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Protein Phosphatase 2A Stabilizes Human Securin, Whose Phosphorylated Forms Are Degraded via the SCF Ubiquitin Ligase

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Sister chromatid segregation is triggered at the metaphase-to-anaphase transition by the activation of the protease separase. For most of the cell cycle, separase activity is kept in check by its association with the inhibitory chaperone securin. Activation of separase occurs at anaphase onset, when securin is targeted for destruction by the anaphase-promoting complex or cyclosome E3 ubiquitin protein ligase. This results in the release of the cohesins from chromosomes, which in turn allows the segregation of sister chromatids to opposite spindle poles. Here we show that human securin (hSecurin) forms a complex with enzymatically active protein phosphatase 2A (PP2A) and that it is a substrate of the phosphatase, both in vitro and in vivo. Treatment of cells with okadaic acid, a potent inhibitor of PP2A, results in various hyperphosphorylated forms of hSecurin which are extremely unstable, due to the action of the Skp1/Cul1/F-box protein complex ubiquitin ligase. We propose that PP2A regulates hSecurin levels by counteracting its phosphorylation, which promotes its degradation. Misregulation of this process may lead to the formation of tumors, in which overproduction of hSecurin is often observed.

Securins are regulatory proteins that associate with a thiol-protease called separase (34). Separase cleaves the Scc1/Mcd1/Rad21 subunit of the cohesin complex and thereby brings about the final resolution of sister chromatid cohesion during the metaphase-to-anaphase transition of mitosis. Securins from different eukaryotes are surprisingly divergent at the primary sequence level, and their physiological importance also varies. Deletion of the budding yeast (*PDS1*) and vertebrate (*PTTG*) securin genes results in increased rates of chromosome loss but is not necessarily lethal (26, 52, 55). On the other hand, the securin proteins of fission yeast (Cut2) and *Drosophila* (PIM) are essential. In the absence of Cut2 or PIM, sister chromatids fail to separate (16, 49). These particular securin proteins, therefore, provide for the first time a function that is absolutely required for sister chromatid separation, perhaps by assisting in separase folding or localization (1, 24, 26). A universal feature of all securin proteins, however, is that they all act as inhibitors of separase activity. In addition, release of this inhibition occurs via their degradation at the metaphase-to-anaphase transition (10, 12, 17, 30, 49, 51, 58). Thus, mitotic securin degradation contributes to the temporal control of separase activity.

Mitotic securin is targeted for degradation by a multisubunit ubiquitin protein ligase known as the anaphase-promoting complex or cyclosome (APC/C) (for a review, see reference 37). During mitosis, APC/C activity is controlled by the mitotic spindle checkpoint pathway. This checkpoint ensures that securin remains stable until all sister chromatids are correctly bi-oriented on the mitotic spindle. The fact that securin stabilization by activation of the mitotic spindle checkpoint prevents premature sister chromatid separation has been clearly shown in budding yeast (10, 55). In contrast, premature sister chromatid separation in the presence of spindle poisons has not been observed in human cells lacking securin (26), presumably because sister chromatid separation in these cells involves additional securin-independent regulatory pathways. Indeed, cyclin-dependent kinase 1 (Cdk1) can directly inhibit separase activity in higher eukaryotes by inhibitory phosphorylation (20, 48). Cdk1 inactivation, resulting from the APC/C-dependent proteolysis of the mitotic cyclins at the metaphase-to-anaphase transition, therefore, presumably leads to separase activation concomitant with securin degradation.

Mammalian securin was originally described as a proto-oncogene because its overexpression in NIH 3T3 transfected cells resulted in an anchorage-independent transformation in vitro and tumor formation when transfectants were injected into athymic nude mice (28, 36, 57). Moreover, securin is highly expressed in many tumors (13, 23, 45, 46). However, the mechanism by which securin overexpression promotes tumorigenesis is still unclear. The fact that securin regulates sister chromatid separation during cell division suggests that overproduction of securin may lead to aneuploidy caused by defective sister chromatid separation, resulting in tumor development (26, 31). Other mechanisms, however, may be involved. We previously reported that securin binds p53 and that this interaction inhibits its apoptotic and transcriptional activities. These results provide a mode by which securin could promote tumorigenesis. The interaction between securin and p53 could lead directly to the accumulation of DNA damage and subsequent development of malignant tumors (2). Furthermore, we have recently provided evidence that human securin, hSecurin, has a novel role in cell cycle arrest after exposure to UV light. Irradiation of cells causes the downregulation of hSecurin protein by, first,

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accelerating its degradation via the proteasome and, second, reducing the efficiency of hSecurin mRNA translation. However, somewhat paradoxically, the presence of at least some hSecurin is necessary for cell proliferation arrest following UV treatment. Thus, an alteration of UV-induced hSecurin downregulation could lead directly to the accumulation of DNA damage (43).

Securin must be strictly regulated to ensure that cell division and the cellular response to DNA damage are correctly accomplished. Nevertheless, compared to the knowledge about separase regulation, there is little information concerning the regulation of securin. The hSecurin/hPTTG protein level has been described as being regulated in a cell-cycle-dependent manner, peaking in mitosis when it is phosphorylated by the mitotic kinase Cdc2 (41). Moreover, hSecurin has been reported to be an in vitro substrate of DNA-protein kinase (44). We now show that hSecurin associates with the heterotrimeric serine/threonine protein phosphatase 2A (PP2A) and that it is a substrate of this phosphatase both in vitro and in vivo. Furthermore, the stability of hSecurin depends on its phosphorylation state, such that hyperphosphorylated forms are rapidly destroyed via the Skp1/Cul1/F-box protein complex (SCF) E3 ubiquitin ligase. We propose that PP2A prevents degradation of hSecurin by removal of phosphate groups that target the protein for ubiquitination and degradation by the proteasome.

MATERIALS AND METHODS

Plasmids, cloning, and sequencing. The pGAD-p27 clone was previously obtained from a two-hybrid screen using hSec-E/A (residues 1 to 163) as a bait (42, 44). DNA sequencing of this clone revealed that it encoded residues 233 to 453 of the human B55δ isoform of the regulatory subunit of the PP2A complex. The p27 insert was amplified by PCR and cloned in frame with the hemagglutinin (HA) tag of the vector pECE (15). The resulting plasmid, pECE-B55δ(233–453), was sequenced to ensure the gene fusion was free of errors. pRSET-A hSec, pRSET-A hSec-E/A, pRSET-RIIα, pCDNA3-hSec VSV (vesicular stomatitis virus), pEF-hSec, and pEF-hSec KAA-DM were previously described (43, 44, 59). pSUPER, pSUPER-Cdh1, and pSUPER-Cdc20 (5) were kindly provided by R. Agami, and pCDNA3 Flag-Cul1(1–452) (54) was provided by Z.-Q. Pan. pCDNA3 and pEGFP-N1 were obtained from Invitrogen and BD Biosciences, respectively.

Yeast two-hybrid screening. *Saccharomyces cerevisiae* strain Hf7c was cotransformed with the indicated constructs by the lithium acetate method (47). Double transformants were plated on yeast dropout medium lacking tryptophan and leucine (47). They were grown for 3 days at 30°C. Colonies were patched on the same medium and replica plated on Whatman 40 filters to test for β-galactosidase activity (4) and for growth on yeast dropout medium lacking tryptophan, leucine, and histidine. Interaction between Snf1 and Snf4 proteins (9) was used as a positive control.

