed in phosphate-buffered saline, replaced (1:3 dilution), and chased with complete DMEM containing 10% fetal calf serum for 48 h. To obtain mitotic cells, 400 ng/ml nocodazole was added to the medium for the last 36 h of the chase. Labeled mitotic cells were collected by shake off, washed three times in prewarmed DMEM, and released into fresh complete medium for 3 h. For metabolic labeling of cells at M/G1, transition of cell cycle, nocodazole-treated mitotic HeLa cells were released into the labeling DMEM in the presence of 50 μCi of [3H]methionine/cysteine for 1 or 3 h. Cells were subsequently subjected to immunoprecipitation analysis with anti-PI3K-C2α (AXIX) antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and visualized by autoradiography and immunoblotting.

Kinase and Protease Inhibitor Treatments—Roscovitine (Calbiochem) was used to inhibit Cdk activity. HeLa cells at late S phase (6 h after release from aphidicolin block) were treated or not with 30 μM roscovitine for 2 h. 400 ng/ml nocodazole was added immediately after the treatment and continued for 15 h. Nonadhering mitotic and adhering G2 cells were collected by mechanical shock and trypsin treatment, respectively. Nocodazole-arrested mitotic HeLa cells were treated with 75 μM roscovitine for 15, 45, and 90 min. For okadaic acid treatment nocodazole-arrested mitotic HeLa cells were treated with 0.5 μM okadaic acid for 30 min then 75 μM roscovitine was added, and treatment continued for 30 min.

SP600125 (Tocris) was used to inhibit JNK activation, and PD98059 and SB221902 (both from Alexis) were used to inhibit ERK and p38 activation, respectively. HeLa cells were pretreated with the inhibitors at concentrations indicated for 30 min before UV irradiation. Irradiated...
cells were cultured for 90 min in the presence of the inhibitors before harvesting.

The specific protease inhibitors MG132, ALLM, and lactacystin were from Calbiochem. HEK-293 cells were UV irradiated as described above. After 2 h of recovery in fresh medium, cells were treated with protease inhibitors (20 μM MG132, 100 μM ALLM, or 50 μM lactacystin) for the indicated times prior to Western blot analysis.

In Vitro Kinase Assay—HsPI3K-C2α was immunoprecipitated from mimosine-treated HeLa cells using affinity-purified anti-HsPI3K-C2α antibody AXIX (1). The antibody-antigen complexes were collected with GammaBind Plus-Sepharose beads and used as substrate for in vitro phosphorylation by cellular extracts. To obtain cellular extracts, pellets of HeLa cells were resuspended in 2 pellet volumes of ice-cold hypotonic buffer (50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 40 mM NaF, 0.5 mM sodium orthovanadate, 40 mM β-glycerophosphate, 5 mM sodium pyrophosphate, and a mixture of protease inhibitors (Complete, Roche)) and disrupted by brief sonication. The resulting homogenates were centrifuged at 400,000 × g for 15 min at 4 °C. Supernatants (~10 mg of protein/ml) were supplemented with 150 mM NaCl and 10 mM MgCl₂ and used as a source of kinases. HsPI3K-C2α immunoprecipitates were mixed with supernatants in a final volume of 100 μl, and phosphorylation assays were initiated by adding 1 μM ATP. Assays were carried out at 30 °C for 1 h and terminated by the addition of ice-cold Tris-buffered saline containing 0.1% of Triton X-100. HsPI3K-C2α immunoprecipitates were collected by centrifugation, washed twice with Tris-buffered saline, and analyzed by Western blotting as described above.

For in vitro phosphorylation GST-AHsPI3K-C2α fusion proteins were expressed in Escherichia coli strain (BL21) and purified by absorption to glutathione-Sepharose beads (Amersham Biosciences). Fusion proteins were left attached to the beads, and phosphorylation reactions with cellular extracts were carried out as described above in the presence of 50 μCi of [γ-³²P]ATP and 1 μM ATP. In vitro phosphorylation of GST-AHsPI3K-C2α and GFP-HsPI3K-C2α fusion proteins by 10 units of purified recombinant human Cdc2-cyclin B (Calbiochem) was performed in Cdc2-kinase buffer (50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 10 mM MgCl₂, 1 mM EGTA) in the presence of 10 μCi of [γ-³²P]ATP and 100 μM ATP. Soluble GST-AHsPI3K-C2α was used as substrate in phosphorylation assays with immunoprecipitated Cdc2. Cdc2 was immunoprecipitated from cytosols of HeLa cells as described above, immunocomplexes bound to GammaBind Plus-Sepharose beads were additionally washed twice in the kinase buffer, and the reaction was initiated by addition of purified GST-AhSsPI3K-C2α and ATP (100 μM ATP, 10 μCi of [γ-³²P] ATP).

