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Securin Is a Target of the UV Response Pathway in Mammalian Cells†

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All eukaryotic cells possess elaborate mechanisms to protect genome integrity and ensure survival after DNA damage, ceasing proliferation and granting time for DNA repair. Securin is an inhibitory protein that is bound to a protease called Separase to inhibit sister chromatid separation until the onset of anaphase. At the metaphase-to-anaphase transition, Securin is degraded by the anaphase-promoting complex or cyclosome, and Separase contributes to the release of cohesins from the chromosome, allowing for the segregation of sister chromatids to opposite spindle poles. Here we provide evidence that human Securin (hSecurin) has a novel role in cell cycle arrest after exposure to UV light or ionizing radiation. In fact, irradiation downregulated the level of hSecurin protein, accelerating its degradation via the proteasome and reducing *hSecurin* mRNA translation, but the presence of hSecurin is necessary for cell proliferation arrest following UV treatment. Moreover, an alteration of UV-induced hSecurin downregulation could lead directly to the accumulation of DNA damage and the subsequent development of malignant tumors.

The segregation of sister chromatids to opposite poles of the cell during anaphase ensures that a complete set of chromosomes is transmitted from one generation to another. Sister chromatid separation is an irreversible process and is therefore tightly regulated. The separation of all sister chromatid pairs is delayed when individual chromosomes are damaged or have failed to attach to the mitotic spindle. Mistakes in such regulatory mechanisms in somatic cells are thought to contribute to the aneuploidy characteristic of many tumor cells (6), whereas defects during meiosis generate trisomies (34).

Sister chromatid cohesion is mediated by a conserved complex called cohesin, which is composed of the Scc1, Scc3, Smc1, and Smc3 proteins in the budding yeast *Saccharomyces cerevisiae* (17). The separation of sister chromatids at the metaphase-to-anaphase transition is triggered by proteolytic cleavage of the Scc1 cohesin subunit by a conserved cysteine protease called Separase (Esp1 in *S. cerevisiae*) (54). Scc1 cleavage destroys the bridge between sister chromatids and thereby enables spindle microtubules to move the sister chromatids toward opposite poles of the cell. For most of the cell cycle, Separase is bound by an inhibitor called Securin (Pds1 in *S. cerevisiae*), which is destroyed by ubiquitin-mediated proteolysis shortly before the metaphase-to-anaphase transition. Moreover, Scc1 cleavage is also regulated by the phosphorylation of Separase recognition sites by the Polo-like kinase Cdc5 (2).

In vertebrates, there are at least two cohesin complexes with different Scc3-like subunits (50). Cohesin is removed from chromosomes in two steps (57). During prophase and prometaphase, the bulk of cohesin dissociates from the arms of condensing chromosomes (31) via a mechanism that depends neither on the Securin-Separase pathway nor on the cleavage of the human ortholog of Scc1 (50). Dissociation appears to be mediated by a Polo kinase-dependent mechanism (51). However, a small amount of cohesin remains in centromeric regions until metaphase and is removed from chromosomes only at the onset of anaphase (57). At least two mechanisms prevent Separase activation, one by causing Separase phosphorylation (cyclin B/Cdk1) and the other by binding to and inhibiting the protease domain (Securin) (48).

Securin is ubiquitinated by a multisubunit ubiquitin protein ligase, the anaphase-promoting complex or cyclosome (APC/C) (8, 14, 62), whose activity is controlled by Mad2, a component of the mitotic checkpoint which ensures that all kinetochores become attached to microtubules (45). In addition, to display full ubiquitin ligase activity, APC/C must bind to the Cdc20 and Cdh1 accessory factors, which are responsible for Securin and cyclin B degradation (36). The role of Securin in Separase activity has been explained in two ways. Securin has been implicated in the subcellular localization of Separase (21, 25) and enables the full catalytic activity of Separase after the destruction of Securin in anaphase (18). That Securin regulates chromatid separation during cell division suggests that aneuploidy caused by defective sister chromatid separation is involved in tumor development (20, 27). In fact, Securin is highly expressed in many tumors that have been analyzed (10, 40, 41).

In *S. cerevisiae*, Securin not only is required for efficient chromosome segregation but also is needed to prevent anaphase in response to spindle and DNA damage (58, 59). In fact, surveillance mechanisms that sense DNA damage arrest cell cycle progression by causing the stabilization of Pds1, thereby blocking sister chromatid separation (52). Moreover, spindle damage blocks sister chromatid separation solely by inhibiting Cdc20-APC/C-dependent Pds1 proteolysis (3). On the other hand, in mammalian cells, Securin interacts with the regulatory subunit of the DNA-dependent protein kinase,

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† This article is dedicated to the memory of Zen. He will live forever in our hearts.

which is involved in nonhomologous end joining (13, 28); this fact suggests that Securin may connect DNA damage response pathways with sister chromatid separation, delaying the onset of mitosis while DNA repair occurs (39).

To identify the mechanisms that involve human Securin (hSecurin) in DNA damage, we studied the effect of UV light on hSecurin. Human cells responded to UV light by rapid proteasome-dependent hSecurin degradation caused by unexpected APC/C activation after UV-induced DNA damage and by specific hSecurin protein synthesis inhibition. Moreover, here we show that hSecurin plays a role in the cellular response to UV radiation, being necessary for cell proliferation arrest after UV treatment.

MATERIALS AND METHODS

Plasmids, cloning, and sequencing. pCDNA3/*hSecurin-VSV*, including a Kozak region, the full-length *hSecurin* cDNA, and the vesicular stomatitis virus (VSV) tag, and pCDNA3/*HA-hSecurin-HA*, including the full-length *hSecurin* cDNA flanked by two hemagglutinin (HA) tags, were obtained by PCR according to Perkin-Elmer's protocol. pEF/*Securin* and pEF/*Securin KAA-DM* were provided by M. Brandeis (Department of Genetics, Silberman Institute of Life Sciences) (63). The 5′ untranslated region (UTR) of *hSecurin* (70 bp upstream of ATG) was identified by primer extension and RNA protection assays (J. A. Pintor-Toro, unpublished results) and cloned by PCR upstream of VSV-tagged *hSecurin* in vector pCDNA3. The region from nucleotide −778 to nucleotide −1 of the *hSecurin* gene (including the minimal region with promoter activity and the *hSecurin* 5′ UTR) was cloned in vector pXP2, a promoterless luciferase reporter vector (35), to obtain pXP2-0.7kb. Sequencing of inserts from cloning vectors was performed on both strands with an automatic sequencer (Amersham Biosciences) by using the dideoxy termination method of Sanger et al. (43).

Cell culture and lysis. The HCT116 human colon carcinoma cell line and a derivative cell line, HCT116 *hSecurin*$^{-/-}$, in which both *hSecurin* alleles have been deleted through homologous recombination (20), were kindly provided by B. Vogelstein (The John Hopkins Oncology Center). The HCT116 cell lines and the Cos-7 and HeLa cell lines (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (the last three supplements from BioWhittaker) in a 5% CO_2 humidified atmosphere at 37°C. The HL-60 cell line (American Type Culture Collection) was grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Stably transfected NIH 3T3 cells with isopropyl-β-D-thiogalactopyranoside (IPTG) regulating the expression of *hSecurin* (5) were maintained at 37°C under 5% CO_2 in monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated newborn calf serum (Gibco), 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. When needed, these cells were treated with 2 mM IPTG (Sigma) diluted in water and added directly to cell media.

Cell lysis was performed with 10^7 to 10^8 cells/ml at 4°C in 150 mM NaCl– 10 mM Tris-HCl (pH 7.5)– 1% Nonidet P-40 (NP-40)– 10% glycerol– 1 mM Na vanadate, 20 mM Na pyrophosphate, 5 mM Na fluoride, 1 mM phenylmethylsulfonyl fluoride– 1 μg of aprotinin/ml– 1 μg of pepstatin/ml– 1 μg of leupeptin/ml– 10 μg of chymostatin/ml (lysis buffer) for 20 min. The extract was centrifuged at 20,000 × *g* for 20 min, and the supernatant was frozen in liquid nitrogen and stored at −80°C. The protein concentration was determined by using the Bradford assay (Bio-Rad Laboratories). To obtain a sonicated total extract, cells were incubated in sodium dodecyl sulfate (SDS) sample buffer, sonicated, and heated at 95°C for 5 min.

Cell synchronization and drugs. HeLa cells enriched in G_1, S, G_2, or M phase were obtained as previously described (22). HeLa G_1 cells were obtained by 16 h of incubation in 6 mM butyrate (Sigma). HeLa G_1/S cells were obtained by a double thymidine block (i.e., two 16-h incubations in 2.5 mM thymidine [Sigma], with an 8-h release). Cells enriched in S phase were harvested 4 h after release from the second block. Cells harvested 8 h after this release were further enriched for the G_2 population by extensive rinsing to remove mitotic cells. Synchronous mitotic cells were obtained by 16 h of incubation in medium containing 5 μM nocodazole. The purity of the phases was confirmed by flow cytometry.

