UV-induced degradation of securin is mediated by SKP1-CUL1-βTrCP E3 ubiquitin ligase

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Summary

Securin is a chaperone protein with bifunctional properties. It binds to separase to inhibit premature sister chromatid separation until the onset of anaphase, and it also takes part in cell-cycle arrest after UV irradiation. At metaphase-to-anaphase transition, securin is targeted for proteasomal destruction by the anaphase-promoting complex or cyclosome (APC/C), allowing activation of separase. However, although securin is reported to undergo proteasome-dependent degradation after UV irradiation, the ubiquitin ligase responsible for securin ubiquitylation has not been well characterized. In this study, we show that UV radiation induced a marked reduction of securin in both the nucleus and cytoplasm. Moreover, we show that GSK-3β inhibitors prevent securin degradation, and that CUL1 and βTrCP are involved in this depletion. We also confirmed that SKP1-CUL1-βTrCP ($SCF^{\beta TrCP}$) ubiquitylates securin in vivo, and identified a conserved and unconventional βTrCP recognition motif (DDAYPE) in the securin primary amino acid sequence of humans, nonhuman primates and rodents. Furthermore, downregulation of βTrCP caused an accumulation of securin in non-irradiated cells. We conclude that $SCF^{\beta TrCP}$ is the E3 ubiquitin ligase responsible for securin degradation after UV irradiation, and that it is involved in securin turnover in nonstressed cells.

Key words: Cell cycle, Degradation, Proteasome, Securin, Ubiquitylation, Ultraviolet radiation

Introduction

Mitosis is a process that results in the segregation of sister chromatids into two newly made cells. During anaphase, sister chromatids synchronously lose cohesion, allowing the replicated chromosomes to segregate to opposite ends of the cell. Separase (official protein symbol ESPL1 for extra spindle poles-like 1) is the key regulator for this segregation. This cysteine protease cleaves the cohesin subunit SCC1-RAD21, which opens the cohesin ring and causes it to dissociate from chromosomes. In vertebrates, separase activation is regulated by both securin (PTTG1 or pituitary tumor-transforming protein 1) and phosphorylation-dependent CCNB1 (cyclin B1) binding (Gorr et al., 2005; Stemmann et al., 2001). Securin is a chaperone protein, which, following chromosome alignment, is ubiquitylated and degraded, leaving separase unbound and active (Hagting et al., 2002; Zou et al., 1999). Securin is degraded by the 26S proteasome at the metaphase-to-anaphase transition via the anaphase-promoting complex/cyclosome (APC/C). APC/C is an E3 ubiquitin ligase that requires the additional function of CDC20 (Cell division cycle protein 20 homologue) or CDH1 (CD20 homologue 1) – two WD40 adaptor proteins (Wasch and Engelbert, 2005). Moreover, securin is degraded not only via APC/C; we have recently demonstrated that some phosphorylated forms of securin are also degraded by the proteasome by the action of the SKP1–CUL1–F-box protein (SCF) complex (Gil-Bernabe et al., 2006). The SCF complex is the other major ubiquitin ligase responsible for the specific ubiquitylation of many regulators of the cell cycle. The receptor component of the SCF complex is one of many F-box proteins, three of which – SKP2, FBW7 and βTrCP – are well characterized.

There is much evidence that securin is important for other cellular functions (Bernal et al., 2002; Ishikawa et al., 2001; Romero et al., 2001). We previously described the role of human securin in cell-cycle arrest after UV irradiation, when it probably prevents cell proliferation during DNA damage repair (Romero et al., 2004). Irradiation causes rapid proteasome-dependent securin degradation and inhibition of specific securin protein synthesis. Thus, it is possible that the presence of securin and its further degradation are necessary conditions to arrest cell proliferation.

In this paper, we revisited securin degradation after UV irradiation, using siRNA, dominant-negative mutants, coimmunoprecipitation experiments, immunofluorescence microscopy, in vivo ubiquitylation assays and point mutations. We found that the proteasome-dependent securin degradation after UV irradiation is mediated by the $SCF^{\beta TrCP}$ ubiquitin ligase, and is prevented by GSK-3β inhibitors. Furthermore, we identified an unconventional motif for βTrCP in securin that is responsible for its degradation. Given that securin overexpression provokes tumor formation and that the protein is highly expressed in many tumors (Saez et al., 1999; Saez et al., 2006; Saez et al., 2002), the knowledge of mechanisms that regulate its stability may help to understand how deregulation of securin is involved in cell transformation.

Results

UV radiation induces securin degradation in both nucleus and cytoplasm

To understand the mechanism involved in securin degradation after UV irradiation, we first studied the subcellular localization of securin and analyzed its loss after irradiation. As securin is degraded in interphase after exposure to UV, and expression of the protein is null or minimal in G1 (Romero et al., 2004), we biochemically synchronized HeLa cells in S and G2 phases, and detected securin expression by immunofluorescence in control and irradiated cells.