Immunofluorescence—Immunofluorescence experiments were performed as described previously (1) using the methanol fixation protocol.

RESULTS

UV-induced and Cell Cycle-dependent Phosphorylation of HsPI3K-C2α—Our previous immunofluorescence studies revealed that in interphase HeLa cells HsPI3K-C2α is localized to nuclear speckles together with the components of the splicing apparatus (1). Inhibition of transcription by actinomycin D or α-amanitin causes subnuclear relocation of the kinase and is accompanied by phosphorylation of the protein, which can be measured as mobility shift of HsPI3K-C2α during SDS-PAGE.

To investigate whether down-regulation of transcription caused by different types of genotoxic stress results in phosphorylation of HsPI3K-C2α, we exposed cells to DNA-damaging treatment such as UV light or ionizing radiation. Exposure of HeLa and MCF7 cells to UV irradiation induced a collapse of nuclear HsPI3K-C2α-positive speckles (Fig. 1A) similar to that observed in actinomycin D-treated cells: speckles lose their irregular shape, become round, and fuse into larger clusters (1). This effect was associated with the increased phosphorylation of HsPI3K-C2α, as measured by its mobility shift on SDS-polyacrylamide gels (Fig. 1B). When cell extracts were treated with λ-phosphatase the appearance of the slower migrating band was abolished (not shown). In contrast to UV-treated cells, exposure of cells to γ-irradiation neither changed subnuclear localization of HsPI3K-C2α (not shown) nor induced its phosphorylation (Fig. 1B). These results suggest that HsPI3K-C2α specifically participates in UV-induced damage response.

The observation that phosphorylation of HsPI3K-C2α correlates with changes in its subnuclear localization let us to speculate that the phosphorylation status of the kinase may also be cell cycle-dependent, because in mitotic cells HsPI3K-C2α-positive speckles dissolve, and the kinase becomes equally distributed over the cytoplasm (Fig. 1A). We used HeLa cells to examine whether HsPI3K-C2α demonstrates different phosphorylation patterns during the cell cycle. Cells were synchronized in different stages of the cell cycle as follows: at late G₁ with mimosine, at M with nocodazole, in early S phase by serum deprivation followed by an aphidicolin block, and cells enriched in G₂ phase were obtained 8 h after release from aphidicolin block (26). Proteins from corresponding cell lysates were fractionated by SDS-PAGE, and HsPI3K-C2α was analyzed by immunoblotting (Fig. 2A). In mimosine-treated cells HsPI3K-C2α was detected as a single band, in cells blocked in S phase a second slower migrating band became visible. Two bands, a faster and a slower migrating, of equal intensity were apparent in cells enriched in G₂ phase. A single slower migrating band was found in prometaphase-blocked mitotic cells. To confirm that altered gel mobility of HsPI3K-C2α was the result of phosphorylation, protein extracts from synchronized HeLa cells were treated with λ-phosphatase (Fig. 2). Phosphatase treatment resulted in the collapse of the slower migrating band of the kinase, indicating that indeed retarded mobility of HsPI3K-C2α during SDS-PAGE is a consequence of phosphorylation. These results demonstrate that HsPI3K-C2α undergoes a cell cycle-regulated phosphorylation that reaches its maximum in mitosis.
Can Be Phosphorylated in Vitro by Kinases Present in HeLa Cell Extracts—The initial strategy to identify residues on which HsPI3K-C2α becomes phosphorylated upon UV irradiation and during cell cycle was to label HeLa cells metabolically in the presence of $^{32}$P. In several attempts we did not succeed to obtain sufficient amounts of $^{32}$P-labeled HsPI3K-C2α by immunoprecipitation to perform phosphopeptide mapping analysis. To overcome this problem, we developed an in vitro phosphorylation assay that allowed the identification of potential phosphorylation sites. As a substrate for phosphorylation we used HsPI3K-C2α fusion protein, which included the region between amino acids 240 and 275 of HsPI3K-C2α, was used for in vitro phosphorylation. Recombinant proteins were bound to glutathione-Sepharose, and the phosphorylation assay was carried out as described above. Proteins were separated on SDS-11% polyacrylamide gels and visualized by Coomassie Blue staining (lower panel).