For some experiments, cells were pretreated with the cell-permeating caspase inhibitors Z-VAD-FMK (20 μM; Sigma) and Ac-DEVD-CHO (100 μM; Sigma), the proteasome and calpain inhibitors Ac-LLnL-CHO (100 μM; Sigma) and Z-LLnV (50 μM; Sigma), or other inhibitors involved in different signaling pathways: wortmannin (phosphatidylinositol 3-kinase, ATM, and DNA-dependent protein kinase [DNA-PK] inhibitor; 5 μM; Sigma), UCN-01 (ATR/Chk1 inhibitor; 1 μM; supplied by the Division of Cancer Treatment and Diagnosis, National Cancer Institute), PD98059 (MEK1 inhibitor; 50 μM; Sigma), SB202190 (p38 inhibitor; 2 μM; Calbiochem), SP600125 (c-Jun N-terminal kinase inhibitor; 25 μM; Calbiochem), caffeine (10 mM; Sigma), cycloheximide (25 μg/ml; Sigma), and aphidicolin (1 μM; Sigma).

Transient transfection and half-life experiments. Constructions containing HA- or VSV-tagged hSecurin as well as wild-type or KAA-DM mutant Securin were transfected by electroporation into Cos-7 cells. At 18 to 24 h posttransfection, cells were harvested or irradiated and later collected. Staining with appropriate antibodies assessed the overexpression of proteins.

Half-life experiments were performed by transient transfection of Cos-7 cells. Expression of the transgene was induced for 18 h. At 1 h after irradiation, cycloheximide was added to the media of control and irradiated cells. Cells were harvested at various times.

UV irradiation and X-irradiation. For UV irradiation, cells were plated on 100-mm culture dishes containing growth medium. After 18 to 24 h, the medium was reduced to 3 ml/dish, and culture dishes were uncovered in a UV cross-linker (model UVC-500; Hoefer). UV irradiation was carried out with 100 J/m^2 (or with various other dosages). Following irradiation, 2 ml of growth medium was added, and the cells were incubated at 37°C for 1 h 30 min (or for various other times) in a CO_2 incubator. For some experiments, cells were pretreated with various drugs.

For X-irradiation, cells were exposed to 25 Gy of X rays and collected 1 h later. Irradiation was performed with a Philips MG 103/2.25 X-ray apparatus emitting at a fixed dose of 0.96 Gy/min, as determined by dosimetry.

Luciferase assays. Cos-7 cells were cotransfected by the DEAE-dextran technique with 1 μg of pXP2 or pXP2-0.7kb and pRL-*Renilla* (50 ng). After 48 h, cells either were left untreated or were UV irradiated (100 J/m^2) as described above. After 1 h 30 min at 37°C, cells were lysed and proteins were quantified by the Bradford assay. Equal amounts of proteins were used for the determination of luciferase activities with a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was determined in triplicate and expressed as a percentage of the expression of pXP2-0.7kb in nonirradiated cells after normalization to the *Renilla* luciferase activity to correct for variations in transfection efficiency.

Measurement of viable cells by trypan blue exclusion. At various time points after treatment, adherent cells were trypsinized and collected together with cells floating in the media. Viable, trypan blue-excluding cells were counted by using a hemocytometer. At least five independent experiments were performed.

Flow cytometry assessment of the cell cycle. At various time points after treatment, adherent cells were trypsinized and collected together with floating cells. A total of 10^6 cells were divided into aliquots and fixed in 70% ethanol for flow cytometry. The remaining cells were processed for protein analysis (see below). Propidium iodide staining of nuclei was performed with a CycleTest Plus DNA reagent kit (Becton Dickinson), and the DNA content was measured with a FACScan instrument (Becton Dickinson). Data were acquired with CellQuest software (Becton Dickinson). ModFit LT2 software (Verity Software House) was used to assess cell cycle status. A total of 20,000 events were analyzed for each sample. Cell cycle and apoptosis were monitored for up to 6 days from the start of treatment.

BrdU incorporation assay. Wild-type and *hSecurin*$^{-/-}$ HCT116 cells were exposed or not exposed to UV radiation (30 J/m^2), labeled for 30 min with 10 μM bromodeoxyuridine (BrdU), harvested, and fixed at 4°C at various times. An in situ cell proliferation kit (Roche Applied Science) was used to detect BrdU incorporated into cellular DNA by flow cytometry. Fluorescence data were displayed as dot plots by using CellQuest software.

Apoptosis assays. For fluorescein isothiocyanate (FITC)-linked annexin V-propidium iodide staining (55), cells were washed with ice-cold phosphate-buffered saline and resuspended in binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 10^6 cells/ml. Five microliters of annexin V-FITC and 10 μl of propidium iodide (50 μg of stock/ml in phosphate-buffered saline) were added to 100 μl of the cell suspension. Cells were gently mixed and incubated in the dark for 15 min. Next, 400 μl of binding buffer was added to each sample prior to analysis by flow cytometry within 1 h. Data were analyzed by using CellQuest software.

For in situ detection of apoptosis, cells were fixed in buffered formalin, pelleted, and embedded in paraffin blocks. Five-micrometer sections were dewaxed and incubated with monoclonal antibody M30 (M30 CytoDeath; Roche Applied Science), which recognizes a specific caspase cleavage site within cytokeratin 18 as an early apoptotic event (26). M30 immunoreactivity was detected by using a streptavidin-biotin-peroxidase method (DakoCytomation) and 3,3′-diaminoben-

zidine as the chromogenic substrate. More than 2,000 cells per block were scored to quantitate M30 immunostaining.

Electrophoresis and Western blot analysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and gels were electroblotted onto nitrocellulose membranes and probed with various antibodies. Immunodetection of Grb2 was used as an internal control for equal loading of the proteins in the gels. Peroxidase-coupled donkey anti-rabbit immunoglobulin G and sheep anti-mouse immunoglobulin G were obtained from Amersham Biosciences. Immunoreactive bands were visualized by using an enhanced chemiluminescence Western blotting system (Amersham Biosciences) according to the manufacturer's protocol.

Antibodies. Proteins were detected with anti-hSecurin polyclonal antibody (10), anti-hSeparase monoclonal antibody (57) (provided by J.-M. Peters, Research Institute of Molecular Pathology), anti-Grb2 polyclonal antibody (Santa Cruz), anti-HA monoclonal antibody (Roche Applied Science), and anti-VSV monoclonal antibody (Sigma).

Northern blotting. RNA was extracted by the guanidium thiocyanate procedure. After precipitation in ethanol at −20°C, RNA pellets were resuspended in water. RNA concentrations were determined by optical density measurements, and samples were frozen at −80°C until use. RNA samples (10 μg of total RNA) were denatured in glyoxal, dimethyl sulfoxide, and phosphate buffer at 60°C for 10 min, size fractionated by electrophoresis through 1% agarose gels, and transferred to nylon membranes. Membranes were hybridized with ^{32}P-labeled *hSecurin* cDNA and washed under stringent conditions. In addition, membranes were also stripped and rehybridized to a human β-*actin* cDNA control probe.

Coimmunoprecipitation experiments. Cellular lysates (1 to 2 mg) were incubated with preimmune serum for 30 min and protein A-Sepharose beads (Amersham Biosciences) for 1 h at 4°C. After centrifugation, beads were discarded; supernatants were incubated for 2 h with anti-hSecurin polyclonal antibody or preimmune serum, followed by protein A-Sepharose beads for 1 h. Beads were washed six times with lysis buffer, and bound proteins were dissolved in SDS sample buffer at 95°C for 5 min and subjected to SDS-PAGE.

RESULTS

UV irradiation decreases hSecurin protein expression. It was previously suggested that hSecurin might be involved in the DNA damage response pathway (39), like Pds1 in *S. cerevisiae* (42). To test this possibility, we examined the effect on the hSecurin protein of exposure of proliferating human HeLa cells to UV-C light. As shown in Fig. 1A, UV irradiation resulted in a reduction in the amount of hSecurin detected by Western blotting. The same result was observed after X-ray irradiation of HL-60 cells (Fig. 2A). The kinetics of hSecurin depletion (Fig. 1B) and the effect of the dose of UV radiation (Fig. 1C) revealed that the decrease in the amount of hSecurin occurs rapidly, within 1 to 2 h after UV exposure, and in a dose-dependent manner. In fact, the application of UV radiation at 15 J/m^2 to HeLa cells covered with medium was sufficient to reduce the amount of hSecurin to 75% at 1 h after treatment (as determined by densitometric quantitation of band intensities). Steady-state amounts of other proteins, such as Grb2, remained unaffected even 10 h after irradiation with UV-C light at 50 J/m^2 (Fig. 1B) (7), indicating the specificity of the phenomenon and, consequently, acting as a loading control.

UV-mediated depletion of hSecurin is not dependent on mRNA downregulation and is dependent on the cell cycle phase. To characterize the effect of UV radiation on hSecurin, we first studied Securin protein expression in several cell lines, including HL-60, HCT116, Cos-7, and NIH 3T3, after UV irradiation, and we observed a reduction in Securin protein expression in all cell lines tested. In addition, the reduction in Securin expression was not an indirect response to global physiological effects of UV damage. We have analyzed the levels of other proteins, such as Grb2, Raf1, hnRNP C, 14-3-3, Separase, and cyclin B1, after UV irradiation and have not observed any decrease in the levels of these proteins after UV irradiation. Moreover, UV-mediated depletion of Securin did not require functional p53, because it could be observed in cell lines expressing wild-type p53 (HCT116 and NIH 3T3 cells), low levels of p53 (HeLa cells), or no p53 (HL-60 and Cos-7 cells) (Fig. 2A). To rule out the possibility that hSecurin downregulation by UV light may be a secondary effect caused by protein translocation to an insoluble extract fraction, we analyzed the sonicated total extracts of irradiated HeLa cells and nonirradiated cells, and we found that UV light again led to a reduction in hSecurin protein expression (Fig. 2A). Next, we examined the steady-state amounts of hSecurin mRNA after UV irradiation, and we found no changes even 3 h after UV irradiation (Fig. 2B). These findings indicate that the UV-induced decrease in hSecurin expression is not dependent on cell type and does not follow the downregulation of mRNA.