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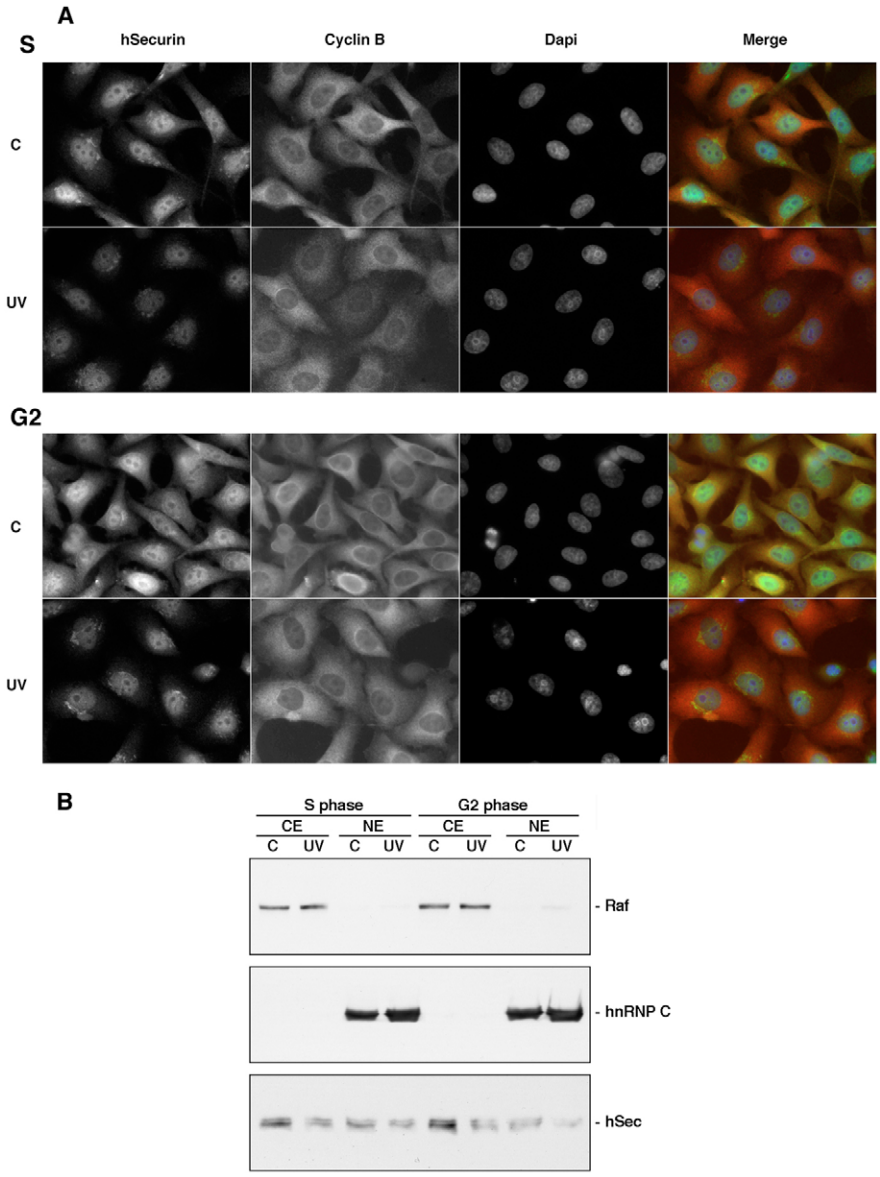

Fig. 1. Reduction in the amount of securin after UV irradiation in the S and G2 phases of the cell cycle occurs in both cytoplasm and nucleus. HeLa cells were synchronized in S and G2 phases, and exposed to UV radiation where indicated. C, synchronized and nonirradiated cells. (A) Immunofluorescence experiments using an immunoaffinity-purified polyclonal anti-securin antibody, a monoclonal anti-CCNB1 (cyclin B) antibody and DAPI. In the merge, securin staining is shown in green, cyclin B in red, and DAPI in blue. Original magnification ×403. (B) Detection of securin in subcellular fractions from synchronized cells. Fractions were separated by SDS-PAGE, transferred, and probed with the indicated antibodies. Anti-Raf and anti-hnRNP C were used to control for purity of the fractions. CE, cytosolic extracts; NE, nuclear extracts.

Securin was present in both nucleus and cytoplasm of S- and G2-arrested HeLa cells (Fig. 1A). Moreover, UV downregulation of securin was observed in both subcellular compartments. Subcellular fractionation studies of the same cells using anti-Raf and anti-hnRNP C antibodies as markers, showed that securin is more abundant in cytosolic than in nuclear extracts, as reported (Dominguez et al., 1998), and that UV-mediated depletion of securin occurred in the nucleus and cytoplasm (Fig. 1B), confirming the immunofluorescence data.