Cell culture and lysis. HCT116, HeLa, and Cos-7 cells (from the American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Invitrogen) and 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker), in a 5% CO_2 humidified atmosphere at 37°C. Cell lysis was performed at 4°C in 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1% Nonidet P-40 (NP40), 10% glycerol, 1 mM sodium vanadate (Na_3VO_4), 20 mM sodium pyrophosphate (PPi), 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 10 μg/ml chymostatin for 20 min. Extracts were centrifuged at 20,000 × *g* for 20 min, and supernatants were frozen in liquid nitrogen and stored at −80°C. The protein concentration was determined using the Bradford assay (Bio-Rad).

Cell synchronization and drugs. HeLa cells enriched in the G_1, S, G_2, or M phase were obtained as previously described (27). HeLa G_1 cells were obtained by incubating cells for 16 h in 6 mM butyrate (Sigma). HeLa G_1/S cells were obtained by performing a double-thymidine block (i.e., two 16-h incubations in 2.5 mM thymidine [Sigma], with an 8-h release in between). Cells enriched in S phase were harvested 4 h after release from the second block. Cells harvested 8 h after release were further enriched for a G_2 population by rinsing extensively to remove mitotic cells. Mitotic arrested cells were obtained by incubation for 16 h in medium containing 5 μM nocodazole. Purity of the phases was confirmed by flow cytometry. For some experiments, cells were pretreated with the proteasome and calpain inhibitor Ac-LLnL-CHO (LLnL; 100 μM [Sigma]) and/or the serine/threonine protein phosphatase inhibitor okadaic acid (1 μM; Sigma).

Electrophoresis, Western blot analysis, and antibodies. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and gels were electroblotted onto nitrocellulose membranes and probed with the following antibodies: anti-hSecurin polyclonal antibody (13); anti-HA monoclonal antibody from Roche; anti-VSV monoclonal antibody from Sigma; anti-Cdc20 and anti-Cdc25A monoclonal and anti-Cul1 polyclonal antibodies from Santa Cruz Biotechnology; anti-Cdh1, anti-PP2A catalytic subunit α (PP2A-cs α), and anti-cyclin B monoclonal antibodies from BD Biosciences; anti-PP2A-B55 α/δ monoclonal antibody from Upstate Biotechnology; and anti-green fluorescent protein (GFP) polyclonal antibody from Immunology Consultants Laboratory. Peroxidase-coupled donkey anti-rabbit immunoglobulin G (IgG) and sheep anti-mouse IgG were obtained from Amersham Biosciences. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) Western blotting system (Amersham Biosciences) according to the manufacturer's protocol.

Affinity chromatography assays. The expression of the six-His fusion proteins was induced in *Escherichia coli* BL21 (DE3) cells by incubation with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h at 37°C. Fusion proteins were purified from bacterial lysates via their affinity to Ni-nitrilotriacetic acid (NTA) agarose (QIAGEN). Cellular lysates (200 to 500 μg) were incubated for 2 h with fusion proteins (100 to 500 ng) bound to the agarose beads. Beads were washed six times in lysis buffer, and bound proteins were eluted by the addition of SDS-sample buffer heated at 95°C for 5 min. Finally, the samples were subjected to SDS-PAGE.

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

Transient transfections and protein half-life experiments. Constructs containing hSec, hSec KAA-DM, hSec VSV, Flag-Cul1(1–452), HA B55δ(233–453), and enhanced GFP (EGFP) or empty vectors were transiently transfected by electroporation into Cos-7 cells, and cells were harvested 18 h posttransfection. Lysates of transfected cells and Western blotting assessed the level of overexpressed proteins. Protein half-life experiments were performed by transient transfection of Cos-7 cells. Expression of the transgenes was induced for 18 h prior to the addition of cycloheximide (50 μg/ml; Sigma) to the culture media and subsequent harvesting of cells at the various times indicated.

Dephosphorylation assays. Cellular extracts or hSecurin immunoprecipitates, prepared without phosphatase inhibitors (NaF, PP_i, and Na_3VO_4), were incubated for 30 min at 30°C with lambda protein phosphatase (λ-PP; Upstate Biotechnology) or at 37°C with commercial PP2A (Promega), B55δ immunocomplexes, or both commercial PP2A and B55δ immunocomplexes. All dephosphorylation assays were carried out in the presence of protease and proteasome inhibitors to avoid nonspecific degradation. After treatment, SDS-sample buffer was added and samples were heated at 95°C for 5 min prior to analysis by SDS-PAGE.

Serine/threonine protein phosphatase activity assays. The activity of serine/threonine phosphatase was determined by using a nonradioactive molybdate dye-based phosphatase assay kit (Promega) according to the manufacturer's recommendations. We first obtained the corresponding immunoprecipitates, as previously described, from lysates prepared in lysis buffer without phosphatase inhibitors (NaF, PP_i, and Na_3VO_4) and washed in PP2A buffer (50 mM imidazole, pH 7.2, 0.2 mM EGTA, 0.02% β-mercaptoethanol, and 0.1 mg/ml bovine serum albumin). Serine/threonine phosphatase activity in these immunoprecipitates was determined by measuring in triplicate the ability of such immune complexes to dephosphorylate a synthetic phosphothreonine peptide, RRA[pT]VA, a preferential substrate of PP2A. The assay mixtures contained PP2A buffer prepared in phosphate-free water, 100 μM phosphopeptide substrate, and okadaic acid, when indicated, in a final volume of 300 μl. The enzyme reaction was started by adding immunoprecipitates in 35 μl of phosphate-free water, and the mixture was allowed to incubate for 2 h at 37°C. The reaction was stopped by the addition of 300 μl molybdate dye-additive mixture, and color was developed by incubating the mixture for 30 min at room temperature. A standard curve was obtained

using inorganic phosphate at concentrations ranging from 500 to 3,000 μM. Absorbance at 600 nm was measured, and the amount of phosphate released was then calculated using the standard curve. Specific hSecurin immunoprecipitate phosphatase activity was calculated by subtracting the phosphatase activity obtained from the preimmune serum mock immunoprecipitation from that obtained from the hSecurin.

siRNA assays. For small interfering RNA (siRNA) assays, we used pSUPER-Cdh1 and pSUPER-Cdc20 (as well as pSUPER as a negative control) to suppress the expression of the endogenous *cdh1* and *cdc20* genes (5). HeLa cells were transiently transfected by electroporation and harvested 48 h posttransfection. Reduction of protein levels was confirmed by Western blotting and hybridization with the appropriate antibodies.

To inhibit *pp2a-cs* α, we used synthetic siRNA oligonucleotides. In this case, transient transfection of HeLa cells was carried out with 100 nM siRNA SMARTpool PP2A-cs α or a nonspecific control pool (Upstate, Dharmacon) using the Oligofectamine method as described by the manufacturer for adherent cells (Invitrogen). Cells were collected 72 h after transfection and analyzed for PP2A-cs α expression.