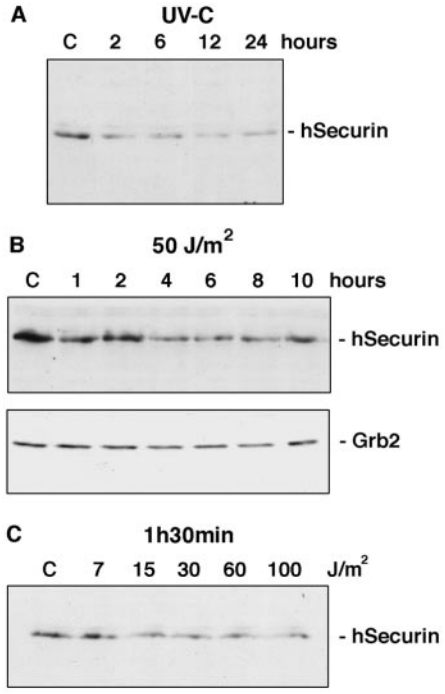

FIG. 1. Effect of UV-C on hSecurin expression. HeLa cells were exposed to UV-C radiation (A, 100 J/m^2; B, 50 J/m^2; C, as indicated) and harvested at the indicated times. Lanes C, untreated cells. Equal amounts of NP-40 extracts were resolved by SDS–12% PAGE. Immunoblotting was performed with polyclonal anti-hSecurin antibodies. Anti-Grb2 antibody was used as a control for equal loading of proteins.

It had been reported that the level of hSecurin protein is upregulated in rapidly proliferating cells, is downregulated in response to serum starvation or cell confluence, and is regulated in a cell cycle-dependent manner, peaking in mitosis (38). To determine whether hSecurin depletion in response to UV light is also cell cycle dependent, we examined the effect of UV exposure during the cell cycle. HeLa cells were biochemically arrested in G_1, S, G_2, and M phases and UV irradiated. At 1 h 30 min after UV treatment, protein samples were obtained and analyzed by immunoblotting; we found that UV light reduced hSecurin protein levels in G_1, S, and G_2 phases and left unal-

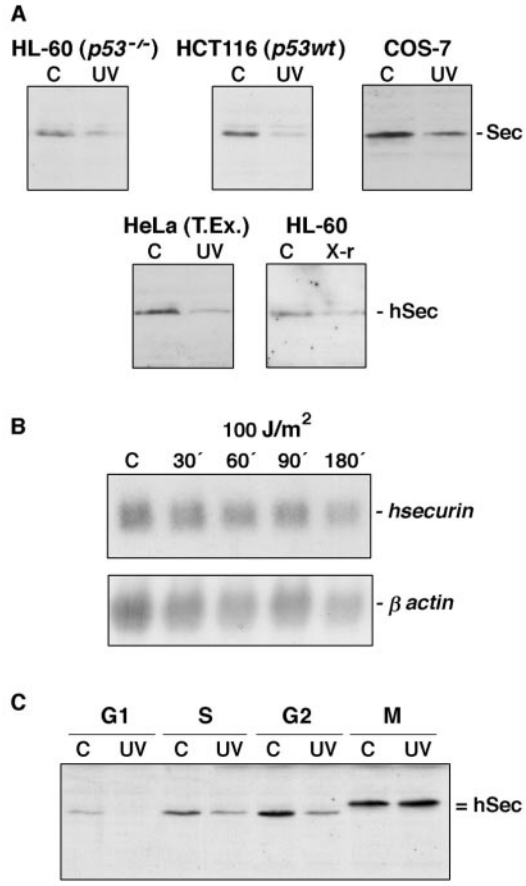

FIG. 2. UV-mediated depletion of hSecurin is independent of p53 and *hSecurin* mRNA expression but is dependent on the cell cycle. (A) Securin expression from different cell lines that were left untreated (lanes C), after UV irradiation (100 J/m^2, harvested at 1 h 30 min after irradiation) (lanes UV), or after X-ray irradiation (25 Gy, harvested at 1 h after irradiation) (lane X-r) was analyzed by Western blotting. Extracts of HeLa cells (T.Ex.) were whole-cell extracts prepared as described in Materials and Methods. *wt*, wild type. (B) Northern blot analysis of *hSecurin* mRNA expression from control (lane C) or UV-treated (100 J/m^2) HeLa cells harvested at the indicated times. Hybridization to a β-*actin* probe is also shown as a control for RNA loading. (C) HeLa cells were arrested in the indicated phases of the cell cycle and harvested (lanes C) or UV irradiated (100 J/m^2, collected 1 h 30 min later). Equal amounts of NP-40 extracts were resolved by SDS–12% PAGE and blotted with anti-hSecurin antibody.

tered the expression of hSecurin protein in nocodazole-induced M phase (Fig. 2C).

Reversal of the UV irradiation effect on hSecurin. In order to make an initial identification of the mechanism involved in hSecurin depletion, UV-treated HeLa cells were incubated in the presence of a variety of inhibitors. We found that the cell-permeating caspase inhibitors Z-VAD-FMK and Ac-DEVD-CHO were unable to inhibit hSecurin depletion (Fig. 3A). Most notably, the proteasome and calpain inhibitors Ac-LLnL-CHO and Z-LLnV increased the hSecurin protein levels under all conditions but were unable to prevent the reduction in hSecurin levels after UV irradiation (Fig. 3B). Similar results were obtained with cells pretreated with the various inhibitors before UV treatment. Next, we tested other inhibitors involved in different signaling pathways: wortmannin (phosphatidylinositol 3-kinase, ATM, and DNA-PK inhibitor), UCN-01 (ATR/Chk1 inhibitor), PD98059 (MEK1 inhibitor), SB202190 (p38 inhibitor), and SP600125 (c-Jun N-terminal kinase inhibitor). However, all of them, used as pretreatment or added at the time of UV irradiation, were unable to prevent the reduction in the amount of hSecurin (Fig. 3C). Only treatment with caffeine restored the amount of hSecurin to its original, unirradiated level (Fig. 3D). Caffeine is a compound that interferes with the activation of Chk1, increases cellular cyclic AMP (cAMP) levels, and prevents apoptosis. Nevertheless, UCN-01, a more specific Chk1 inhibitor, 8-Br-cAMP, a membrane-permeating cAMP analogue, and the caspase inhibitors were not able to prevent hSecurin diminution (Fig. 3A and C and data not shown), suggesting that another mechanism regulated by caffeine is responsible for hSecurin downregulation.

hSecurin turnover becomes accelerated in response to UV light. To further explore alternative mechanisms of hSecurin depletion, we transiently transfected Cos-7 cells with VSV-tagged hSecurin expressed from a heterologous promoter (cytomegalovirus promoter). We found that the level of hSecurin-VSV also was decreased after UV irradiation, and protein turnover measurements demonstrated shortening of the hSecurin half-life in these cells exposed to UV light (Fig. 4A). However, when we assayed the inhibitors mentioned above, we found not only that caffeine abolished the reduction in the level of hSecurin-VSV but also that treatment with the proteasome inhibitors Ac-LLnL-CHO and Z-LLnV immediately before UV irradiation restored the amount of ectopic hSecurin-VSV to its original level (Fig. 4B). The same results were obtained with HA-tagged hSecurin or with another heterologous promoter (EF promoter) (Fig. 4C and data not shown).

To confirm that downregulation by UV irradiation of ectopic hSecurin was due to the activation of protein degradation via the proteasome, we used a nondegradable hSecurin mutant protein. hSecurin degradation is mediated by an RXXL destruction box (D-box) and a KEN box and is inhibited only when both sequences are mutated (63). Cos-7 cells transfected with an expression vector for the nondegradable hSecurin KAA-DM mutant were irradiated, and the level of mutant protein was analyzed by Western blotting. Figure 4C shows that hSecurin KAA-DM was completely stable after UV irradiation, indicating that UV-induced hSecurin degradation is mediated by the APC/C pathway of ubiquitin-mediated proteolysis.

The fact that the proteasome inhibitors were able to abolish the effect of UV irradiation on the ectopic but not on the endogenous protein suggested to us that in nontransfected cells, UV irradiation was acting on something other than protein stability. Therefore, we decided to block protein synthesis to avoid the possible interference of UV light with the contribution of newly synthesized protein and to examine, under these conditions, the ability of the proteasome inhibitors to restore the amount of hSecurin to the original, unirradiated level. As shown in Fig. 4D, fifth and sixth lanes, UV-induced hSecurin depletion of cycloheximide-pretreated HeLa cells was completely abolished by the proteasome inhibitors. Therefore, UV light accelerates hSecurin degradation by the ubiquitin-proteasome pathway.