APC/C activity is not involved in securin degradation after UV exposure

In vertebrates, the APC/C ubiquitin ligase associates with CDC20 and CDH1 activators in a cell-cycle-dependent manner (Wasch and Engelbert, 2005). To determine whether securin depletion in response to UV is mediated by APC/C, we suppressed expression of the endogenous *CDC20* and *CDH1* genes, using siRNA. HeLa cells were transiently transfected with pSUPER-Cdc20 and pSUPER-Cdh1, and expression of securin after UV irradiation was analyzed by western blotting (Fig. 2A). However, although the expression level of securin slightly increased in control conditions, as well as in other APC/C substrates, such as CCNB1 (data not shown), RNA interference of *CDC20* and *CDH1* did not prevent securin degradation after UV radiation. However, despite the fact that securin degradation mediated by APC/C involves CDH1 or CDC20 (Zur and Brandeis, 2001), to eliminate the possibility that securin might also bind directly to core subunits of APC/C in a CDC20- and CDH1-independent manner, we inactivated APC/C using a pool of three target-specific siRNAs designed to knock down *CDC27/APC3* expression. APC/C contains at least 10 different, evolutionarily conserved components, and CDC27/APC3 is one of these core components (King et al., 1995; Lamb et al., 1994; Sikorski et al., 1990; Tugendreich et al., 1995). *CDC27* siRNA efficiently reduced the level of endogenous CDC27 and, coincident with this reduction, the amount of securin was slightly increased in nonirradiated cells (Fig. 2B). By contrast, scrambled sequences of siRNA (mock) had no effect on the CDC27 level, and did not alter the level of securin protein. However, CDC27 depletion did not prevent securin degradation after UV exposure. Together, these results indicate that the APC/C ubiquitin ligase is not involved in securin downregulation after UV irradiation.

SKP1-CUL1-βTrCP ubiquitin ligase is involved in UV-mediated degradation of securin

The other major class of ubiquitin ligases that has a central role in cell-cycle regulation is the SCF complex. We assessed whether SCF plays a key role in securin degradation after UV exposure. To this end, we transfected COS-7 cells with a plasmid containing a cullin 1 (CUL1) fragment ($CUL1_{1-452}$), which exerts a dominant-negative effect by forming SCF complexes lacking RING-box protein 1 (RBX1). These mutated complexes bind to the substrates but are unable to catalyze their ubiquitylation (Wu et al., 2000). In fact, this truncated form of CUL1 led to an accumulation of CDC25A (Gil-Bernabe et al., 2006), a known SCF substrate (Donzelli et al., 2002). Transfected cells were irradiated, and securin expression was studied by western blotting. Expression of $CUL1_{1-452}$

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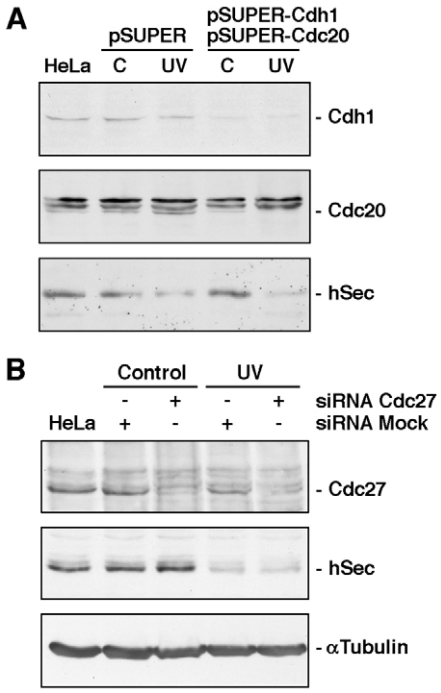

Fig. 2. UV-induced degradation of securin is not mediated by APC/C. (A) HeLa cells were transiently transfected with pSUPER-Cdh1 and pSUPER-Cdc20 or pSUPER alone, and harvested 48 hours after transfection. Where indicated, cells were irradiated 1 hour 30 minutes before harvesting. Extracts were Western blotted for the detection of CDH1, CDC20 and securin (hSec). Note that the western blot for CDC20 shows three bands, the upper two (and most prominent) of which are crossreactive and not specific to CDC20. (B) HeLa cells were transiently transfected with siRNA oligonucleotides against CDC27/APC3 or nonspecific control oligonucleotides (mock) and, after 48 hours, were irradiated or left untreated, and collected 1 hour 30 minutes later. Western blots of extracts were analyzed for CDC27/APC3, securin and α-tubulin levels. HeLa, lysates from nontransfected HeLa cells.

was able to block securin degradation after UV irradiation (Fig. 3A). Similar results were obtained when a securin vesicular stomatitis virus (VSV)-containing plasmid was also cotransfected with pCDNA3-Flag-CUL1$_{1-452}$ to prevent difficulties in interpretation of results because of a reduction in endogenous securin mRNA translation after UV irradiation (Romero et al., 2004) (Fig. 3B). Therefore, the SCF complex is involved in loss of securin after irradiation.