We also transfected HeLa cells with expression vectors for hSec VSV, HA B55δ(233–453), or empty vectors, along with either siRNA SMARTpool PP2A-cs α or a control pool siRNA using the Lipofectamine (Invitrogen) method. Cells were collected 48 h after transfection. When indicated, LLnL was added 24 h before harvesting of the cells.

RESULTS

hSecurin associates with protein phosphatase PP2A. A yeast two-hybrid screen was used to identify proteins that interacted with hSecurin, (residues 1 to 163) (44). Among the positive clones isolated from a Jurkat cDNA library, we identified one which encoded the human isoform δ of the B55 family of PP2A regulatory subunits (residues 233 to 453) (Fig. 1A). Furthermore, in vitro binding experiments demonstrated that hSecurin also associates with the PP2A catalytic subunit (PP2A-cs). Lysates from HeLa cells were incubated with either full-length 6His-hSecurin or truncated 6His-hSecurin(1–163) bound to Ni-NTA agarose. After washing the resin, proteins were Western blotted for the detection of PP2A-cs (Fig. 1B). PP2A from cellular extracts associated with both full-length and truncated 6His-hSecurin proteins, whereas there was no interaction with an irrelevant fusion protein (6His-RIIα protein). Finally, we demonstrated that endogenous hSecurin forms a complex with the PP2A catalytic and B55δ regulatory subunits by coimmunoprecipitation experiments. Lysates from HeLa cells were incubated with anti-hSecurin polyclonal antibody or with preimmune serum. The immunoprecipitated material was detected for the presence of the PP2A catalytic and B55δ regulatory subunits (Fig. 1C). hSecurin coimmunoprecipitated with both PP2A subunits. Similar results were obtained with extracts from human colorectal HCT116 cells (data not shown).

hSecurin associates with enzymatically active PP2A. We tested whether PP2A associated with hSecurin is catalytically active. To this aim, anti-hSecurin and mock preimmune serum immunoprecipitates were obtained from lysates of HCT116 cells. Immunocomplexes were incubated with a PP2A phosphopeptide substrate, and the amount of released phosphate was measured. Phosphatase activity was markedly greater in anti-hSecurin than in mock preimmune serum immunoprecipitates (Fig. 2A). This activity was highly sensitive to okadaic acid treatment, with an inhibition of 50% obtained with 1 nM concentration (Fig. 2B). Since the 50% inhibitory concentration (IC_{50}) is close to the known sensitivity of PP2A, we conclude that the PP2A bound to hSecurin is catalytically active.

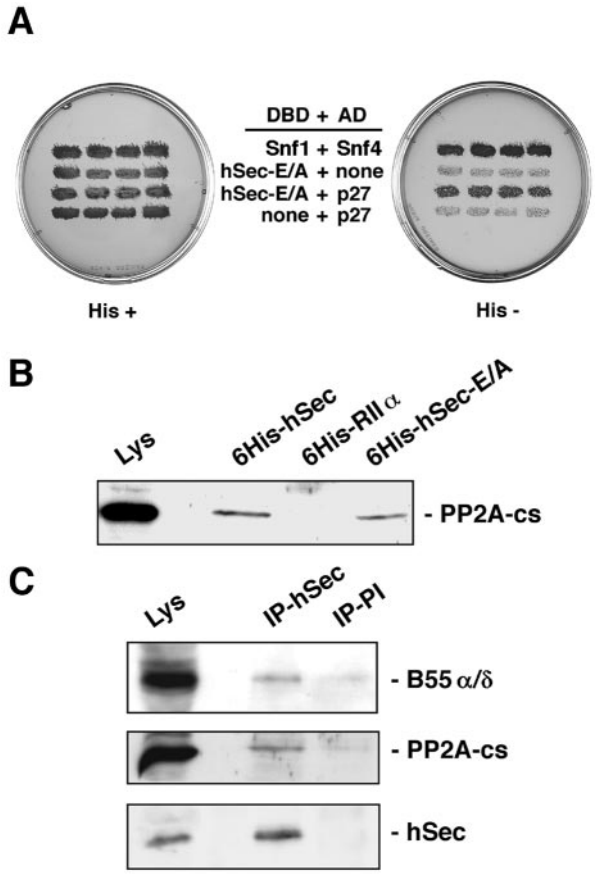

FIG. 1. hSecurin interacts with the serine/threonine PP2A phosphatase. (A) hSec-E/A (residues 1 to 163 of hSecurin) interacts with p27 (residues 233 to 453 of PP2A-B55δ) in a yeast two-hybrid assay. Hf7c reporter strain was cotransformed with the indicated constructs. The interaction between the two-hybrid proteins is indicated by growth in the absence of histidine (dark gray patch). DBD, fusion with the DNA-binding domain of Gal4; AD, fusion with the activation domain of Gal4; none, empty vector. Snf1-Snf4 interaction was used as a positive control. (B) hSecurin interacts with PP2A-cs in vitro. Six-His fusion proteins were incubated with NP40 extracts from HeLa cells, purified on an Ni-NTA agarose column, and their association with PP2A-cs was determined by immunoblotting. RIIα is an unrelated protein. Lys, lysate from HeLa cells. (C) hSecurin binds to the PP2A complex in vivo. Anti-hSecurin and preimmune (PI) sera were used in immunoprecipitations from NP40 extracts of HeLa cells. The immunoprecipitates (IP) were probed for the presence of PP2A-B55α/δ, PP2A-cs, and hSecurin. Lys, lysate from HeLa cells.

hSecurin interacts with PP2A in all phases of the cell cycle. We wanted to determine whether PP2A associated with hSecurin throughout the cell cycle. To this end, HeLa cells were arrested in G_1, S, G_2, and M phases and the level of PP2A that associated with hSecurin in each phase was tested by coimmunoprecipitation. PP2A-cs associated with immunoprecipitated hSecurin throughout the cell cycle, and the level of coimmunoprecipitated material was relative to that of hSecurin, reaching a peak in metaphase-arrested cells (Fig. 3A). Likewise, phosphatatase activity was also observed in hSecurin immunoprecipitates at each cell cycle phase (Fig. 3B). The level of activity was dependent on the amount of immunoprecipitated

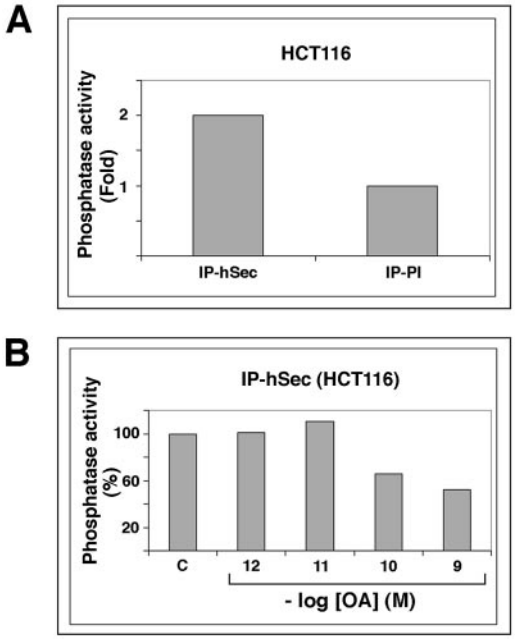

FIG. 2. hSecurin immunoprecipitates contain phosphatase activity sensitive to okadaic acid. (A) hSecurin was immunoprecipitated from NP40 extracts of HCT116 cells. Preimmune serum (PI) was used as a control. Phosphatase activity level was standardized to that obtained from immunoprecipitates (IP) using the preimmune serum. (B) Immunoprecipitates of hSecurin from NP40 extracts of HCT116 cells were incubated with increasing concentrations of okadaic acid (OA). Activities are represented as a percentage of that observed in the control containing no OA (C). Similar findings were obtained in five separate experiments.