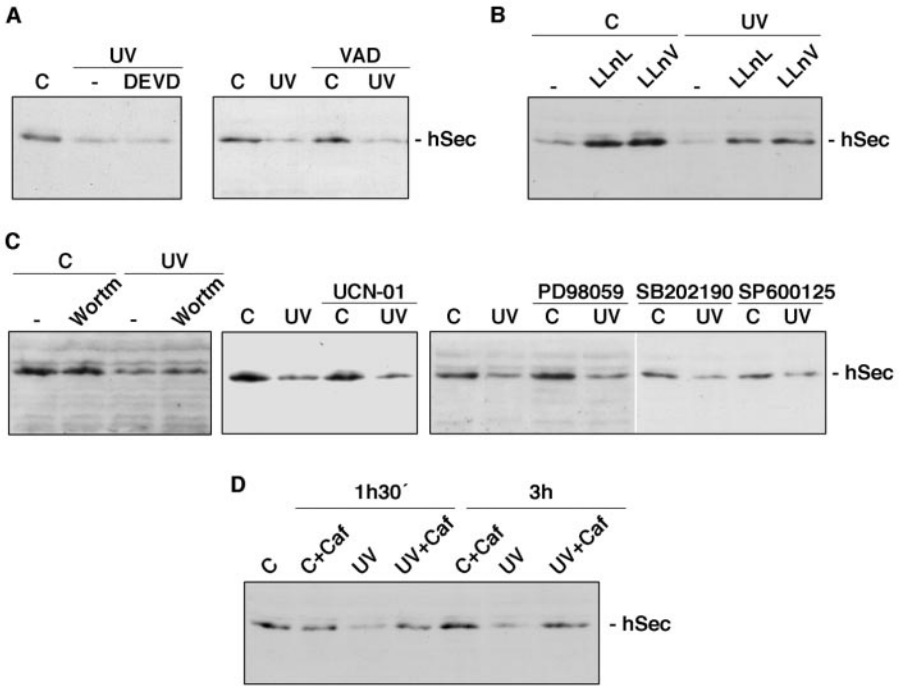

FIG. 3. Caffeine inhibits the effect of UV light on hSecurin expression. HeLa cells were left untreated (lanes C) or UV irradiated (100 J/m^2, harvested at 1 h 30 min after irradiation) in the presence or absence of various compounds to inhibit the effect of UV light on hSecurin expression. Equal amounts of NP-40 extracts were resolved by SDS–12% PAGE. Immunoblotting was performed with polyclonal anti-hSecurin antibodies. (A) The caspase inhibitors Ac-DEVD-CHO (100 μM) (DEVD) and Z-VAD-FMK (20 μM) (VAD) were added 1 h before to irradiate HeLa cells. (B) The proteasome inhibitors Ac-LLnL-CHO (100 μM) (LLnL) and Z-LLnD (50 μM) (LLnV) were added 30 min before to irradiate HeLa cells. (C) Wortmannin (5 μM) (Wortm), UCN-01 (1 μM), PD98059 (50 μM), SB202190 (2 μM), and SP600125 (25 μM) were added between 30 min and 1 h before to irradiate cells. (D) Caffeine (10 mM) (Caf) was added 1 h before UV treatment, and cells were collected after 1 h 30 min or 3 h.

UV irradiation provokes specific hSecurin protein synthesis inhibition. The results described above, showing that in the absence of protein synthesis the inhibition of the proteasome is able to reverse completely the effect of UV light (Fig. 4D), whereas when protein synthesis proceeds the inhibition of protein degradation does not reverse the effect of UV light (Fig. 3B), suggest that UV light also may be involved in hSecurin protein synthesis repression. Mammalian cells subjected to UV irradiation actively repress not only DNA replication and transcription but also mRNA translation (12, 19, 30). The features of an mRNA that mediate translational control are found both in the 5′ and in the 3′ UTRs. In the 5′ UTR, the secondary structure, the binding of proteins, and the presence of upstream open reading frames can interfere with the association of initiation factors with the cap or with scanning of initiation complexes. The 3′ UTR can mediate translational activation by directing cytoplasmic polyadenylation and can confer translational repression by interfering with the assembly of initiation complexes (9). To investigate whether UV radiation provokes specific endogenous hSecurin protein synthesis inhibition, we cloned the 5′ UTR of *hSecurin* (see Materials and Methods) upstream of VSV-tagged *hSecurin* instead of the Kozak region. Next, we transfected this construction into Cos-7 cells and tested UV treatment in the presence or absence of the proteasome inhibitor Ac-LLnL-CHO. Figure 5A shows how this inhibitor was unable to reverse the reduction in the level of hSecurin-VSV, whereas it completely inhibited the reduction in the protein level when *hSecurin* had a cloned Kozak region. However, when we cloned the 3′ UTR of *hSecurin* downstream of hSecurin and transfected and irradiated Cos-7 cells, no change was observed compared with the results obtained for hSecurin without this region (data not shown). Therefore, UV light induces specific endogenous hSecurin protein synthesis inhibition mediated by the 5′ UTR of *hSecurin*.

To confirm these results and eliminate the effect of hSecurin proteasome-dependent degradation, we generated a chimeric luciferase reporter construction that included the *hSecurin* promoter, the entire *hSecurin* 5′ UTR, and the firefly *luciferase* cDNA (plasmid pXP2-0.7kb). This construction was transfected into Cos-7 cells by the DEAE-dextran method. After 48 h, cells were UV irradiated, and luciferase activity was measured in triplicate in cell extracts obtained in three experiments. Figure 5B shows that UV light reduced the luciferase activity of pXP2-0.7kb to 17%, indicating that the 5′ UTR of *hSecurin* inhibits translation of the chimeric reporter mRNA in vivo. Transcription of firefly luciferase constructions detected by Northern blotting and translation of *Renilla* luciferase measured by luciferase activity were not affected by UV irradiation (data not shown). Altogether, these results demonstrate that UV radiation downregulates the hSecurin protein level in two ways, by accelerating its degradation via the proteasome and by reducing its mRNA translation level.

UV irradiation activates APC/C-dependent hSecurin degradation in S and G_2 phases. Two regulatory proteins, Cdc20 and Cdh1, activate APC/C. The activity of APC/C is high from late mitosis until late in G_1 phase but low in S and G_2 phases and

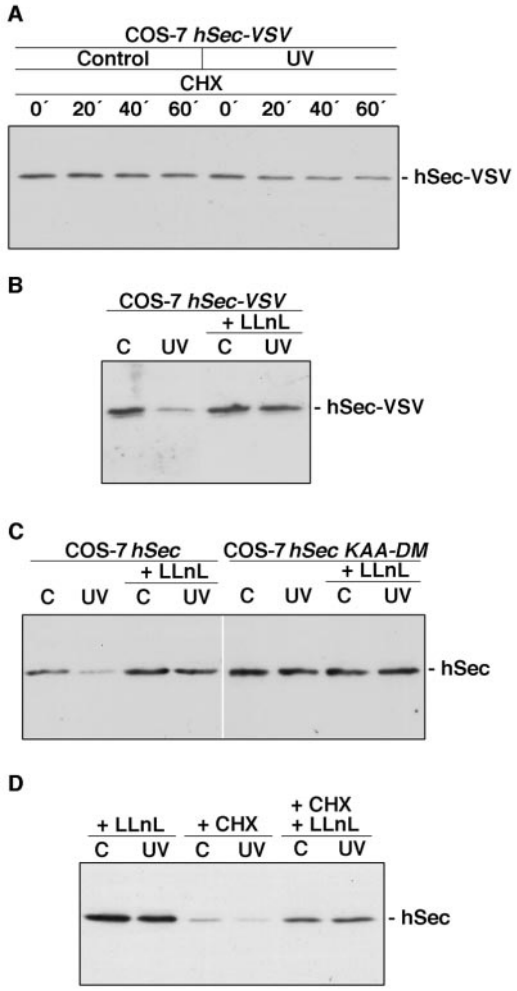

FIG. 4. UV light accelerates hSecurin degradation by the ubiquitin-proteasome pathway. (A) Half-life of hSecurin protein in Cos-7 cells. Cells were transiently transfected with hSec-VSV, and expression was induced for 18 h. Cycloheximide (CHX, 25 μg/ml) was added to the medium at 1 h after irradiation (100 J/m^2), and cells were collected at the indicated times. hSec-VSV was detected by immunoblotting with anti-VSV monoclonal antibody. (B) Ectopic hSec-VSV is degraded in a proteasome-dependent manner in UV-irradiated Cos-7 cells. Cells were transfected as described for panel A. Ac-LLnL-CHO (100 μM) (LLnL) was added to the culture medium of control (lanes C) or irradiated (100 J/m^2) cells immediately after treatment. Cells were harvested at 1 h 30 min after treatment. Samples were allowed to migrate and were immunoblotted. (C) A nondegradable hSecurin mutant is completely stable after UV irradiation. Cos-7 cells were transfected with *hSecurin* or *hSecurin KAA-DM* and treated as described for panel B. Western blotting was performed with anti-Securin antibodies. (D) The effect of UV light on endogenous hSecurin expression is inhibited by proteasome inhibitors in HeLa cells with a block in protein synthesis. Cells were preincubated with LLnL (100 μM) or CHX (25 μg/ml) or both for 1 h and irradiated as described above. Immunoblotting were performed with anti-hSecurin antibodies.