Fig. 2. Phosphorylation of HsPI3K-C2α during the cell cycle. A, HeLa cells were synchronized in different phases of the cell cycle. Cells enriched in late G1, were obtained by treatment with mimosine (G1); serum starvation followed by aphidicolin block yielded cells in S phase (S); cells enriched in G2 phase were obtained 8 h after release from aphidicolin block (G2); mitotic cells were obtained after nocodazole arrest (M). Cellular extracts were either not or with λ-phosphatase (λ PPase), separated on SDS-6% PAGE, and Western blots were probed with anti-HsPI3K-C2α antibody AXIII B, in vitro phosphorylation of HsPI3K-C2α with cellular extracts. Immunoprecipitated HsPI3K-C2α immobilized on GammaBind Plus-Sepharose was incubated with extracts prepared from either asynchronously grown (A) or mitotic (M) HeLa cells in the presence of 1 mM ATP at 30 °C for 1 h. As a control for autophosphorylation, immunoprecipitated HsPI3K-C2α was incubated under the same conditions without cell extract. When indicated, HsPI3K-C2α immunoprecipitates were treated with λ-phosphatase (λ PPase) after termination of the phosphorylation assay. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-HsPI3K-C2α antibody AXIX (upper panel). GST-ΔHsPI3K-C2α fusion protein, which included the region between amino acids 240 and 275 of HsPI3K-C2α, was used for in vitro phosphorylation. Recombinant proteins were bound to glutathione-Sepharose, and the phosphorylation assay was carried out as described above. Proteins were separated on SDS-11% polyacrylamide gels and visualized by Coomassie Blue staining (lower panel).
In vivo phosphorylation of HsPI3K-C2α by Cdc2—The G2/M transition of the cell cycle is triggered by activation of a protein kinase cascade. The major kinase, p34\(^{cdc2}\), required for promoto...
Fig. 5. In vivo phosphorylation of HsPI3K-C2α is sensitive to inhibition of Cdc2 activity by roscovitine. A, HeLa cells synchronized in late S were treated or not with 30 μM roscovitine for 2 h, nucodazole was then added, and the treatment continued for 15 h. Adherent cells (G0) and nonadherent mitotic (M) cells were separately harvested and analyzed. Comparable amounts of protein from each sample were separated by SDS-PAGE. B, analysis of protein extracts prepared from nucodazole-arrested mitotic HeLa cells that were treated with 75 μM roscovitine for 15, 45, and 90 min. C, nucodazole-arrested mitotic HeLa cells treated with 0.5 μM okadaic acid for 30 min or left untreated prior to addition of 75 μM roscovitine for 30 min. Cell extracts were analyzed by SDS-PAGE followed by immunoblotting with anti-HsPI3K-C2α antibody AXXIII.

Phosphorylation of HsPI3K-C2α in G2 phase. Immunoblot analysis revealed that roscovitine-treated cells were nonadherent mitotic cells, arrested in mitosis. In contrast, only approximately 10% of roscovitine-treated cells were nonadherent mitotic cells, whereas the majority remained adherent because of the arrest in G2 phase. Therefore we performed pulse-chase experiments to determine whether HsPI3K-C2α, which was phosphorylated in mitosis, becomes dephosphorylated or degraded upon reentry of cells into subsequent G1 phase. Fig. 7A (left panel) shows that HsPI3K-C2α metabolically labeled with [35S]methionine during interphase becomes fully phosphorylated and remains stable in prometaphase-arrested cells. However, upon release of mitotic cells from the nucodazole block, the level of the [35S]-labeled phosphorylated HsPI3K-C2α decreases. Concomitantly unphosphorylated kinase appears, which is not labeled with [35S]methionine, and therefore represents de novo synthesized protein (Fig. 7A, right panel). To confirm that HsPI3K-C2α is indeed newly synthesized at the G1 transition of the cell cycle, unlabeled mitotic cells were released from nucodazole block in the presence of [35S]methionine (Fig. 7B). Progression into G1 phase resulted in the appearance of the unphosphorylated [35S]methionine-labeled form of HsPI3K-C2α.