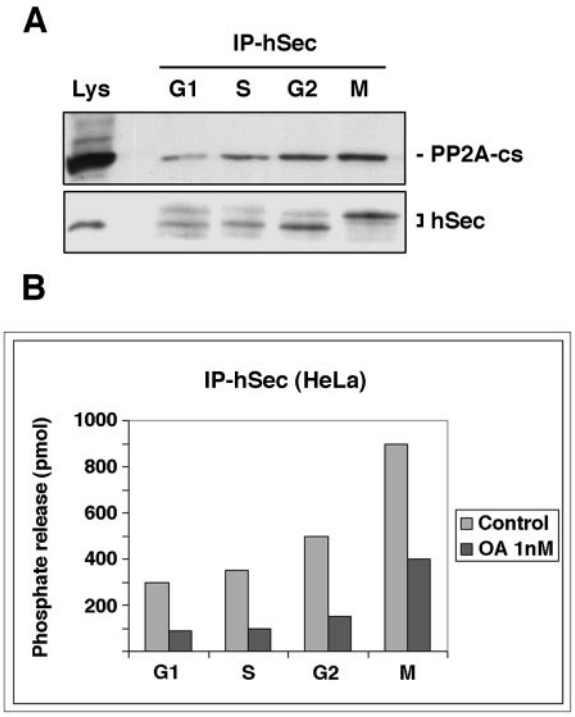

FIG. 3. The phosphatase activity associated with hSecurin immunoprecipitates is dependent on the stage of the cell cycle and inhibited by okadaic acid. (A) hSecurin was immunoprecipitated from extracts of synchronized HeLa cells harvested at various cell cycle phases. Immunoprecipitates (IP) were probed for the presence of hSecurin and PP2A-cs. Lys, lysate from asynchronous HeLa cells. (B) Specific phosphatase activity of hSecurin immunoprecipitates, obtained as described for panel A, was measured in presence or absence of 1 nM okadaic acid (OA). Phosphatase activity was calculated as the amount of phosphate released from a synthetic phosphothreonine peptide. When indicated, OA was added to assay mixtures. Similar findings were obtained in five separate experiments.

hSecurin and was inhibited at low concentrations of okadaic acid in all cases.

hSecurin is a substrate of PP2A both in vitro and in vivo. Little is known about the regulation of hSecurin phosphorylation in vivo. It has been previously published that the main phosphorylated form of hSecurin appears in cells arrested in metaphase after treatment with nocodazole and is Cdc2 dependent (41). Mitotically phosphorylated hSecurin is observed as a band whose migration is retarded in SDS-polyacrylamide gels. We explored the possibility that phosphorylated mitotic hSecurin may be a substrate of PP2A. To this end, hSecurin was immunoprecipitated from nocodazole-treated HeLa cell extracts and incubated with commercially obtained PP2A phosphatase. After treatment, there appeared little difference in the relative levels of phosphorylated and nonphosphorylated forms of hSecurin. However, lambda protein phosphatase (λ-PP) was able to dephosphorylate the mitotic phosphorylated form of hSecurin (Fig. 4A). Then, to confirm that the mitotic phosphorylated form of hSecurin is not a substrate of PP2A, we immunoprecipitated B55δ from HeLa cell extracts and tested its ability to dephosphorylate the mitotic phosphorylated form of hSecurin. Nevertheless, no effect was detected, even when commercial PP2A was also added (Fig. 4B, upper panel), while B55δ immunoprecipitates were able to dephosphorylate other phosphorylated forms of hSecurin (Fig. 4B, lower panel). Therefore, we conclude that PP2A does not dephosphorylate the main mitotic phosphorylated form of hSecurin.

To further investigate whether hSecurin is a substrate of PP2A, we treated cells with okadaic acid in order to observe hyperphosphorylated forms of hSecurin. Cell lysates of HeLa cells incubated in the presence of 1 μM okadaic acid for 1 h 30 min prior to harvesting were immunoblotted for hSecurin detection. Surprisingly, very little hSecurin could be detected (Fig. 4C). The addition of the proteasome inhibitor Ac-LLnL-CHO (LLnL) to cells incubated with okadaic acid, however, revealed the presence of several retarded bands. Incubation of cell extracts, from LLnL- and okadaic acid-treated cells, with λ-PP exposed these bands as being hyperphosphorylated forms of hSecurin. Thus, the proteasome inhibitor prevents the degradation of the phosphorylated forms of hSecurin in the presence of okadaic acid. At the concentration used in this experiment, okadaic acid mainly inhibits PP2A phosphatase activity, but we cannot rule out the possibility that it could also inhibit other phosphatases. In a more direct experiment to determine whether PP2A has a role in the phosphorylation status of hSecurin, we analyzed the appearance of phosphorylated bands when the expression level of the PP2A-cs was reduced by using siRNA. As shown in Fig. 4D, the interference of PP2A-cs