Of the ~70 F-box proteins identified in humans, SKP2 (S-phase kinase-associated protein 2) and βTrCP (also known as FBW1A) are thought to be involved in cell-cycle control. To discover whether one of these proteins mediates securin degradation, we used dominant-negative variants of SKP2 and βTrCP lacking the F-box domain (Margottin et al., 1998; von der Lehr et al., 2003), which couples F-box proteins to the SCF complexes. βTrCP, but not SKP2, was involved in securin degradation after UV irradiation (Fig. 3). These results were obtained on both endogenously (Fig. 3C,E) and ectopically expressed (Fig. 3D,F) securin. Taking these results together, we demonstrate that Skp1-Cul1-βTrCP is the ubiquitin ligase that mediates the UV-induced degradation of securin.

βTrCP association with securin in vivo increases after UV irradiation

To confirm that the SCF$^{\beta TrCP}$ complex is directly involved in the ubiquitylation and degradation of securin, and does not act via an intermediate regulator, we studied the βTrCP-securin interaction in vivo. COS-7 cells were transfected with pCS2-HA-βTrCP, and extracts were used to immunoprecipitate securin in the presence of the leucine-leucine-norleucine (LLnL) proteasome inhibitor to prevent securin degradation. Western blots revealed that HA-βTrCP coimmunoprecipitated with securin (Fig. 4A). Securin also coimmunoprecipitated with the dominant-negative form of βTrCP (βTrCPΔF), and this association increased after UV irradiation (Fig. 4B). These results implicate the SCF$^{\beta TrCP}$ complex in securin turnover in control cells, and explain the fact that UV radiation provokes an increase of securin degradation. Indeed, as shown in Fig. 3E, βTrCPΔF reversed the endogenous securin depletion observed after UV irradiation and increased the level of securin under nonirradiated conditions.

βTrCP mediates ubiquitylation of securin

Next, we tested in vivo ubiquitylation of securin by βTrCP. For this purpose, COS-7 cells were transiently transfected with pCDNA3-2HA-hSec and plasmids containing MT-ubiquitin or both MT-ubiquitin and MT-βTrCP (Fig. 5A). Transfected cells were incubated with LLnL for 4 hours, and lysed under strong denaturation conditions favoring preservation of ubiquitylated proteins. Filters were blotted with anti-HA to detect 2HA-hSec and its ubiquitylated forms. Fig. 5A shows that 2HA-hSec was effectively ubiquitylated and that ubiquitylation was strongly stimulated by βTrCP overexpression. Furthermore, UV radiation increased ubiquitylation of 2HA-hSec (Fig. 5B). Together, these findings demonstrate that securin is ubiquitylated by SCF$^{\beta TrCP}$ in nonstressed cells, and that this E3 ubiquitin ligase is responsible for securin degradation after UV irradiation.

Identification of a new securin destruction motif

βTrCP usually recognizes a phosphorylated DSGx$_{2-4}$S motif to target proteins for ubiquitylation and further destruction (Fuchs et al., 2004). However, many other proteins have a nonphosphorylated DSGX$_{2-4}$S-like motif (Busino et al., 2003; Kanemori et al., 2005; Watanabe et al., 2004). In fact, alignment of βTrCP-binding sites in recently identified substrates has led to the proposal of a revised consensus βTrCP-binding motif: **D**/E/S-**S**/E/D-**G**/A-**x$_{2-4}$**-**S**/E/D (Tempe et al., 2006).

Securin does not have a DSGx$_{2-4}$S motif. However, it does have a single site in accordance with the revised consensus motif (Fig. 6A). This sequence is conserved among the three isoforms of securin and in various mammalian species. To determine whether this unconventional motif is responsible for the UV-induced securin degradation, we decided to mutate Asp109 (this residue sometimes mimics the negative charge of a phosphoserine) to Gly. COS-7 cells were transfected with a plasmid containing this mutant, and irradiated. The securin mutant was not degraded after UV irradiation (Fig. 6B), in contrast to the endogenous Cdc25A protein. Therefore, we have identified the motif necessary for securin degradation mediated by βTrCP.

GSK-3β inhibitors prevent UV-induced securin degradation

We have previously demonstrated that the effects of UV radiation on securin expression were reversed with caffeine, an efficient inhibitor of cellular DNA repair (Romero et al., 2004). However, when we tested other inhibitors in signaling pathways that could be disrupted with caffeine (UCN-01 for ATR-Chk1 pathway, 8-Br-cAMP for cAMP levels, and caspase inhibitors for apoptosis), we were unable to prevent the reduction in the amount of the protein.