To determine whether mitotic destruction HsPI3K-C2α is caused by the proteasome activity, the proteasome inhibitor MG132 (29) was added to mitotic cells during the release from nucodazole block. Fig. 7C shows that addition of MG132 caused significant stabilization of the phosphorylated form of the kinase as well as stabilization of cyclin B1, a known target of proteasome degradation in late mitosis. These results suggest that M/G1 transition of the cell cycle triggers a proteasome-dependent degradation of phosphorylated HsPI3K-C2α.
Fig. 6. HsPI3K-C2α is directly phosphorylated on Ser259 by Cdc2 kinase in vitro. A, purified GST-HsPI3K-C2α was subjected to in vitro phosphorylation with Cdc2 kinase immunoprecipitated with anti-Cdc2 antibody from HeLa cells that were asynchronously grown (A), synchronized in G1 phase (G2), and arrested in mitosis (M). When indicated (+), samples were pretreated with 10 μM roscovitine for 20 min before the addition of [γ-32P]ATP. The top panel shows the autoradiograph of [32P] incorporation into GST-HsPI3K-C2α; the corresponding Coomassie Blue-stained SDS-polyacrylamide gel is depicted in the middle panel. The amount of Cdc2 present in the phosphorylation assays was analyzed by immunoblotting (bottom panel). B and C, mutation of Ser259 abrogates in vitro phosphorylation of HsPI3K-C2α by Cdc2 kinase. B, wild-type GST-HsPI3K-C2α (Wt) and the mutant GST-HsPI3K-C2α/S259A (S259A) and GST-HsPI3K-C2α/S259D (S259D) fusion proteins were incubated with purified Cdc2-cyclin B (10 units) for 40 min in the presence of [γ-32P]ATP. Proteins were separated on SDS-polyacylamide gel and visualized by autoradiography (32P). C, full-length wild-type HsPI3K-C2α (Wt) and the mutant HsPI3K-C2α/S259A (S259A) and HsPI3K-C2α/S259D (S259D) were transiently expressed as GFP fusion proteins in COS-7 cells. GFP fusion proteins were immunoprecipitated with anti-GFP antibody from cell extracts and used as substrates for in vitro kinase assays with purified Cdc2-cyclin B as described above. After SDS-PAGE and electrophoresis, phosphorylated proteins were visualized by autoradiography (32P) and immunoblotted with anti-HsPI3K-C2α antibody (WB).

HsPI3K-C2α-GFP and HsPI3K-C2α/S259A-GFP (Fig. 8C) gave similar results, showing that UV-induced phosphorylation of HsPI3K-C2α-GFP at Ser259 is sensitive only to SP600125.

To examine further whether JNKs are implicated in the phosphorylation of HsPI3K-C2α, we tested whether the transient expression of JNKK2/MKK7, a specific JNK-activating MAPKK, influences the steady-state level of the phosphorylation of HsPI3K-C2α. Transfection of JNKK2 into HEK-293 cells stably expressing HsPI3K-C2α-GFP or HsPI3K-C2α/S259A-GFP resulted in a significant increase of the steady-state level of phosphorylated HsPI3K-C2α-GFP, which was close to that seen in UV-irradiated cells. As expected, the mutant HsPI3K-C2α/S259A-GFP failed to show any change in the electrophoretic mobility upon UV irradiation (Fig. 9, upper panel). The efficiency of JNK activation caused by transfection of JNKK2 was similar in both cell lines as judged by the increase in c-Jun phosphorylation at Ser73 (Fig. 9, lower panel).