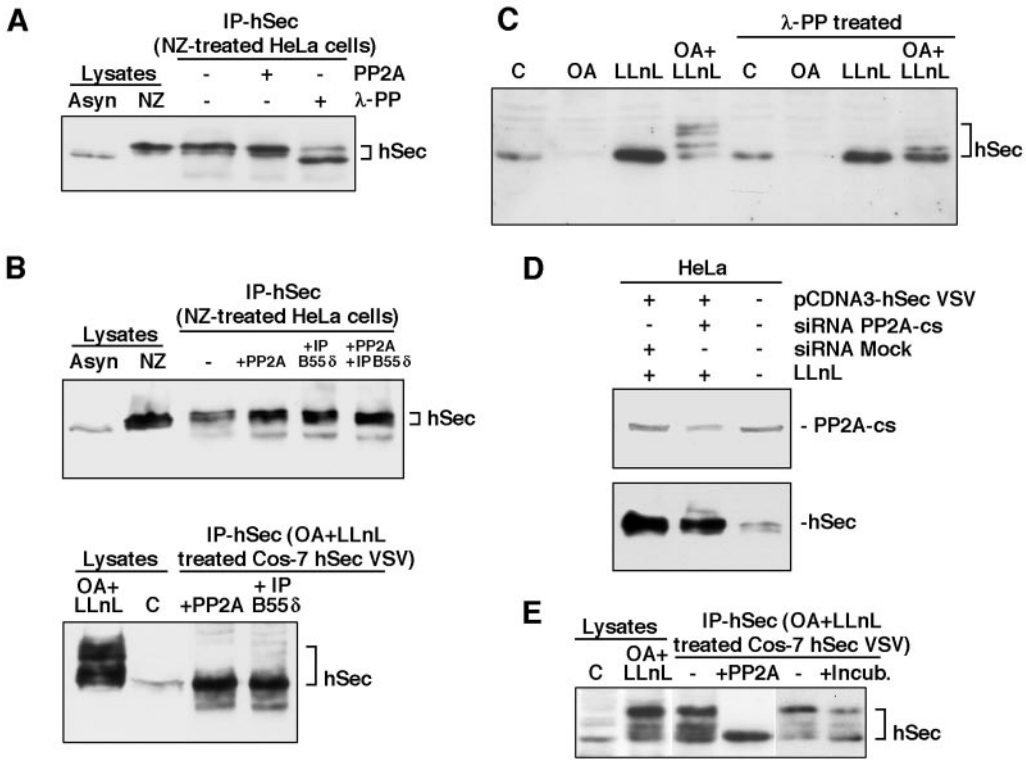

FIG. 4. Specific phosphorylated forms of hSecurin, which are degraded by the proteasome in the presence of okadaic acid, are substrates of PP2A phosphatase. (A) Phosphorylated forms of hSecurin from nocodazole-treated cells are not substrates of PP2A phosphatase. hSecurin immunoprecipitates (IP) from nocodazole-treated HeLa cells were incubated with or without commercially obtained PP2A or lambda protein phosphatase (λ-PP) prior to Western blotting for detection of hSecurin. Asyn, lysate from asynchronous HeLa cells; NZ, lysate from nocodazole-treated HeLa cells. (B) In the upper panel, similarly to panel A, hSecurin immunoprecipitates from nocodazole-treated HeLa cells were incubated with commercial PP2A (+PP2A), B55δ immunocomplexes (+ IP B55δ), or both commercial PP2A and B55δ immunocomplexes, prior to Western blotting for detection of hSecurin. −, hSecurin immunoprecipitates from nocodazole-treated HeLa cells; Asyn, lysate from asynchronous HeLa cells; NZ, lysate from nocodazole-treated HeLa cells. (Lower panel) Cos-7 cells were transiently transfected with epitope-tagged hSecurin VSV (hSec VSV) prior to treatment with both okadaic acid (OA), and the proteasome and calpain inhibitor Ac-LLnL-CHO (LLnL). hSecurin immunoprecipitates from these cells were incubated either with commercial PP2A or B55δ immunocomplexes. Lysates and immunoprecipitates were Western blotted for hSecurin detection. Lane C, lysates from nontreated transfected Cos-7 cells; OA+LLnL, lysates from okadaic acid- and LLnL-treated transfected Cos-7 cells. (C) HeLa cells were treated with okadaic acid, LLnL, or both for 1 h 30 min prior to treatment with or without λ-PP for 30 min. Lysates were subsequently Western blotted for the detection of hSecurin. Lanes C, lysate from control untreated HeLa cells. (D) HeLa cells were transiently transfected with pCDNA3-hSec VSV along siRNA oligonucleotides against PP2A-cs α or nonspecific control oligonucleotides (mock) and collected after 48 h. LLnL was added 24 h after transfections. Extracts were Western blotted for PP2A-cs and hSecurin detection. −, lysate from nontransfected and nontreated HeLa cells. (E) Cos-7 cells were transiently transfected with hSec VSV prior to treatment with both okadaic acid and LLnL. hSecurin immunoprecipitates from these cells were incubated either with (+PP2A) or without (+Incub.) commercial PP2A. Lysates and immunoprecipitates were Western blotted for hSecurin detection. −, nonincubated hSecurin immunoprecipitates. Lane C, lysates from nontreated transfected Cos-7 cells; OA+LLnL, lysates from okadaic acid-and LLnL-treated transfected Cos-7 cells.

in the presence of LLnL revealed a retarded band that was not present in the control using scrambled sequences of siRNA. This result strongly supports the idea that hSecurin is a substrate of PP2A.

Next, we wished to determine whether the hyperphosphorylated forms of hSecurin observed in the presence of okadaic acid were indeed substrates of PP2A. In order to better detect hSecurin, we transiently transfected Cos-7 cells with epitope-fused hSecurin VSV. Subsequently, these cells were treated with both okadaic acid and LLnL prior to immunoprecipitation of hSecurin. Like with endogenous hSecurin, hSecurin VSV became hyperphosphorylated. However, when incubated with commercial PP2A, only the faster-migrating band was observed, showing that hSecurin is a substrate of PP2A in vitro. Also, when incubated with itself, the relative ratio of slowly migrating bands to the fastest migrating band decreased, strongly suggesting that hSecurin is an in vivo substrate of PP2A.

Phosphorylation of hSecurin triggers its degradation via the SCF E3 ubiquitin ligase. The instability of hSecurin in cells treated with okadaic acid, which in addition was reversed in the presence of the proteasome inhibitor, prompted us to investigate whether either the APC/C or the SCF E3 ubiquitin ligases were required for targeting hSecurin to the proteasome for destruction. We first tested the involvement of the APC/C in this process, since hSecurin is a known substrate of this ubiquitin ligase. hSecurin degradation by APC/C is mediated by two degradation motifs, an RXXL destruction box (D-box) and a KEN-box, and is only completely inhibited when both sequences are mutated (59). Cos-7 cells, transiently transfected