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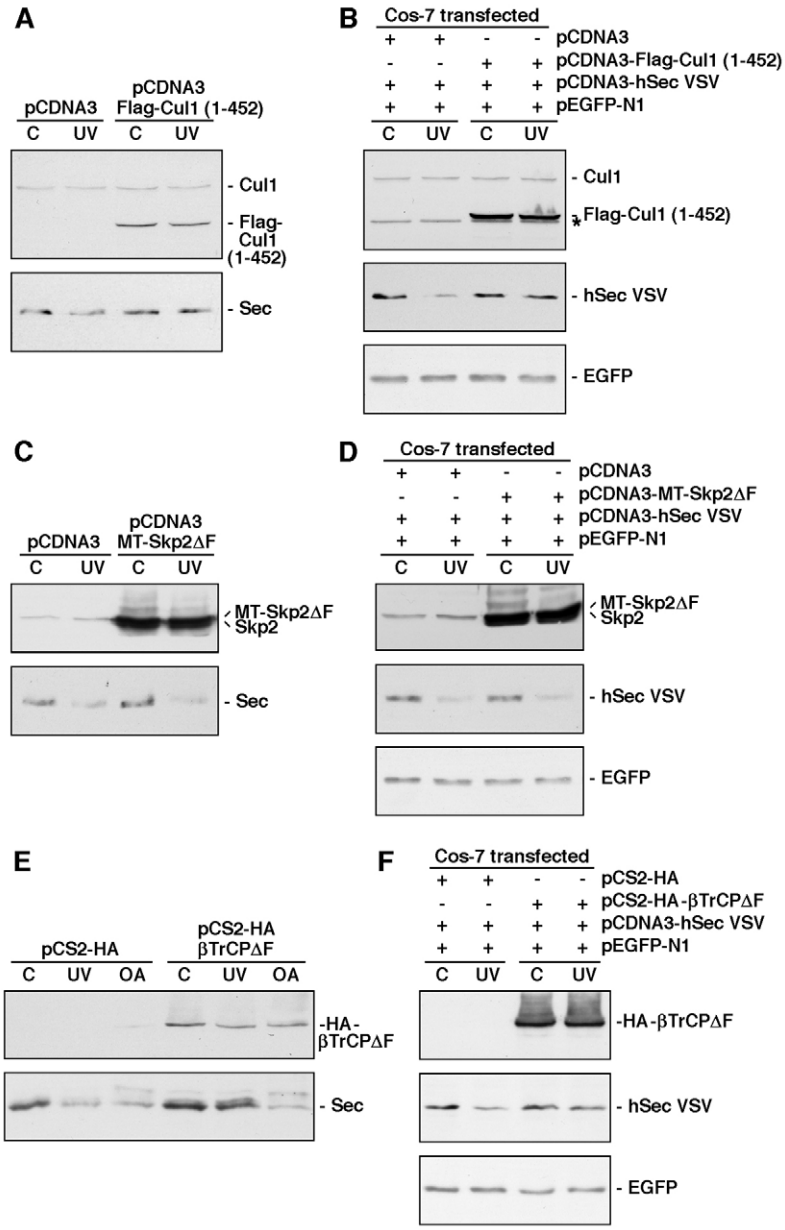

Fig. 3. SKP1-CUL1-βTrCP is the E3 ubiquitin ligase that mediates the UV-induced degradation of securin. (A) COS-7 cells transiently transfected with pCDNA3 or pCDNA3-Flag-CUL1$_{1-452}$ were irradiated (UV) or left untreated (C) 1 hour 30 minutes before harvesting. NP40 extracts were prepared and western blotted for CUL1, Flag-CUL1$_{1-452}$ and securin detection. (B) COS-7 cells were transiently transfected with the indicated plasmids and treated as above. Western blots were probed for the presence of CUL1, Flag-CUL1$_{1-452}$, securin-VSV and EGFP. The asterisk indicates an nonspecific band. (C) COS-7 cells were transiently transfected with pCDNA3 or pCDNA3-MT-Skp2ΔF, and irradiated (UV) or left untreated (C) 1 hour 30 minutes before harvesting. Extracts were western blotted for SKP2, MT-SKP2ΔF, and securin detection. (D) COS-7 cells were transfected with the indicated plasmids, treated as described, and blotted with anti-SKP2, anti-VSV, and anti-GFP. (E) COS-7 cells transiently transfected with pCS2-HA or pCS2-HA-βTrCPΔF were left untreated (C), irradiated (UV), or treated with okadaic acid (OA) 1 hour 30 minutes before harvesting. NP40 extracts were prepared and western blotted for the detection of the indicated proteins. Cells treated with OA were used as a negative control of the experiment because phosphorylated forms of securin after OA treatment are degraded via the SCF complex (Gil-Bernabe et al., 2006), but βTrCPΔF does not prevent this degradation. (F) Transfected COS-7 cells with the indicated plasmids were treated as described, and blotted for the presence of HA-βTrCPΔF, securin-VSV, and EGFP.

To further investigate the signaling molecules mediating UV-induced securin degradation, the effect was assessed of other kinase inhibitors, such as roscovitine (a potent and selective inhibitor of the cyclin-dependent kinases), rapamycin (an mTOR kinase inhibitor), Bis I (bisindolylmaleimide I hydrochloride, a protein kinase C inhibitor) and lithium chloride (a GSK-3β inhibitor). Of these, only lithium chloride prevented the UV-induced securin downregulation (Fig. 7A). Similar results were obtained with cells pretreated with cycloheximide to block protein synthesis and to prevent the interference of UV with newly synthesized protein (data not shown). To confirm the role of GSK-3β in securin expression after UV irradiation, we used TDZD-8, a specific GSK-3β inhibitor with little effect on other kinases (Martinez et al., 2002). TDZD-8 prevented securin degradation after UV irradiation (Fig. 7B) and revealed a phosphorylated securin form migrating as a retarded band on SDS-polyacrylamide gels, which was more marked after UV irradiation. This phosphorylated form may correspond to priming phosphorylation events that are characteristic of GSK-3β substrates (Forde and Dale, 2007). In fact, this retarded band disappeared after lambda protein phosphatase treatment (Fig. 7B, right panel). Therefore, we conclude that GSK-3β is involved in securin degradation.