UV Irradiation Induces Phosphorylation-dependent Degradation of HsPI3K-C2α—Our observation that mitotic phosphorylation of HsPI3K-C2α was followed by its degradation at the M to G1 transition of the cell cycle led us to speculate that phosphorylation at Ser259 might be a common signal required to activate HsPI3K-C2α proteolysis. Therefore, we examined the rate of disappearance of phosphorylated HsPI3K-C2α after UV irradiation. HEK-293 cells expressing HsPI3K-C2α-GFP or HsPI3K-C2α/S259A-GFP were exposed to UV light and allowed to recover for 2–24 h. Whole cell extracts from equivalent numbers of cells were prepared and analyzed on Western blots. Fig. 10A illustrates that the slower migrating phosphorylated forms of HsPI3K-C2α and HsPI3K-C2α-GFP are induced within 2 h after UV irradiation and diminish thereafter until they become barely detectable after 24 h (compare recovery time between 2 and 24 h). In contrast, the levels of faster migrating unphosphorylated forms of these proteins remain unaffected during the recovery period. Over the time course after UV irradiation the levels of both HsPI3K-C2α and HsPI3K-C2α-GFP diminish by about 50% compared with those detected in unirradiated cells. Phosphorylation-deficient HsPI3K-C2α/S259A-GFP remains stable after UV irradiation (Fig. 10A), suggesting that mutation of Ser259 to alanine protects the protein from proteolysis. We used the reversible inhibitor MG132 to demonstrate that the reduction of the levels of phosphorylated HsPI3K-C2α and HsPI3K-C2α-GFP is caused by subsequent degradation by the proteasome. MG132 is a potent inhibitor of the proteasome but also inhibits calpains. We therefore examined the effects of additional protease inhibitors on the HsPI3K-C2α turnover. As shown in Fig. 10B, treatment of cells with MG132 (29) or with lactacystin, an irreversible inhibitor specific for proteasome (39, 40), for 16 h after UV irradiation resulted in a significant stabilization of the phosphorylated forms of HsPI3K-C2α and HsPI3K-C2α-GFP. Conversely, ALLM, a peptide aldehyde that is more selective for calpain than to the proteasome (41), caused only marginal effects. Neither of the inhibitors affected stability of HsPI3K-C2α/S259A-GFP.
**DISCUSSION**

It has been reported that class II PI 3-kinases can be phosphorylated (1, 11, 24). However, identities of phosphorylation site(s) and the kinase(s) involved remained elusive. It has been also not clear how phosphorylation modulates the properties of these enzymes. Here we investigated cell cycle-dependent and genotoxic stress-induced phosphorylation of HsPI3K-C2α and demonstrated that HsPI3K-C2α becomes phosphorylated on Ser259 in cellular response to UV irradiation and in dividing cells during mitosis. Phosphorylation on Ser259, which is located next to a proline, induces a conformational change in the phosphorylated form of the kinase which leads to the decrease in its electrophoretic mobility on SDS-polyacrylamide gels.

The phosphorylation status of HsPI3K-C2α during the cell cycle is under the direct control of Cdc2. Several lines of evidence support this conclusion. First, marginal phosphorylation of HsPI3K-C2α is detected during interphase, whereas the phosphorylated form of the kinase appears at the G1/S transition phase and reaches a maximum in mitosis, which coincides with the timing of Cdc2 activation. Second, mitotic phosphorylation of HsPI3K-C2α in both in vivo and in vitro assays was sensitive to roscovitine, a highly selective inhibitor of cyclin-dependent kinases (27, 28). Several cyclin-dependent kinases, including Cdc2-cyclin B and Cdk2-cyclin A or E, are sensitive to roscovitine. However, the main target of roscovitine in mitosis is Cdc2-cyclin B because Cdk2-cyclin E and Cdk2-cyclin A kinases are active at the G1/S transition and during S phase, respectively. Furthermore, purified activated Cdc2/cyclin B was able to phosphorylate HsPI3K-C2α fusion proteins on Ser259 in vitro. Mutations of Ser259 to either alanine or asparagine abolished Cdc2-mediated phosphorylation of HsPI3K-C2α.