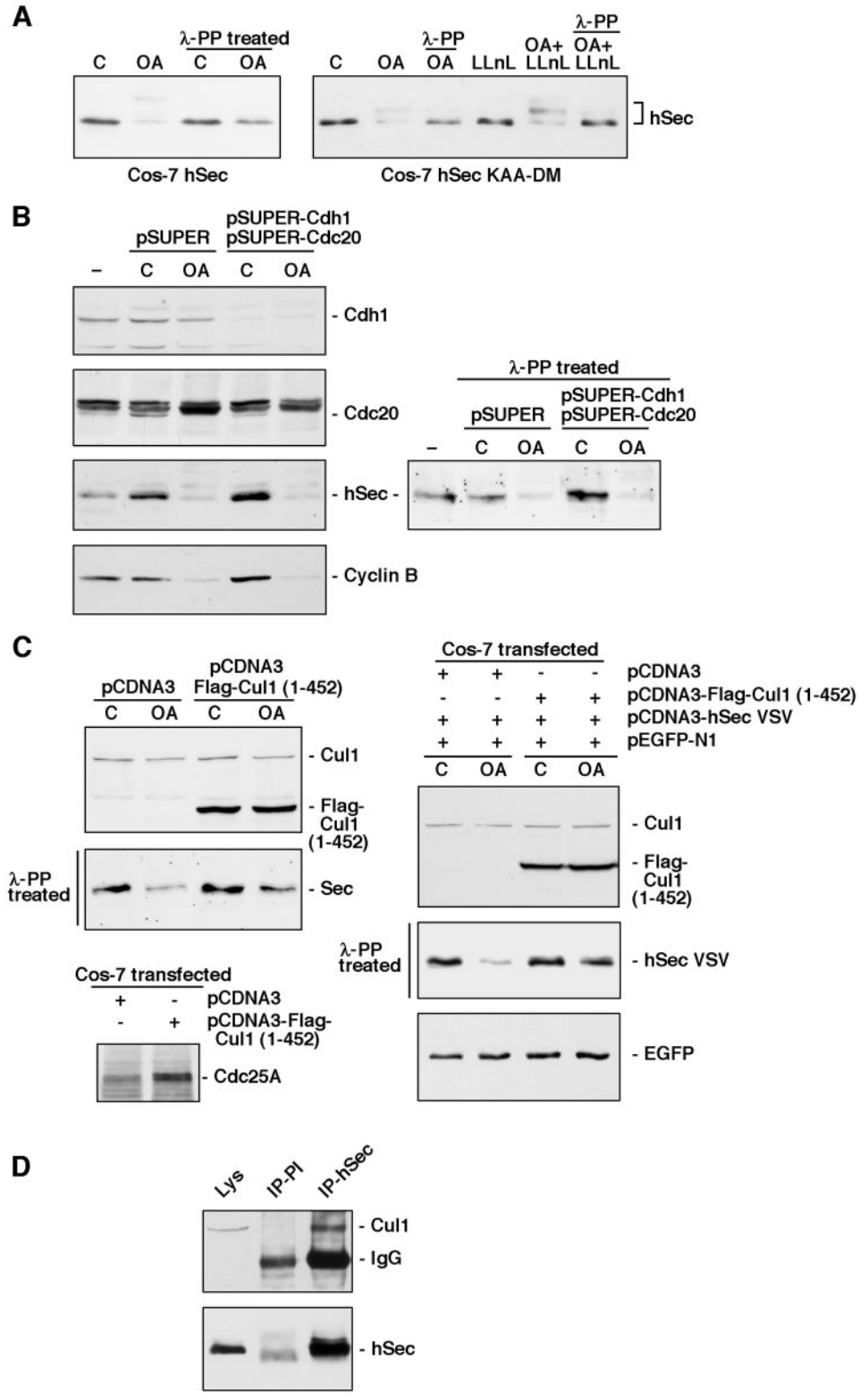

FIG. 5. Phosphorylated forms of hSecurin induced by okadaic acid are not APC/C substrates but are degraded via the SCF E3 ubiquitin ligase. (A) Cos-7 cells transiently transfected with either hSecurin (hSec) or hSecurin with KEN-box and D-box mutations (hSec KAA-DM) were treated with okadaic acid (OA), LLnL, or both. Extracts, where indicated, were treated with λ-PP for 30 min. Western-blotted extracts were detected for hSecurin. Lanes C, lysates from nontreated transfected Cos-7 cells. (B) HeLa cells were transiently cotransfected with pSUPER-Cdh1 and pSUPER-Cdc20 or with pSUPER alone and harvested 48 h after transfection. When indicated, okadaic acid was added 1 h 30 min before cells were harvested. Extracts were Western blotted for the detection of Cdh1, Cdc20, hSecurin, and cyclin B. Note that the Western blot for Cdc20 shows three bands, the upper two (and most prominent) of which are cross-reactive and not specific to Cdc20. The right panel shows a Western

with an expression vector for the nondegradable form of hSecurin (hSecurin KAA-DM) as well as wild-type hSecurin, were treated with okadaic acid, and the level of hSecurin was analyzed by Western blotting. Both hSecurin and hSecurin KAA-DM levels were similarly reduced after okadaic acid treatment, compared to cells that were not treated with okadaic acid or with both okadaic acid and LLnL (Fig. 5A). This observation is consistent with the notion that the APC/C is not involved in the degradation of hSecurin in cells treated with okadaic acid.

Degradation of hSecurin via the APC/C is mediated by two accessory proteins, Cdc20 and Cdh1. To confirm the above result, both Cdc20 and Cdh1 transcripts were depleted by RNA interference. HeLa cells, transiently transfected with both pSUPER-Cdh1 and pSUPER-Cdc20 (5), were incubated either with or without okadaic acid, and the level of hSecurin was analyzed by Western blotting (Fig. 5B). Both Cdh1 and Cdc20 levels were reduced, leading to an accumulation of hSecurin and cyclin B in the absence of okadaic acid. However, lack of both APC/C activators failed to increase the stability of hyperphosphorylated hSecurin in okadaic acid-treated cells. λ-PP treatment of extracts from transfected or interfered cells, in order to pack all potential phosphorylated forms of hSecurin into one band, confirmed the previous results. Thus, we conclude that the APC/C is not involved in targeting hSecurin for destruction in okadaic acid-treated cells.

We subsequently entertained the possibility that hSecurin was targeted for degradation by the proteasome via the SCF E3 ubiquitin ligase. To this end, we used an N-terminal Cul1 fragment that blocks SCF-mediated degradation due to the formation of catalytically inactive complexes (54). We transiently transfected Cos-7 cells with a plasmid containing a Cul1 fragment encoding residues 1 to 452 [pCDNA3 Flag-Cul1(1–452)] and confirmed that the truncated form of Cul1 led to an accumulation of its known substrate, Cdc25A (14) (Fig. 5C, lower panel). Next, we examined whether it was able to block securin degradation after okadaic acid treatment. The expression of Flag-Cul1(1–452) led reproducibly to an increase of securin after okadaic acid treatment that was especially visible after the retarded phosphorylated forms of securin were reduced to a single band after λ-PP treatment. Similar results were obtained by using ectopically expressed hSecurin (Fig. 5C). These results are consistent with the notion that securin is a substrate of the SCF complex that targets phosphorylated forms of securin for destruction. In fact, endogenous hSecurin and Cul1 proteins coimmunoprecipitated from HeLa cells (Fig. 5D).

PP2A stabilizes hSecurin via the B55δ regulatory subunit. Two observations point to an involvement of PP2A in stabilizing cellular levels of hSecurin. First, PP2A containing the B55δ regulatory subunit directly binds to hSecurin. Second, okadaic acid, a potent inhibitor of PP2A phosphatase, both prevents dephosphorylation of hSecurin and promotes its proteasomal degradation (see above). To address this possibility, we first analyzed the level of securin in cells overexpressing B55δ regulatory protein. If B55δ directly targets PP2A-cs to hSecurin, its overexpression should direct a larger portion of the cellular pool of catalytic PP2A subunits to dephosphorylate and thus stabilize hSecurin. We directly compared the amount of securin in mock transiently transfected cells (pECE) with that in cells transiently transfected with a construct containing a truncated form of B55δ comprising residues 233 to 453 [pECE-B55δ(233–453)]. This region included the conserved binding domain 2 that is able to bind the scaffold A subunit of PP2A, which in turn binds to PP2A-cs (32). Overexpression of B55δ(233–453) caused a dramatic and specific increase in the level of endogenous securin, while cyclin B levels were not altered (Fig. 6A, left panels). To discount the possibility that this accumulation was due to increased transcriptional or translational activity of the endogenous hSecurin gene, we analyzed the effect of the overexpression of B55δ on ectopically expressed hSecurin, using a constitutive heterologous promoter. Ectopic hSecurin also accumulated to high levels in such cells (Fig. 6A, right panels).

Next, we measured the hSecurin half-life in cells overexpressing B55δ(233–453). To this end, Cos-7 cells were transiently cotransfected with pCDNA3-hSec VSV and either pECE or pECE-B55δ(233–453) plasmid. Expression was induced for 18 h, cycloheximide was added to the media, and cells were collected over a 2-h period. As expected, the half-life of hSecurin VSV was higher in cells expressing B55δ than in mock-treated cells (Fig. 6B). Thus, increased levels of the B55δ form of PP2A are sufficient to lead to an accumulation of hSecurin due to promoting its stability.