early mitosis (24). In the metaphase-to-anaphase transition, Cdc20 activates APC/C, allowing sister chromatid separation. On the other hand, Cdh1 is believed to maintain APC/C activity from the end of mitosis until the end of G_1. However, after UV irradiation, hSecurin is degraded both in S and in G_2 phases (Fig. 2C). To determine whether APC/C is activated in S and G_2 after UV irradiation, we synchronized HeLa cells in S and G_2 and tested hSecurin expression before and after UV irradiation in the presence or absence of a proteasome inhibitor. We found that hSecurin expression in nonirradiated S-phase cells does not change after 1 h 30 min in the presence of Ac-LLnL-CHO, indicating that APC/C is not activated under these conditions (Fig. 6A, second and third lanes). In fact, recent reports have shown that Cdh1-APC/C is inactivated from S phase until midmitosis through phosphorylation by cyclin A-Cdk2 or cyclin B-Cdk1 in mammalian cells (32). For nonirradiated cells in G_2 phase, we could not compare the hSecurin expression levels between cells treated and cells not treated with the proteasome inhibitor because after 1 h 30 min, the cells entered anaphase and hSecurin protein was degraded (Fig. 6B, second and third lanes). However, for irradiated cells in both S and G_2 phases, downregulation of hSecurin was essentially inhibited by Ac-LLnL-CHO (Fig. 6A and B, fourth and fifth lanes). These results indicate that UV radiation induces APC/C activation in the cell cycle phases where it is not activated under control conditions.

hSecurin-hSeparase complexes are maintained after UV irradiation. The activation of hSecurin degradation in S and G_2 phases in response to UV irradiation was an absolutely unex-

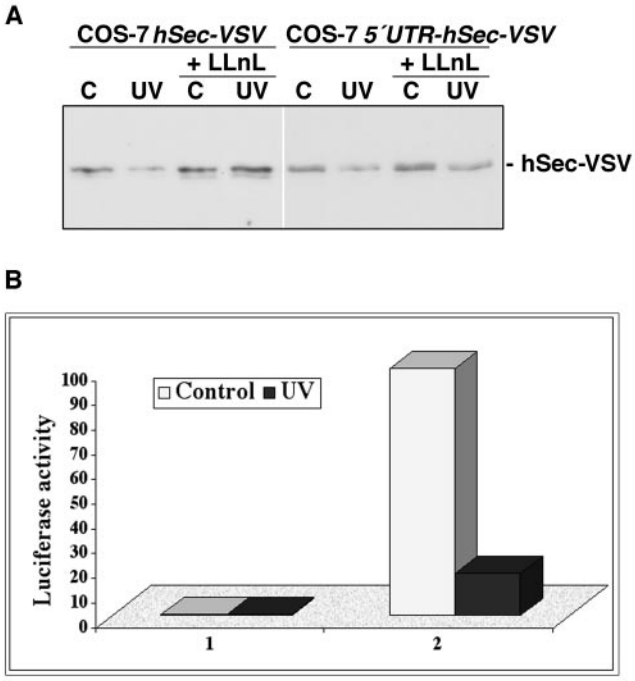

FIG. 5. UV irradiation provokes specific hSecurin protein synthesis inhibition. (A) Cos-7 cells were transfected with *hSec-VSV* or *5′ UTR-hSec-VSV* and treated as described in the legend to Fig. 4B. (B) Cos-7 cells were cotransfected with pXP2 (including the firefly luciferase cDNA without a promoter) and pRL-*Renilla* (columns labeled 1) or with pXP2-0.7kb (including the hSecurin promoter, the entire *hSecurin* 5′ UTR, and the firefly luciferase cDNA) and pRL-*Renilla* (columns labeled 2). After 48 h, cells were irradiated (100 J/m^2) or not and then were collected. Equal amounts of proteins were used for the determination of luciferase activities, and after normalization, the results were expressed as a percentage of the expression of pXP2-0.7kb in nonirradiated cells. These results are representative of three independent experiments.

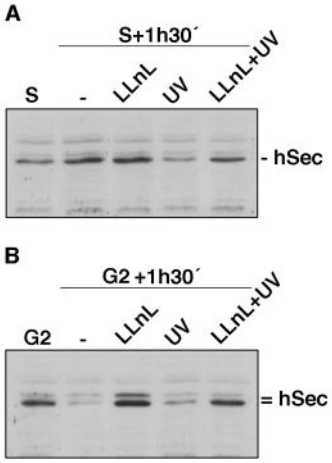

FIG. 6. UV irradiation actives APC/C-dependent hSecurin degradation in S and G_2 phases. HeLa cells were synchronized in S (A) and G_2 (B) phases, and the expression of hSecurin was studied. hSecurin expression was also analyzed at 1 h 30 min after synchronization in cells that were left untreated (lane −), cells in the presence of Ac-LLnL-CHO (100 μM) (LLnL) or UV irradiation (100 J/m^2), or cells in the presence of both LLnL and UV irradiation. Western blots were developed with anti-Securin antibodies.

pected result. The loss of Securin before anaphase and with DNA damage caused by UV light would lead to premature sister chromatid separation, which would be catastrophic for the cell. In fact, in *S. cerevisiae*, Pds1 is stabilized in response to UV irradiation, ensuring that sister chromatids are not separated before DNA repair occurs. Nevertheless, we have shown that UV radiation reduces hSecurin expression, but it is noteworthy that approximately 15 to 20% of the total hSecurin protein remains unaltered even at a high dose of UV light.

Next, we investigated whether the hSecurin remaining after UV treatment interacted with hSeparase to inhibit premature sister chromatid separation. To this end, HeLa cells were synchronized and hSecurin-hSeparase complexes were tested by coimmunoprecipitation experiments in all phases of the cell cycle. Figure 7 shows that similar amounts of hSeparase were immunoprecipitated with anti-hSecurin antibodies from extracts of both control and irradiated cells; these data indicated that hSecurin-hSeparase complexes were maintained after UV irradiation, preventing sister chromatid separation before the repair of UV-damaged DNA.

Loss of hSecurin causes premature entry into mitosis after UV irradiation. Previous observations indicated that cells lacking hSecurin grow somewhat more slowly than wild-type cells, but the cell cycle distributions of unsynchronized cells and the apoptotic fraction and the percentages of cells in mitosis are essentially identical for *hSecurin*$^{+/+}$ and *hSecurin*$^{-/-}$ clones (20). In fact, the growth curve for *hSecurin*$^{-/-}$ cells is almost indistinguishable from that for wild-type cells, which divide approximately every 18 h (Fig. 8A). To determine the involvement of hSecurin in cell cycle progression after UV irradiation, we tested whether the loss of *hSecurin* affected the proliferative properties of HCT116 cells. Wild-type and *hSecurin*$^{-/-}$ HCT116 cells were irradiated and monitored by using growth curves and cell cycle analysis. First, cells were exposed to UV radiation (30 J/m^2), and cell survival was measured every day as described in Materials and Methods. Wild-type cells arrested proliferation for approximately 4 days (Fig. 8B) and began normal proliferation later. However, *hSecurin*$^{-/-}$ cells proliferated from the beginning, but more slowly than the corresponding nonirradiated cells (Fig. 8C). Figure 8D compares wild-type and *hSecurin*$^{-/-}$ HCT116 cell proliferation after UV irradiation. It clearly shows that hSecurin is necessary to arrest cell proliferation after UV irradiation.

Next, we studied the cell cycles of *hSecurin*$^{+/+}$ and *hSecurin*$^{-/-}$ cells after UV irradiation. Fluorescence-activated cell sorting (FACS) analysis of wild-type and *hSecurin*$^{-/-}$ cells at different time points after release from an aphidicolin-induced G_1/S block showed that the cell cycle distributions were the same for both genotypes; these data suggested that hSecurin deficiency does not impair the transition from S phase to mitotic entry in normal cell cycle progression (Fig. 9A). However, when cells released from an early-S-phase block were UV irradiated at mid-S phase and the cell cycle progression of wild-type and *hSecurin*$^{-/-}$ cells was analyzed, we observed a significant increase in the G_2/M fraction of *hSecurin*$^{-/-}$ cells at 12 to 24 h after treatment and a corresponding increase in the number of cells with G_1-phase DNA content; however, the vast majority of wild-type cells appeared to arrest in S phase, as determined with ModFit LT2 sofware (Fig. 9B and C). To confirm these results, we carried out a higher-resolution two-dimensional analysis with a BrdU pulse to identify the replicating (S-phase) population. After exposure of control and irradiated cells to a 30-min pulse of BrdU, we monitored the progression of BrdU-labeled S-phase cells throughout the cell cycle by measuring DNA content (Fig. 10). The progression of nonirradiated wild-type cells was very similar to that of *hSecurin*$^{-/-}$ cells. However, after irradiation, wild-type HCT116 cells showed a higher percentage of cells arrested in S phase than did *hSecurin*$^{-/-}$ HCT116 cells (Fig. 10A). Accordingly, wild-type cells had a lower percentage of labeled cells in G_1 phase (Fig. 10B). These results are in agreement with the results of our cell cycle analyses following synchronization with aphidicolin.

In addition, we analyzed the sub-G_1 populations in both cell lines after UV irradiation and observed that cell death took place shortly after UV irradiation in both wild-type and *hSecurin*$^{-/-}$ cells. However, *hSecurin*$^{-/-}$ cells showed an in-

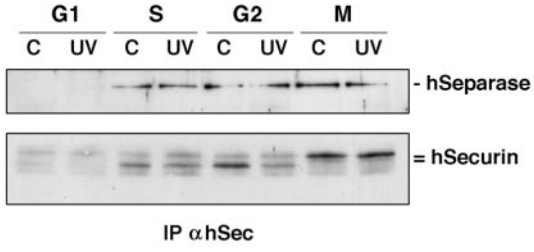

FIG. 7. Effect of UV irradiation on hSecurin-hSeparase complexes. Extracts from HeLa cells synchronized in various phases of the cell cycle (lanes C) or synchronized and UV irradiated (100 J/m^2, harvested at 1 h 30 min after irradiation) were used to immunoprecipitate (IP) hSecurin. Coimmunoprecipitated proteins were transferred to nitrocellulose filters, and Western blotting was performed with anti-hSeparase antibodies. The same filters were decorated with anti-Securin antibodies.