Discussion

Securin (*PTTG1*) has been identified as a proto-oncogene because its overexpression induces cell transformation in fibroblasts and tumor formation in nude mice (Dominguez et al., 1998; Pei and Melmed, 1997). Furthermore, the amount of securin protein in most primary tumors is very high compared with that in normal tissues (Saez et al., 1999; Saez et al., 2006; Saez et al., 2002). In addition, securin is involved in the cell response to DNA damage (Bernal et al., 2002; Romero et al., 2004; Romero et al., 2001). In budding yeast, Pds1/securin contributes to cell-cycle arrest in response to DNA damage (Wang et al., 2001). In fission yeast, Cut2/securin is essential for separase stability and for the proper repair of DNA damaged by UV, X-ray, and γ-ray radiation (Nagao et al., 2004). We demonstrated that in human cells UV radiation downregulates the level of securin protein, inducing a cell-cycle arrest (Romero et al., 2004). In that study, we found that a securin mutant in D and KEN boxes (hSec KAA-DM) was stable after UV exposure, suggesting that securin degradation is mediated by APC/C (Romero et al., 2004). However, in light of recent results showing that some phosphorylated forms of securin are degraded via the SCF complex (Gil-Bernabe et al., 2006), we decided to revisit this question. In this report, we used siRNA against several APC/C subunits, dominant-negative variants of several SCF proteins, coimmunoprecipitation experiments, immunofluorescence microscopy, in vivo ubiquitylation assays and securin point mutants, and we demonstrated that $SCF^{\beta TrCP}$ is the E3 ubiquitin ligase responsible for securin degradation after UV irradiation. Furthermore, we found that GSK-3β inhibitors prevent this degradation, suggesting that GSK-3β might be involved

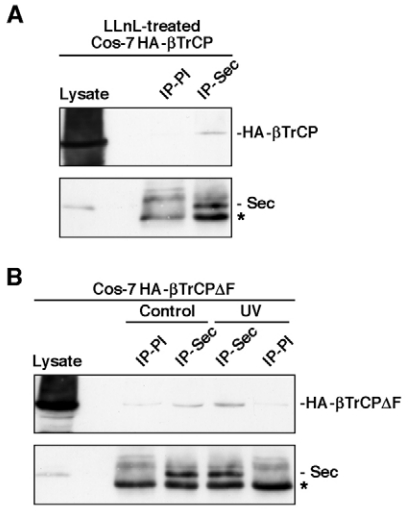

Fig. 4. Securin binds to βTrCP in vivo. (A) Anti-securin and preimmune (PI) sera were used in immunoprecipitations from NP40 extracts of COS-7 cells transiently transfected with pCS2-HA-βTrCP and treated with LLnL for 4 hours before harvesting. The immunoprecipitates were probed for the presence of HA-βTrCP and securin. Lysate, lysate from LLnL-treated COS-7 pCS2-HA-βTrCP cells. (B) COS-7 cells transiently transfected with pCS2-HA-βTrCPΔF were left untreated (Control) or irradiated (UV) before harvesting. Extracts were used for coimmunoprecipitation experiments. Lysate, lysate from COS-7 pCS2-HA-βTrCPΔF cells. Asterisk indicates IgG light chains.

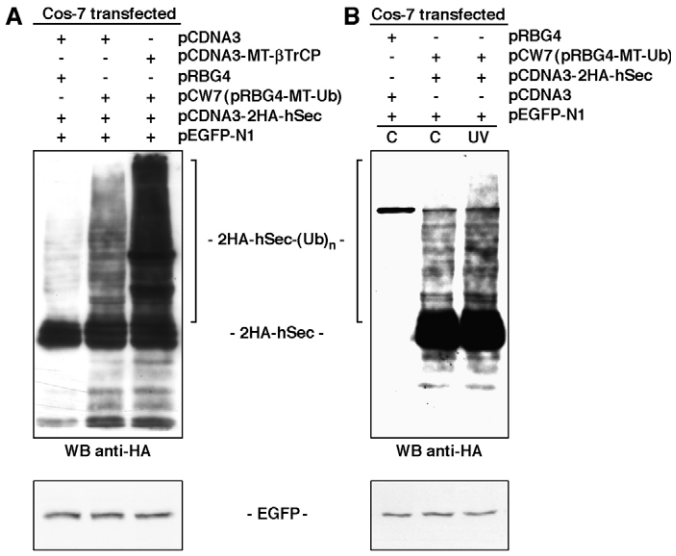

Fig. 5. Securin is ubiquitylated by $SCF^{\beta TrCP}$. (A) COS-7 cells were transfected with the indicated plasmids, and treated with LLnL for 4 hours before harvesting. Extracts were prepared as indicated in the Materials and Methods. Proteins were separated by SDS-PAGE, electroblotted and probed with anti-HA and anti-GFP antibodies. (B) Transfected cells were left untreated (C) or irradiated (UV) 1 hour 30 minutes before harvesting.