Although we previously showed that okadaic acid treatment of HeLa cells resulted in reduced levels of hSecurin, it is possible that not only was PP2A phosphatase activity inhibited, but so was that of other phosphatases that are also sensitive to this drug. To test the specific role of PP2A in hSecurin stability, we analyzed the level of hSecurin when PP2A expression was specifically inhibited by using RNA interference. siRNA directed against the catalytic subunit of PP2A efficiently reduced the level of endogenous PP2A-cs. Coincident with this reduction, the amount of hSecurin was dramatically decreased (Fig. 6C). In contrast, scrambled sequences of siRNA (mock) had no effect on the level of the PP2A-cs and did not alter the hSecurin level. Moreover, cyclin B levels were again unaltered. In contrast, scrambled sequences of siRNA (mock) had no effect on the level of the PP2A-cs and did not alter the hSecurin level. Moreover, cyclin B levels were again unaltered.

blot of extracts which were treated with λ-PP. −, lysates from nontransfected HeLa cells. (C) In the left panels, Cos-7 cells transiently transfected with pCDNA3 or pCDNA3 Flag-Cul1(1–452) were treated either with (lanes OA) or without (lanes C) okadaic acid before harvesting. NP40 extracts were prepared and were Western blotted for Cul1, Flag-Cul1(1–452), and securin detection. Where indicated, extracts were treated with λ-PP. Expression of Cdc25A was also tested in Cos-7-transfected cells. (Right panels) Cos-7 cells transiently transfected with the indicated plasmids were treated either with (lanes OA) or without (lanes C) okadaic acid before harvesting. NP40 extracts were prepared and were Western blotted for Cul1, Flag-Cul1(1–452), hSec VSV, and EGFP detection. Where indicated, extracts were treated with λ-PP. (D) Anti-hSecurin and preimmune (PI) sera were used in immunoprecipitations from NP40 extracts of LLnL-treated Cos-7 cells. The immunoprecipitates (IP) were probed for the presence of Cul1 and hSecurin. Lys, lysate from LLnL-treated Cos-7 cells; IgG, the IgG heavy chains.

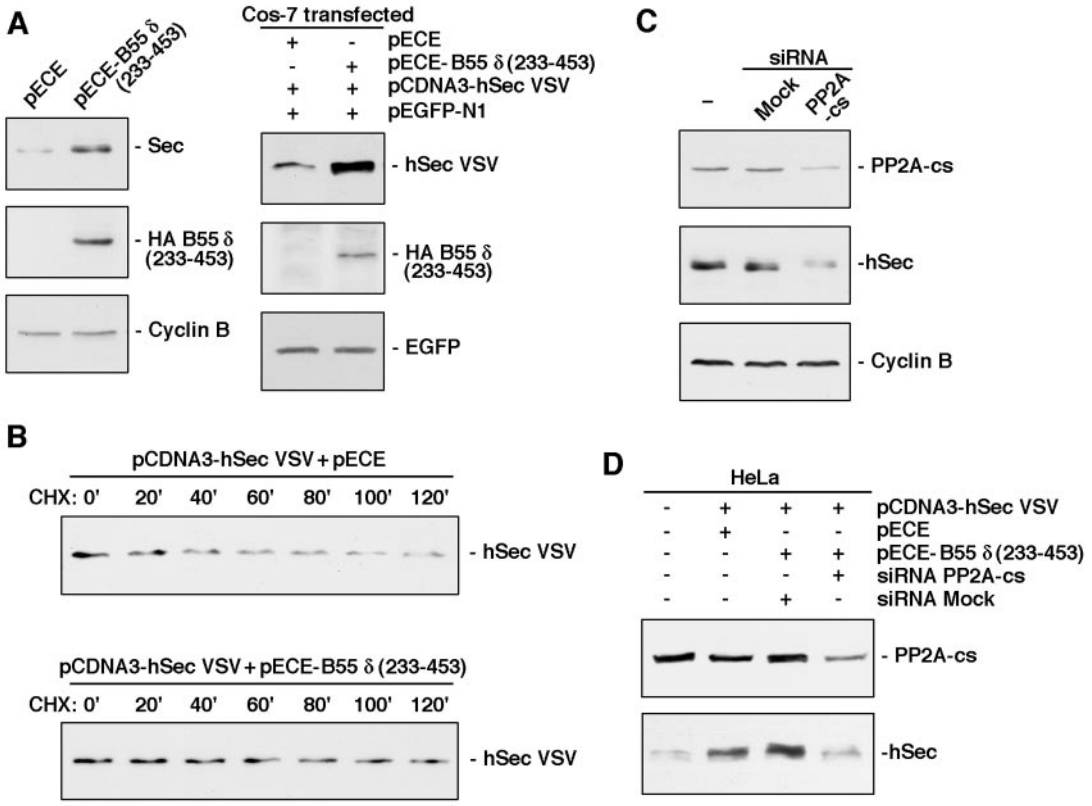

FIG. 6. The PP2A phosphatase complex stabilizes hSecurin. (A) Cos-7 cells were transiently transfected with either empty vector (pECE) or pECE-B55δ(233–453). Endogenous securin levels (upper left panel) or cotransfected hSecurin VSV (pCDNA3-hSec VSV; upper right panel) was detected in immunoblotted lysates. EGFP (pEGFP-N1; right panel) was used as an internal control for transfection efficiency. (B) Cos-7 cells were transiently cotransfected with hSecurin VSV (hSec VSV) and either HA B55δ [pECE-B55δ(233–453)] or empty vector (pECE), and their expression was induced for 18 h. Cycloheximide (CHX; 50 μg/ml) was added to the media to inhibit protein synthesis, and cells were collected at the indicated times after addition of CHX. hSecurin VSV levels in cell lysates were detected by immunoblotting with anti-VSV monoclonal antibody. (C) HeLa cells were transiently transfected with siRNA oligonucleotides against PP2A-cs α or nonspecific control oligonucleotides (mock) and collected after 72 h. Western blots of extracts were analyzed for PP2A-cs, hSecurin, and cyclin B levels. −, extract from noninterfered HeLa cells. (D) HeLa cells were transiently transfected with the indicated plasmids along siRNA oligonucleotides against PP2A-cs α or nonspecific control oligonucleotides (mock) and collected after 48 h. Extracts were Western blotted for PP2A-cs and hSecurin detection.

Finally, we investigated whether the hSecurin stabilization observed after overexpression of B55δ was simply due to its binding to hSecurin or to its protection against phosphorylation. To this aim, we analyzed the effect of the B55δ regulatory subunit on hSecurin stability under conditions that do not allow PP2A-cs to act over hSecurin. B55δ was overexpressed, and simultaneously PP2A-cs was interfered with by siRNA. As shown in Fig. 6D, the overexpression of B55δ not only did not increase the hSecurin level, but even this level was lower than the basal one. Taken together, these results demonstrate that PP2A contributes to stabilization of hSecurin by dephosphorylating phospho-residues that promote its degradation through the SCF ubiquitin pathway.