**Fig. 7.** Phosphorylated HsPI3K-C2α is degraded at the M/G1 transition of the cell cycle. A, asynchronously growing HeLa cells were labeled with [35S]methionine/cysteine overnight (A) and thereafter incubation was continued in [35S]-free medium for 48 h, norepin was added to the medium for the last 36 h of chase to obtain mitotic cells (M). Labeled mitotic cells (M) were released from norepine block and added to the medium for the last 36 h of chase to obtain mitotic cells in medium containing 0.01% Me2SO or 20 μM HeLa cells (M) were released from norepine block into the fresh medium containing [35S]methionine/cysteine for 1 h and 3 h. Subsequent interphase. Unlabeled mitotic nocodazole-arrested HeLa extracts were analyzed by immunoblotting with anti-HsPI3K-C2α antibody and anti-c-Jun (pSer73) antibody.

**Fig. 8.** Inhibition of UV-induced phosphorylation of HsPI3K-C2α on Ser259 by the JNK inhibitor SP600125, but not by inhibitors of MEK1 and p38 kinase. Cells were pretreated with 50 μM PD98059 or 10 μM SB202190 (A) or with the indicated concentrations of SP600125 (B) for 30 min, afterward UV irradiated (300 J/m²), and then cultured in the presence of the inhibitors for 90 min. Cell extracts were analyzed by SDS-PAGE followed by immunoblotting with anti-HsPI3K-C2α antibody AXXIII and anti-c-Jun (pSer259) antibody. C, HEK-293 cells that stably expressed full-length wild-type HsPI3K-C2α or mutant HsPI3K-C2α S259A as GFP fusion proteins were pretreated with the indicated inhibitors for 30 min, UV irradiated, and analyzed as described above.

**Fig. 9.** JNKK2 overexpression stimulates the basal level of phosphorylation of HsPI3K-C2α on Ser259. Stable clones of HEK-293 cells expressing either HsPI3K-C2α-GFP (Wt) or HsPI3K-C2α S259A-GFP (S259A) were transiently transfected with JNKK2 expression vector (JNKK2) or empty vector (mock). 24 h after transfection, cells were left untreated (−) or UV irradiated (+). Cell extracts were analyzed by SDS-PAGE followed by immunoblotting with anti-GFP antibody and anti-c-Jun (pSer397) antibody.
in vitro. In addition, Cdc2 activity appears to be indispensable for maintaining phosphorylation of HsPI3K-C2α during mitosis, probably by antagonizing an okadaic acid-sensitive phosphatase.

In interphase cells HsPI3K-C2α is concentrated in nuclear speckles (1), which represent a subnuclear compartment enriched in small nuclear ribonucleoprotein particles and other splicing factors. In mitosis, at the end of prophase the breakdown of the nuclear envelope is accompanied by the disassembly and dispersal of all major nuclear structures and down-regulation of transcription and splicing. Following the changes in the nuclear structure, at the metaphase-anaphase transition HsPI3K-C2α is found to disperse throughout the cytoplasm concomitantly with its complete phosphorylation. A mechanism for mitotic repression of transcription is phosphorylation, which leads to inactivation of transcription factors and causes their release from mitotic chromatin (42, 43). Whether phosphorylation of HsPI3K-C2α in early mitosis plays a similar role and controls the enzyme activity in vivo is not clear. The finding that in vitro lipid kinase activity of mitotic, fully phosphorylated HsPI3K-C2α is similar to that isolated from interphase cells suggests that phosphorylation per se does not alter the activity of the enzyme. In addition, the activity of phosphorylation-deficient mutants (S259A or S259D) of HsPI3K-C2α is similar to that of the wild-type enzyme. Therefore, it is conceivable that phosphorylation of HsPI3K-C2α induces modifications in protein-protein or protein-nucleic acid interactions which affect localization of HsPI3K-C2α and perhaps prevent the kinase from reaching potential substrates.

Analysis of the protein turnover suggests that mitotic phosphorylation on Ser259 indirectly controls the activity of HsPI3K-C2α by facilitating its degradation at the M/G1 transition of cell cycle. Several examples of regulated proteolysis have been characterized. In many cases, modification of the substrate by phosphorylation provides a recognition signal for specific E3 ubiquitin-protein ligases, followed by subsequent substrate by phosphorylation provides a recognition signal for specific E3 ubiquitin-protein ligases, followed by subsequent ubiquitination which targets the substrate for degradation via the proteasome. Cell cycle-regulated proteolysis in anaphase depends on anaphase-promoting complex, a multisubunit ubiquitin ligase (E3) (45). Targets of the anaphase-promoting complex contain destruction boxes necessary for ubiquitination-mediated proteolysis (46). Sequences which could represent putative destruction boxes (RXxxL) are found in HsPI3K-C2α, one of them in close vicinity to the phosphorylation site Ser259.