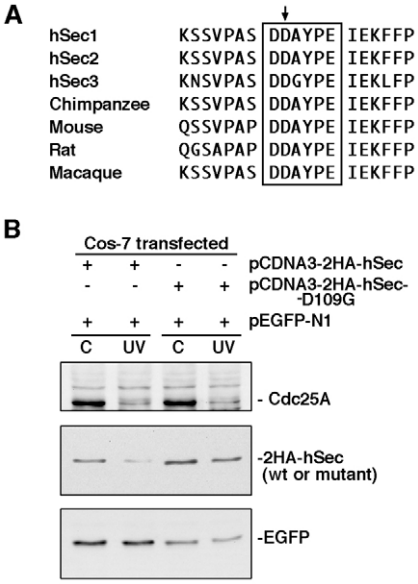

Fig. 6. Identification of a new securin destruction motif. (A) Alignment of βTrCP-binding motifs in securin proteins of various species. The arrow indicates Asp109 of securin (hSec1) that was mutated to Gly. (B) COS-7 cells were transfected with the indicated plasmids, and left untreated (C) or irradiated (UV) before harvesting. NP40 extracts were prepared and western blotted for the detection of the indicated proteins.

in securin phosphorylation and stability. At present, we do not know why the hSec KAA-DM mutant is not destroyed after UV exposure. It is possible that these mutations alter the structure of the protein in such a way that it cannot be degraded.

It is known that the most (or one of the most) important role(s) of securin is to bind to separase to inhibit its proteolytic activity (Zou et al., 1999). In this context, it is reasonable to propose that securin-induced tumor formation might be the result of aneuploidy caused by defects in sister chromatid separation. In fact, both *PTTG1* overexpression and *PTTG1* depletion (at least transiently) are involved in the induction of aneuploidy (Pfleghaar et al., 2005; Yu et al., 2000).

The findings reported in this work have the additional interest that they might contribute to explaining the increased amount of securin in tumors, especially in low-proliferation tumors, in which securin expression cannot be explained by the cell proliferation rate. To date, no genomic aberrations in securin (i.e. promoter mutations or amplification) have been detected (Kanakis et al., 2003). Perhaps an increased stability of securin by an insufficient degradation is one of the causes of these cancers. In fact, aberrations in removal of N-Myc, Myc, Fos, Jun, Src or the adenovirus E1A proteins may derail the cell cycle and result in malignant transformation (Ciechanover, 2003). If this is so in the case of securin, its accumulation and subsequent oncogenic activation could result from one (or both) of two distinct mechanisms: mutations in kinases or phosphatases that alter the phosphorylation status of securin, or mutations in the targeting machinery involved in the securin degradation.

Direct binding of βTrCP is usually mediated by a phosphorylated $DSGx_{2\text{-}4}S$ motif in ubiquitylation substrates. Securin does not have this classical motif, but it does have a recently described motif in which phosphorylatable serines may be substituted by acidic amino acids (Tempe et al., 2006). Our results confirm this revisited consensus motif, and contribute with a new motif sequence to the set of βTrCP-binding proteins. In fact, this motif is conserved in the securin amino acid sequence of several mammalian species. However, it has been reported that βTrCP can bind to the entirely nonphosphorylated βTrCP-binding motif in CDC25A (Kanemori et al., 2005). Under our conditions, we cannot rule out the possibility that securin is partially phosphorylated, especially on nearby

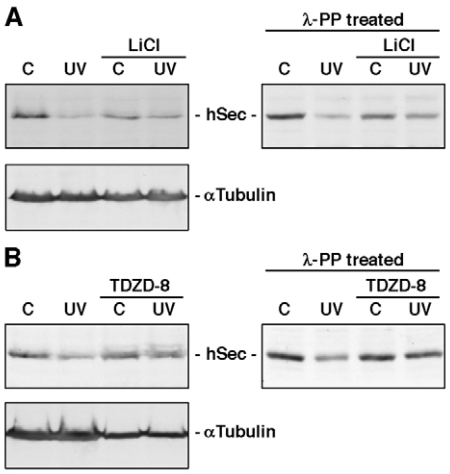

Fig. 7. GSK-3β inhibitors reverse the effect of UV radiation on securin expression. HeLa cells were left untreated (C) or UV-irradiated (UV) in the presence or absence of LiCl (A) or TDZD-8 (B). Inhibitors were added 1 hour before irradiation. Where indicated, extracts were treated with λ-PP. Equal amounts of extracts were resolved by SDS-PAGE. Immunoblotting was performed with anti-securin and anti-α-tubulin antibodies.

residues of the βTrCP-binding motif. In any case, UV radiation exposure increases securin ubiquitylation, suggesting that UV might induce phosphorylation of the protein. This question will be addressed in the near future.