DISCUSSION

The experiments described in this paper show that human securin can associate with the fully assembled, enzymatically active PP2A holoenzyme. We identified through a yeast two-hybrid screen, using hSecurin as bait, the B55δ regulatory subunit of PP2A. Pull-down and coimmunoprecipitation assays demonstrated that hSecurin interacts specifically with the PP2A complex both in vitro and in vivo and that this association does not depend on the cell cycle phase. Among the proteins shown to associate with PP2A and regulate its activity are the small t antigens encoded by SV40 and polyomavirus, the adenovirus E4orf4 protein, casein kinase II, and Hox II (19). In some cases, the interaction results in a negative regulation of PP2A activity. Although we have not determined its effect on the specific activity of PP2A, hSecurin clearly does not block its enzymatic activity.

Our discovery that hSecurin associates with enzymatically active PP2A provided the impetus for testing whether phosphorylated hSecurin might serve as a substrate of PP2A. Our data strongly support the conclusion that this is the case. Experiments performed in vitro using a commercial source of PP2A and phosphorylated hSecurin as a substrate revealed that PP2A is able to dephosphorylate the various forms of phosphorylated hSecurin obtained from okadaic acid-treated cells but not the main phosphorylated form of hSecurin obtained from mitotically arrested nocodazole-treated cells. Thus, the ability of PP2A to dephosphorylate

hSecurin is probably dependent on the specific phosphorylated residue.

The observation that okadaic acid treatment of cells results in the destabilization of hSecurin suggests that PP2A may regulate hSecurin stability. Okadaic acid has been shown to preferentially inhibit PP2A at the low concentration used in these experiments. However, we cannot rule out that okadaic acid does not inhibit to some degree the other major serine-threonine phosphatases PP1, PP2B/calcineurin, and PP2C or other less-well-characterized phosphatases (11). Nevertheless, two observations strongly implicate PP2A in the regulation of hSecurin stability. First, decreasing cellular PP2A activity, as a result of using RNA interference against the catalytic subunit of PP2A, leads to a reduction in hSecurin levels. Second, increasing the cellular levels of the PP2A regulatory subunit B55δ promotes the accumulation of hSecurin by reducing its turnover, and which is very meaningful, this B55δ effect requires the phosphatase activity of PP2A. Moreover, the effect of PP2A on the stabilization of hSecurin is not a consequence of the influence of PP2A on the cell cycle progression, since cyclin B levels remain unchanged in either cells overexpressing B55δ or cells with reduced levels of PP2A-cs. In conclusion, we propose that the association of PP2A to hSecurin leads to hSecurin stabilization via its dephosphorylation.

It has been well documented that PP2A activity is important for the regulation of the stability of the proto-oncogenic transcription factor c-Myc. The fine tuning of c-Myc expression occurs at the protein level through modulation of its stability (3, 21). The half life of c-Myc polypeptides is determined largely by phosphorylation of two specific residues found in all mammalian Myc family members, which results in its degradation via the ubiquitin proteasome pathway (21, 40). PP2A was shown to be required to dephosphorylate c-Myc at serine 62, leading to its proteolysis (56). Another example of proteins in which PP2A regulates their stability is the Pim family of protein kinases. The Pim kinases associate with the catalytic subunit of PP2A in vivo and are substrates of PP2A phosphatase in vitro. Overexpression of PP2A decreases the steady-state levels of the Pim proteins, and the inhibition of PP2A activity by okadaic acid enhances the stability of Pim-1 (33).

In an attempt to determine the effect of okadaic acid treatment, and thus PP2A action, on the phosphorylation status of hSecurin, we found that not only was hSecurin hyperphosphorylated, but the various hyperphosphorylated forms were extremely unstable, being rapidly degraded by the proteasome. Knowing that the APC/C E3 ubiquitin ligase targets hSecurin for destruction during anaphase and mitotic exit, we naturally studied its involvement in okadaic acid-mediated instability of hSecurin. To be fully active, APC/C requires two additional subunits that regulate its target specificity, Cdc20 and Cdh1, thus forming two distinct complexes (APC/C^{Cdc20} and APC/C^{Cdh1}) (37). Target specificity is due to the ability of Cdc20 and Cdh1 to recognize two specific destruction sequences on APC/C substrates, namely the D-box and KEN-box motifs (18, 39). hSecurin degradation is catalyzed by both Cdc20 and Cdh1, is mediated by a D-box and a KEN-box, and is inhibited only when both sequences are mutated (59). Our results demonstrate that degradation of phospho-hSecurin, produced during okadaic acid treatment, is not dependent on the APC/C. First, the nondegradable form of hSecurin (with both D- and KEN-box motifs mutated) is not resistant to okadaic acid-mediated degradation. Second, the depletion of both APC/C activators, Cdc20 and Cdh1, by siRNA was not able to block the degradation of phospho-hSecurin. We thus turned to investigate the involvement of other ubiquitin ligases in this degradation process. Knowing that the action of the SCF ubiquitin ligase complex is tightly coupled to substrate phosphorylation status, we decided to study whether the SCF plays a role in the degradation of phospho-hSecurin. The SCF ligase has a broad function in many physiological processes by ubiquitinating proteins involved in, for example, cell cycle regulation, transcription, and signal transduction (22, 29, 38). Recruitment of substrates to the SCF complex occurs via one of a variety of F-box protein subunits, which function as molecular adaptors (8, 53). F-box proteins interact via the F-box motif with the Skp1 subunit, which can bridge the F-box to the cullin (35), which in turn serves as a scaffold to bring the catalytic RING finger protein to the substrate (50). Substrate recognition by the F-box protein is mediated in most cases by phosphorylation of target proteins (22, 25).

To test the possible involvement of the SCF in mediating hSecurin degradation in okadaic acid-treated cells, we made use of a dominant-negative N-terminal Cul1 mutant that interferes with the degradation of SCF substrates (54). Upon expression of this mutant by transient transfection, hSecurin was substantially stabilized in okadaic acid-treated cells. This result provides evidence that the SCF is involved in the proteolysis of phospho-hSecurin and indicates that the abundance of hSecurin in the cell is regulated not only by the APC/C during metaphase and mitotic exit, but also by the SCF at other cell cycle stages. Such a dual mode of degradation has been described for the Cdc25A phosphatase, an activator of cell cycle progression. Both the APC/C and the SCF are involved in Cdc25A turnover. While the APC/C degrades Cdc25A during mitotic exit, SCF regulates its abundance in the S and G_2 phases. Moreover, in response to DNA damage or to stalled replication, the activation of the ATM and ATR protein kinases results in Chk1 and Chk2 activation and Cdc25A hyperphosphorylation that stimulate SCF-mediated ubiquitination of Cdc25A and its proteolysis (6, 7, 14). In this context, it is worth mentioning that hSecurin is also degraded in response to irradiation (43). The new findings reported here raise additional important questions that will need to be addressed in the future. These include the nature of the phosphorylation events that trigger hSecurin degradation and the role of SCF ubiquitination in regulating hSecurin levels during a normal cell cycle as well as in response to DNA damage. Unraveling the mechanisms involved in regulating hSecurin stability is of additional interest in that it may contribute to explaining the cause of the high hSecurin levels detected in many tumors, since no mutations in its promoter or in its open reading frame have been found to date, and it may further our understanding of how such high levels lead to oncogenesis.

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