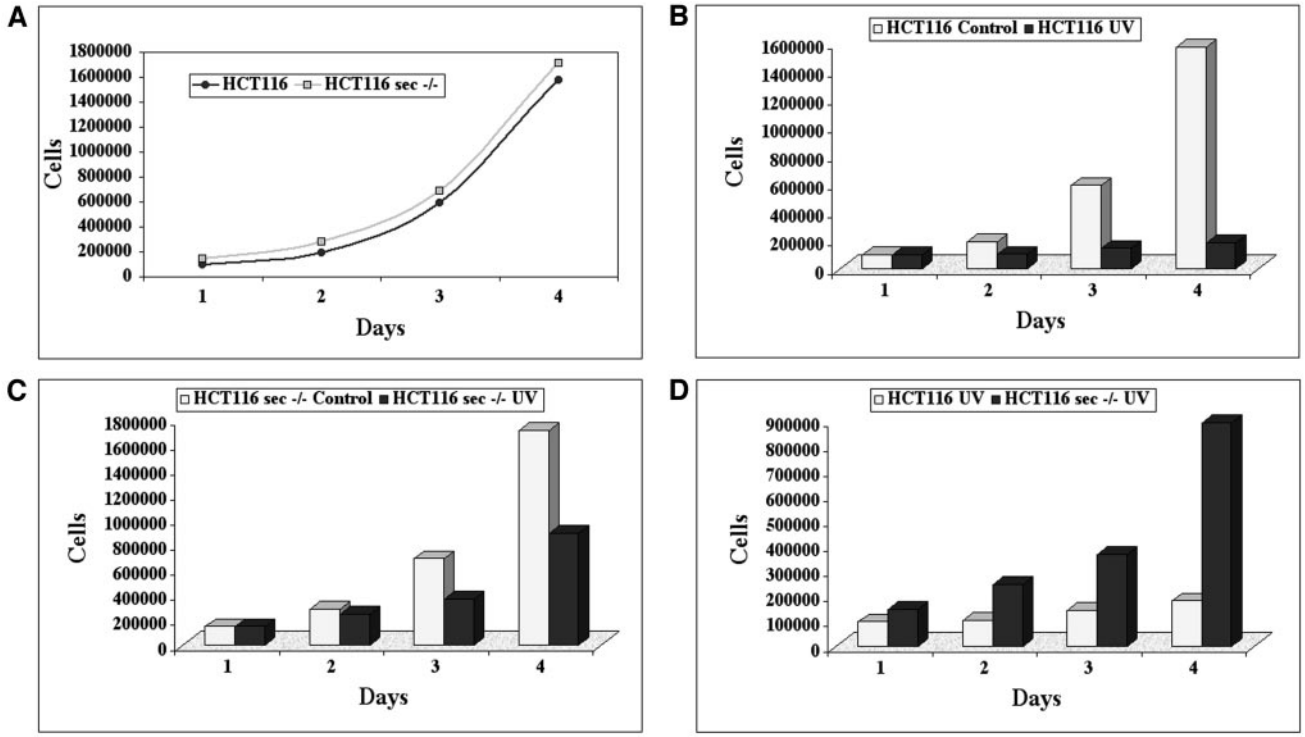

FIG. 8. Cell proliferation studies of wild-type and *hSecurin*$^{-/-}$ HCT116 cells after UV irradiation. (A) Representative growth curves for wild-type (filled circles) and *hSecurin*$^{-/-}$ (empty squares) HCT116 cells under control conditions. (B) HCT116 cells were exposed to UV irradiation (30 J/m^2), and their proliferation was compared with that of nonirradiated cells. Viable cells were measured as described in Materials and Methods. (C) *hSecurin*$^{-/-}$ HCT116 cells were treated as described for panel B. (D) Comparison of the cell proliferation of wild-type and *hSecurin*$^{-/-}$ HCT116 cells after UV irradiation (30 J/m^2).

crease in the number of cells having a sub-G_1 DNA content that was very visible 6 days after UV treatment. In fact, apoptosis assays were performed at day 6 after UV treatment, and detectable apoptotic events were detected more frequently (5 to 10%) in *hSecurin*$^{-/-}$ HCT116 cells than in wild-type HCT116 cells. When we used annexin V, which binds phosphatidylserine in the outer plasma membrane during apoptosis, the increases in the number of apoptotic events were 25% in *hSecurin*$^{-/-}$ cells and 15% in wild-type cells, with respect to nonirradiated control cells (Fig. 11A). In situ detection of apoptosis by monoclonal antibody M30, which recognizes a neoepitope in cytokeratin 18 that becomes available at early caspase cleavage, revealed that among over 2,000 cells analyzed per slide, wild-type cells averaged 108 apoptotic cells and *hSecurin*$^{-/-}$ cells averaged 210 apoptotic cells (5.4 and 10.4% apoptotic wild-type and *hSecurin*$^{-/-}$ cells, respectively [Fig. 11B]). All of these findings suggest that *hSecurin*$^{-/-}$ cells cause a premature entry into mitosis after UV irradiation that provokes a corresponding increase in cell death.

DISCUSSION

For successful eukaryotic mitosis, sister chromatid pairs remain linked after replication until their kinetochores have been attached to opposite spindle poles by microtubules. Cohesion between sister chromatids also has another role: to provide a substrate for postreplicative DNA repair. In fact, disruption of the *Scc1* gene not only causes premature sister chromatid separation but also results in defects in the repair of spontaneous and induced DNA damage (47). These data implicate cohesin in genome stability at both the DNA level and the chromosome level. Moreover, in *S. cerevisiae*, Pds1 is stabilized in response to DNA damage, delaying the onset of anaphase to allow repair before sister sequences are segregated from each other. Pds1 is stabilized by phosphorylation, mainly through the action of Chk1 kinase. In addition, Rad53 is also required both to maintain active Cdk1 (Cdc28) and to prevent anaphase entry (42).

In this study, we provide evidence that hSecurin is involved in the DNA damage response pathway. We found that, unlike in yeast cells, X-rays and UV light induced a rapid reduction in Securin protein levels in mammalian cells. This effect was produced by specific Securin protein synthesis inhibition and proteasome-dependent Securin degradation. The existence of two distinct pathways regulating the level of Securin in response to UV light is deduced from several observations. First, proteasome inhibitors provided only partial reversal of the UV downregulation of endogenous hSecurin, whereas they inhibited completely the effect of UV light on ectopic hSecurin expressed from a heterologous promoter. Second, when new protein synthesis was blocked by cycloheximide, proteasome inhibitors were able to inhibit totally the effect of UV light on endogenous hSecurin. Third, proteasome inhibitors protected only partially the UV-induced decay of ectopic hSecurin expressed from a heterologous promoter but including the 5′ UTR of *hSecurin* mRNA.