In addition, a computer-assisted sequence analysis revealed a putative PEST motif (amino acids 519–531). PEST sequences, which are found in numerous short lived proteins, are assumed to target proteins for degradation via the proteasome, although the exact mechanism is unclear (47). We are currently investigating whether HsPI3K-C2α is a substrate for ubiquitination and which domains of the protein are important for degradation.

Further studies are necessary to resolve the concise function of HsPI3K-C2α in cell cycle progression. Our findings suggest that elimination of the kinase at the exit from mitosis could be necessary to ensure a proper entry into subsequent G1 phase. This view is consistent with our observation that overexpression of wild-type HsPI3K-C2α and its phosphorylation-deficient mutants (S259A and S259D) in COS-7 and Chinese hamster ovary cells leads to mitotic defects such as multipolar spindle assembly, which results in aberrant cytokinesis and formation of multinucleated cells. A similar effect of defective

---

* S. A. Didichenko and M. Thelen, unpublished data.
cytokinesis was observed in cells expressing constitutively active class I PI 3-kinase (p110CAAX) (48). Thus, it is tempting to speculate that down-regulation of PI 3-kinase activity could be an essential step for execution of the mitotic program.

Cellular responses to UV irradiation include the activation of MAP kinase signaling pathways. By using selective inhibitors of different MAP kinases, we demonstrate that UV-induced phosphorylation of HsPI3K-C2α on Ser259 appears to be dependent on the activation of JNK signaling pathway and does not involve ERKs and p38. Accordingly, ectopic expression of JNK1/2, which is dependent on the activation of JNK signaling pathway and does not involve ERKs and p38, corroborates the involvement of JNKs in UV irradiation-induced phosphorylation. UV responses mediated by JNK include transcriptional output in the nucleus (49, 50) and antiapoptotic signaling events in the cytoplasm (51, 52). Prominent changes in the subnuclear localization of HsPI3K-C2α after UV irradiation and the observation that only a fraction of HsPI3K-C2α is accessible to phosphorylation suggest that JNK might target only nuclear HsPI3K-C2α.

Similar to mitosis, UV-induced phosphorylation serves as a signal for activation of proteasome-dependent HsPI3K-C2α proteolysis. This conclusion is supported by the following observations: (i) there is an apparent preferential disappearance of phosphorylated form of HsPI3K-C2α over time after UV irradiation; (ii) treatment of UV-irradiated cells with proteasome inhibitors resulted in a significant stabilization of the phosphorylated form; (iii) the level of phosphorylation-deficient mutant GFP-HsPI3K-C2α/S259A remained unaffected after UV irradiation.

It is well known that RNA synthesis is down-regulated in response to DNA damage to allow transcription-coupled repair. Inhibition of transcription caused by DNA-damaging agents, such as α-amanitin, actinomycin D, cisplatin, and UV irradiation, leads to the degradation of the polymerase II LS (53, 54). The irreversible disassembly of transcription complexes as consequence of the degradation of polymerase II LS has been proposed as a mechanism for down-regulation of transcription (55). UV irradiation, similar to α-amanitin and actinomycin D treatment, leads to morphological changes of HsPI3K-C2α-positive speckles and induces its phosphorylation, suggesting that these events are linked to transcriptional repression. The fact that HsPI3K-C2α follows the fate of the polymerase II LS and is degraded upon cell exposure to UV irradiation could therefore reflect the disassembly of transcriptional complexes.

In summary, our findings suggest that the phosphorylation of HsPI3K-C2α on Ser259 is critical for its subnuclear localization and turnover and that two distinct signaling pathways phosphorylate Ser259 depending on the physiological state of the cell. Identification of proteins that associate with HsPI3K-C2α and analysis of the regulation of these interactions by phosphorylation are necessary to determine the physiological role of serine phosphorylation in HsPI3K-C2α function.