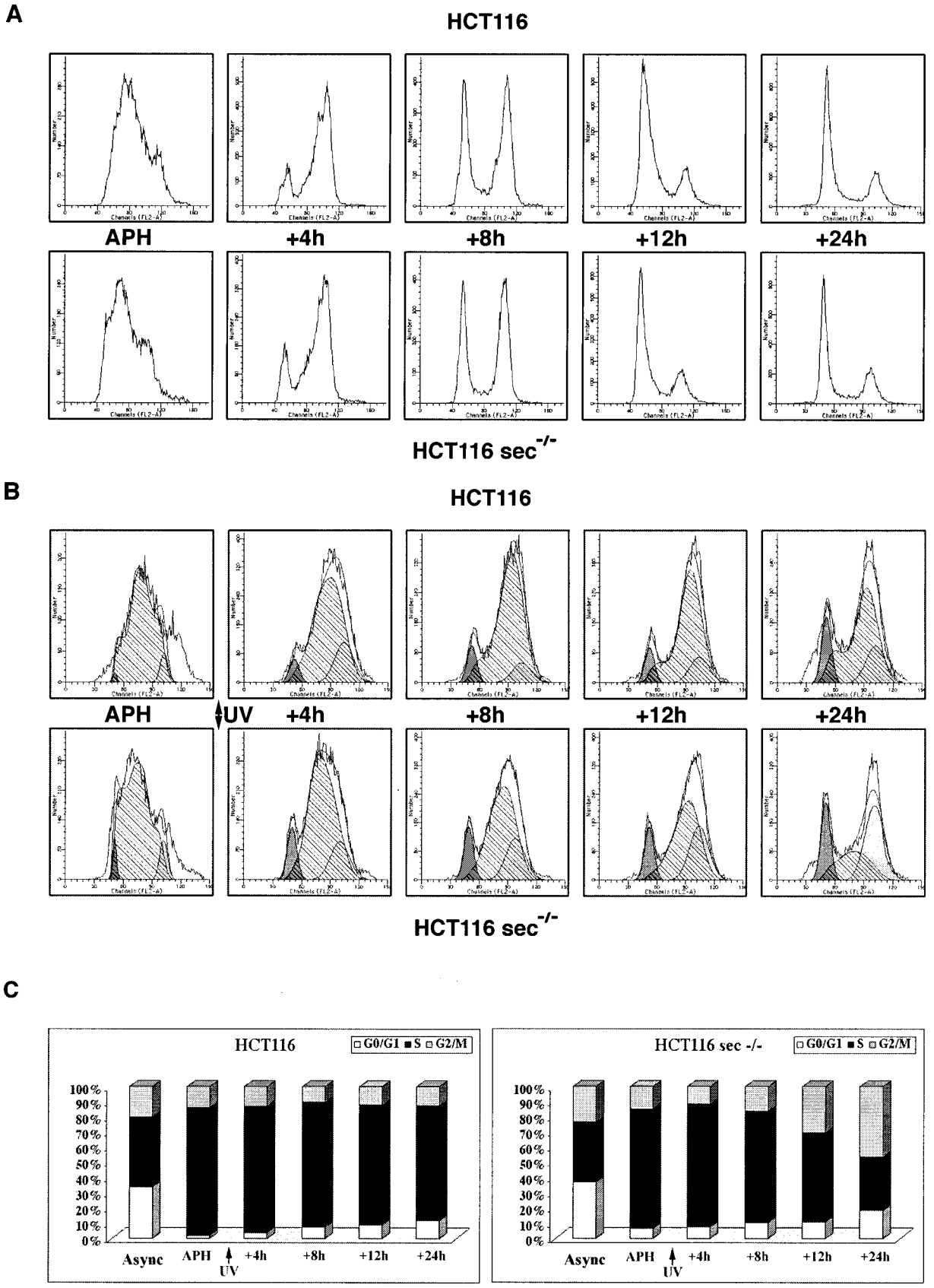

FIG. 9. Effect of UV light on the cell cycle of HCT116 cells and *hSecurin*$^{-/-}$ HCT116 cells. (A) Cells were synchronized by treatment with aphidicolin (APH) for 18 h. At the indicated time points after release from the APH block, cells were harvested and analyzed by FACS. (B) Cells were synchronized as described for panel A and, at 1 h after release from the APH block, were UV irradiated (30 J/m^2), harvested at the indicated times, and analyzed by FACS. (C) Percentages of cells in each cell cycle phase. Async, asynchronous cells.

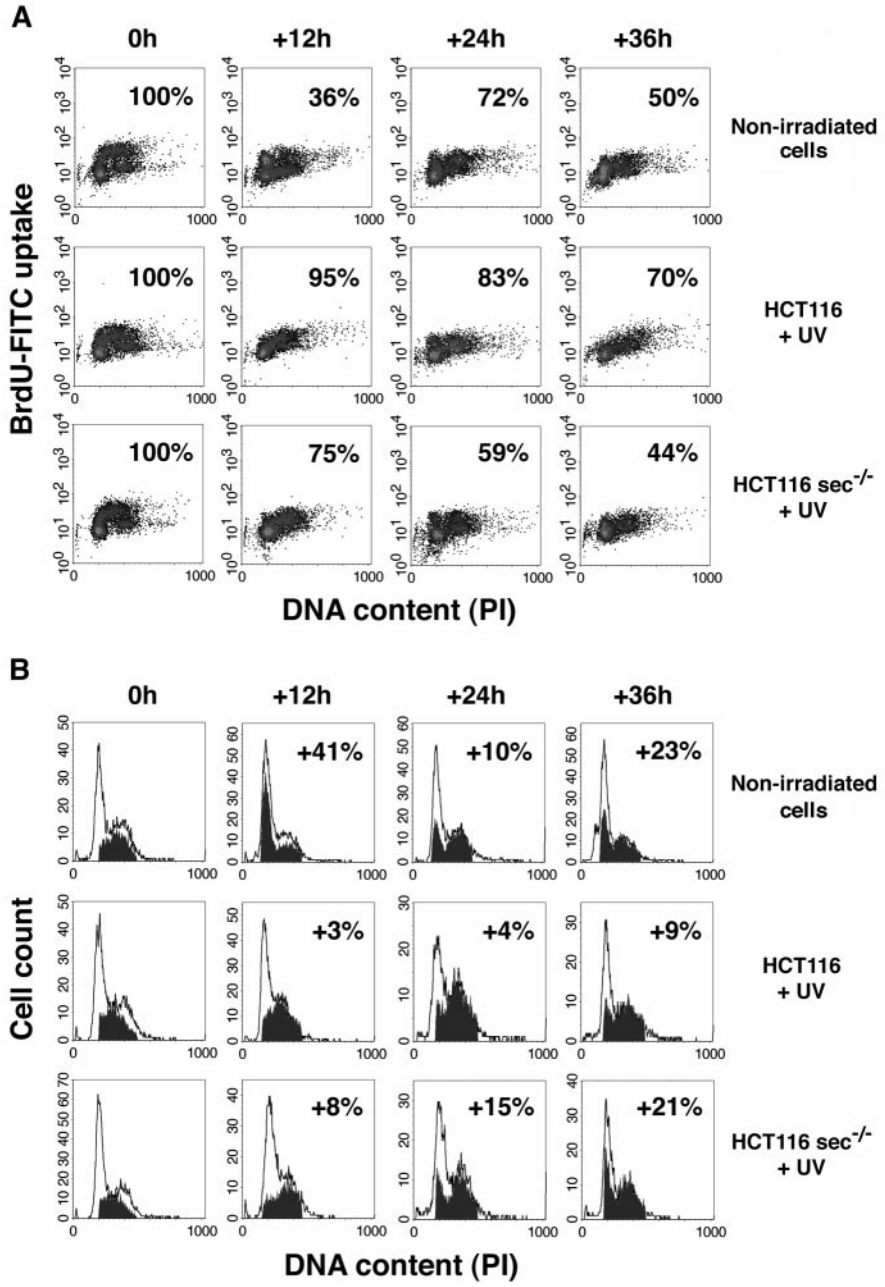

FIG. 10. BrdU pulse-chase analysis of the cell cycle kinetics of wild-type and *hSecurin*$^{-/-}$ HCT116 cells exposed to UV radiation. (A) After UV irradiation of asynchronous cell cultures, S-phase cells were labeled with a 30-min pulse of BrdU. The transit of labeled cells through the cell cycle was monitored by measuring the percentage of cells in S phase at intervals after BrdU pulse-labeling (time zero). The relative percentages of cells were calculated as (percentage of cells in S phase at each time interval/percentage of cells in S phase at time zero) × 100. (B) Histograms showing the DNA content of cells used in the BrdU pulse-chase experiment described for panel A. Areas filled in black correspond to BrdU-labeled cells and overlay global areas, i.e., labeled plus unlabeled cells (unfilled areas). The percentages represent increments of BrdU-labeled cells in G_1 at each time interval relative to the percentage of labeled cells under the G_1 peak at time zero. PI, propidium iodide.

Securin downregulation after UV irradiation is a general phenomenon that was observed in all cell lines tested and did not require functional p53. Equivalent results were obtained in other studies on the expression of Cdc25A, a phosphatase involved in the G_1/S progression, S phase, and the G_2/M transition, in response to DNA damage (11, 33). Other work with long-term doxorubicin or bleomycin treatment (36 h) proposed that, under these conditions, *hSecurin* is a p53-targeted gene (61).

The effects of UV irradiation on hSecurin expression were reversed by caffeine, an efficient inhibitor of cellular DNA repair. Thus, caffeine was able to prevent both hSecurin protein synthesis inhibition and proteasome-dependent hSecurin degradation. However, when we used other inhibitors, such as UCN-01, 8-Br-cAMP, and caspase inhibitors, all of which are involved in signaling pathways with which caffeine interferes, we were unable to avoid the reduction in the amount of either

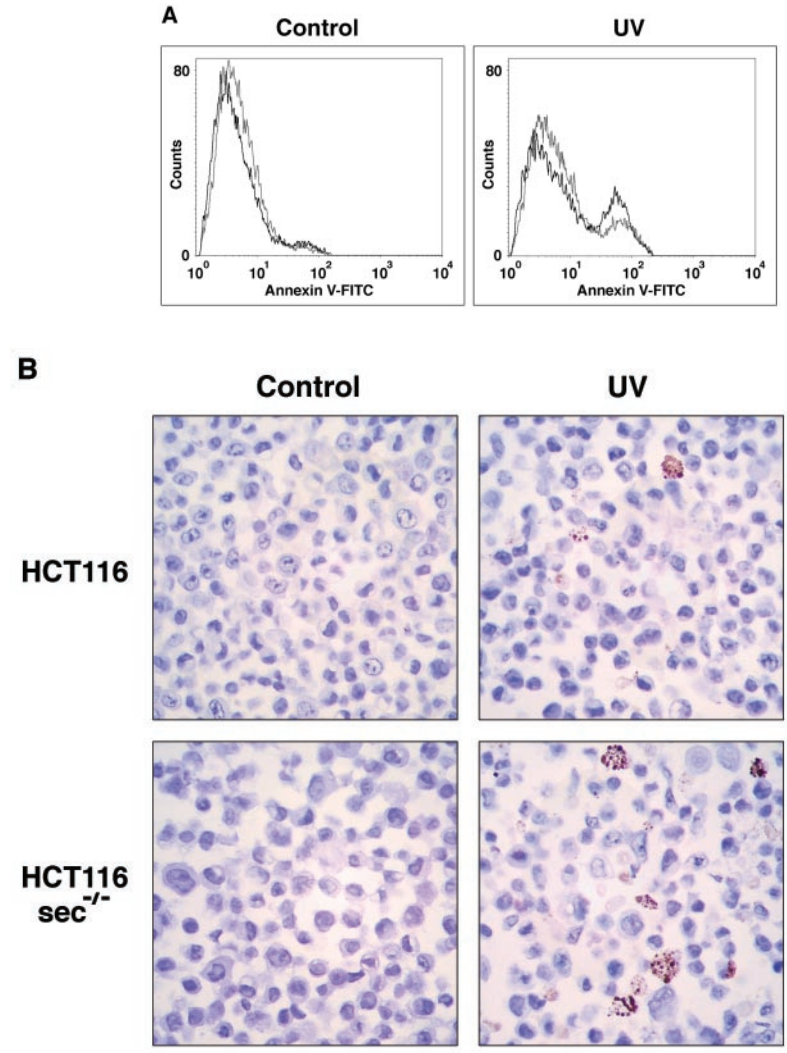

FIG. 11. UV light-mediated apoptosis in wild-type and *hSecurin*$^{-/-}$ HCT116 cells. (A) Cells were synchronized and irradiated as described in the legend to Fig. 9B. Control cells were not irradiated. After 6 days, cells were harvested and assayed for apoptotic events. Histogram overlays show FITC-annexin V binding of control and irradiated wild-type (grey line) and *hSecurin*$^{-/-}$ (black line) HCT116 cells. (B) In situ detection of apoptosis in control and irradiated wild-type and *hSecurin*$^{-/-}$ HCT116 cells at day 6 after UV irradiation. Apoptotic cells (brown) were detected after monoclonal antibody M30 immunostaining. Original magnification, ×70.

endogenous or ectopic hSecurin protein. These data suggest that another mechanism regulated by caffeine is responsible for hSecurin downregulation. Alternatively, it is also conceivable that two or more pathways are involved in hSecurin regulation by DNA damage. Further experiments will be necessary to determine exactly the signaling pathway(s) involved in hSecurin downregulation.

UV-induced hSecurin degradation was mediated by two APC/C-dependent ubiquitination signals, the D-box and the KEN box. Interestingly, UV radiation activated hSecurin degradation in S and G_2 phases of the cell cycle where, under normal conditions, APC/C was not active (24). APC/C plays a crucial role in the regulation of cell cycle progression and is activated by two types of WD40 repeat-containing proteins, Cdc20 and Cdh1. It has been suggested that Cdc20-APC/C is required for both initiation of anaphase and exit from mitosis (29, 53, 60), while Cdh1-APC/C is required from the end of mitosis until the end of G_1 (44, 46, 56). However, it was also reported recently that DNA damage causes immediate and p53-independent G_1 arrest mediated by the rapid proteolysis of cyclin D1, suggesting that APC/C may be activated in response to DNA damage and contribute to checkpoint activation (1). Similarly, it was also reported that APC/C is activated during the G_2-phase trough interaction with Cdh1 in response to DNA damage (49). Our data agree with these results showing that destruction of hSecurin is a consequence of APC/C activation in S and G_2 phases after UV irradiation. This destruction is probably mediated by the activation by Cdh1 of APC/C because an hSecurin DM mutant (D-box mutant hSecurin and KEN-box wild-type hSecurin) is also degraded after UV irradiation (data not shown). In addition, only Cdh1-APC/C is able to recognize proteins with a KEN box, whereas Cdc20-APC/C recognizes proteins that contain a D-box (37).

The loss of *Cdh1* causes premature entry into mitosis after

DNA damage, indicating that Cdh1 plays a role in the DNA damage-induced G_2 checkpoint. The substrates of X-ray-activated Cdh1-APC/C were not mitotic cyclins, and it was suggested that the degradation of other potential substrates induces G_2 arrest (49). To explore the possibility that UV-induced degradation of hSecurin is involved in this Cdh1-APC/C-dependent G_2 checkpoint, we studied the effect of UV radiation on the cell cycle of *hSecurin*$^{-/-}$ HCT116 cells. We expected a prolonged arrest in G_2 phase after UV irradiation, but the results were absolutely surprising. In fact, the loss of *hSecurin* caused premature entry into mitosis after UV irradiation.

hSecurin protein is necessary for cell proliferation arrest, as determined by FACS and proliferation studies of wild-type and *hSecurin*$^{-/-}$ cells. *hSecurin*$^{+/+}$ HCT116 cells cease to proliferate in response to UV irradiation, while *hSecurin*$^{-/-}$ cells continue to proliferate, although more slowly. These observations are consistent with those of an earlier study of *Pds1* mutants of *S. cerevisiae*; that study demonstrated that irradiated *Pds1* mutant cells undergo mitosis, inappropriately initiating anaphase after gamma irradiation and failing to inhibit cytokinesis, DNA replication, and bud formation (59).

Using FACS analysis to compare the cell cycle status of *hSecurin*$^{+/+}$ and *hSecurin*$^{-/-}$ cells after UV irradiation at mid-S phase, we observed that at 12 to 24 h after treatment, the majority of wild-type cells were arrested in S phase, while *hSecurin*$^{-/-}$ cells exhibited an apparent escape to G_2/M phase. Furthermore, analysis of the movement of the BrdU-labeled cell population showed that after irradiation, wild-type cells had a higher percentage of cells arrested in S phase than did *hSecurin*$^{-/-}$ cells. Moreover, cell death took place shortly after irradiation in both wild-type and *hSecurin*$^{-/-}$ cells; however, until day 4, the proliferation rate was higher than the death rate in *hSecurin*$^{-/-}$ cells, while it was lower in wild-type cells. These data explain why, although some wild-type cells entered G_1 phase, the population did not proliferate. After day 4, the proliferation rate in wild-type cells surpassed their death rate. In fact, dead cells were diluted and a normal proliferation rate was reestablished. On the other hand, a normal rate was not reestablished in *hSecurin*$^{-/-}$ cells because a population of apoptotic cells was being generated continuously, suggestive of defects in DNA repair. Even at 6 days after irradiation, when apoptotic cells are expected to be diluted, cell death was 10% higher in *hSecurin*$^{-/-}$ cells than in wild-type cells. These data support our hypothesis of continuous apoptosis in damaged *hSecurin*$^{-/-}$ cells over the long term.

Cells can reduce the rate of ongoing DNA synthesis when exposed to DNA-damaging conditions (see reference 4 for a review). UV light is an agent that leads to protracted replication arrest. DNA damage detected during S phase is apt to be repaired via homologous recombination mechanisms involving sister chromatids (23). Ionizing radiation exposure during S phase activates the ATM kinase, which initiates a complex response to delay DNA replication. Defects in ATM or its substrates Nbs1 and Chk2 cause radioresistant DNA synthesis. The ATR kinase also functions as a critical regulator of the cellular DNA damage response. In fact, ATR is required for the G_2/M DNA damage checkpoint as well as the DNA replication checkpoint, which suppresses mitosis in the presence of unreplicated DNA. ATR mediates responses to a broad spectrum of genotoxic stimuli, including ionizing radiation, UV light, DNA replication inhibitors, and agents that induce DNA interstrand cross-links. Recent studies have indicated that, in mammalian cells, ATM and ATR may represent two parallel branches of the DNA damage response pathway; however, other studies have demonstrated that the ATM/Chk2 and ATR/Chk1 pathways show a high degree of cross talk and connectivity (15). The phenotype showed by *hSecurin*$^{-/-}$ cells after UV irradiation was similar to the radioresistant DNA synthesis phenotype caused by defective ATM, suggesting that hSecurin is involved in S-phase checkpoint arrest.

Condering all of the data, we conclude that hSecurin is a target of the DNA damage response pathway in higher eukaryotes and that its presence is necessary for UV irradiation-induced S-phase arrest. The biological significance of its decay in response to UV light remains to be determined. To answer this question, we have attempted to obtain a stably transfected cell line in which *hSecurin* is not under translational control or depleted after UV irradiation, although we have not yet done so successfully. In fact, other authors have shown that this kind of cell line is most troublesome at sister chromatid separation (16). It is possible that hSecurin has a dual role in the control of the progression of the cell cycle in response to UV treatment, in that both its presence and its further destruction are necessary to arrest cell proliferation. This notion may appear paradoxical but is not surprising. The role of Securin as an inhibitor of sister chromatid separation before anaphase has been well characterized, and previous studies provided evidence of the dual mechanism of Separase regulation by Securin. In human cells, the effect of Securin deletion, rather than leading to premature chromatid separation, is to retard chromosome separation, because Securin is necessary for the proper functioning and processing of Separase (20). In *S. cerevisiae*, Securin is required to support Separase activity in anaphase and, at the same time, Securin must be destroyed before Separase becomes active (18).

Although in human cells hSecurin is not stabilized in response to UV light but rather is destabilized, there is no consequence on its role as a sister chromatid separation inhibitor, because hSecurin-Separase complexes are maintained unaltered after UV treatment. There are mechanisms to stabilize those complexes by preventing sister chromatid segregation before DNA is repaired. On the other hand, it will be interesting to search for complexes of hSecurin and other putative partners that are disrupted after UV treatment. The knowledge of such complexes will shed light on the novel role of hSecurin in the cellular DNA damage response.

Finally, it was recently demonstrated that hSecurin interacts with p53, represses its transcriptional activity, and reduces its ability to induce cell death in vivo (5); these data suggest a new functional mechanism by which Securin can promote tumorigenesis. It is possible that the nondegradation of Securin after DNA damage provokes tumor formation by blocking p53 functions.

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