It seems clear that to ensure both sister chromatid separation and cell response to UV radiation, securin functions are tightly regulated by several mechanisms, including abundance in the cell, phosphorylation status, subcellular localization, and even the existence of several isoforms. In fact, all of these mechanisms may overlap in some way.

The securin levels at each stage of the cell cycle depend on the balance between protein synthesis and degradation. Other cell-cycle regulators, such as the CDC25 phosphatases, are similarly controlled. With regard to degradation, CDC25A behaves similarly to securin, which is degraded at the end of mitosis by the proteasome via APC/C (Donzelli et al., 2002), whereas the $SCF^{\beta TrCP}$ complex is responsible for its degradation during S and G2 phases and in response to DNA damage (Busino et al., 2003; Falck et al., 2001). As alluded above, CDC25A also has a nonphosphorylated βTrCP-binding motif (Kanemori et al., 2005). It is possible that both proteins are simultaneously involved in the cell-cycle arrest after UV irradiation, because to arrest proliferation, the cell machinery could degrade both CDC25A and securin. It would also be interesting to know whether CDC25A is a phosphatase for securin, but, for the moment, the only identified phosphatase involved in securin stability is PP2A (Gil-Bernabe et al., 2006). In any case, multiple mechanisms are in place to regulate securin functions during the normal cell cycle and after UV radiation exposure, and a better understanding of the ubiquitylation machinery will provide new insights into the regulatory biology of cell-cycle transitions and the development of anticancer drugs.

Materials and Methods

Plasmids, point mutations and sequencing

pCDNA3-hSec VSV, pCDNA3-2HA-hSec, pSUPER-Cdh1, pSUPER-Cdc20, pCDNA3 Flag-Cul1 (1-452), pCS2-HA-βTrCP, pCDNA3-MT-βTrCP, pCS2-HA-βTrCPΔF, pCDNA3-MT-Skp2ΔF, pCW7 (pRBG4-MT-Ubiquitin) and empty vectors were described previously (Brummelkamp et al., 2002; Gil-Bernabe et al., 2006; Margottin et al., 1998; Romero et al., 2004; von der Lehr et al., 2003; Ward et al., 1995; Wu et al., 2000). hSec-D109G was constructed using the Transformer Site-Directed Mutagenesis Kit from BD Biosciences. Sequencing of point mutations was performed on both strands with an automatic sequencer.

Cell culture, transient transfection and UV irradiation

Routinely, HeLa and COS-7 cells were grown in Dulbecco's modified Eagle's medium (BioWhittaker) as described (Romero et al., 2004). HeLa cells enriched in S or G2 phases were also obtained as previously described (Jin et al., 1998). 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), lithium chloride (LiCl, 100 mM Sigma), or TDZD-8 (100 μM Calbiochem).

DNA constructs were transiently transfected by electroporation into COS-7 cells, and cells were harvested 18 hours post transfection. Where indicated, cells were UV-irradiated with 100 J/m^2 and harvested 1 hour 30 minutes later (Romero et al., 2004).

Subcellular fractionation and lysis

Subcellular fractionation of HeLa cells was carried out as described (Romero et al., 1998). Cell lysis was performed at 4°C in Nonidet P-40 (NP40) buffer. When necessary, extracts were treated with lambda protein phosphatase (λ-PP) (Gil-Bernabe et al., 2006). Extracts for interference experiments were prepared using an NP40 buffer with 420 mM NaCl. Protein concentration was determined using the Bradford assay (Bio-Rad).

Western blot analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and gels were electroblotted onto nitrocellulose membranes and probed with the following antibodies: immunoaffinity-purified anti-securin polyclonal antibody (Dominguez et al., 1998), anti-hnRNP C monoclonal antibody (Romero et al., 1998), anti-HA-peroxidase monoclonal antibody (Roche), anti-VSV and anti-α-tubulin monoclonal antibodies (Sigma), anti-Cdc20, anti-Cdc25A and anti-Cdc27 monoclonal and anti-Cul1 and anti-Raf1 polyclonal antibodies (Santa Cruz Biotech), anti-Cdh1 and anti-cyclin B monoclonal antibodies (BD Biosciences), and anti-GFP polyclonal antibody (Immunology Consultants Laboratory). Peroxidase-coupled donkey anti-rabbit IgG and sheep anti-mouse IgG were obtained from Amersham Biosciences. Immunoreactive bands were visualized using the Enhanced Chemiluminescence Western blotting system (ECL, Amersham Biosciences).

Coimmunoprecipitation experiments

Cell lysates (1-2 mg) were incubated with preimmune serum for 30 minutes, and subsequently with protein-A-Sepharose beads (Amersham Biosciences) for 1 hour at 4°C. After centrifugation, beads were discarded, and supernatants incubated for 2 hours with polyclonal anti-securin or preimmune serum, followed by protein A-Sepharose beads for 1 hour. Beads were washed, and bound proteins were solubilized by the addition of SDS-sample buffer heated at 95°C for 5 minutes.

In vivo ubiquitylation assays

COS-7 cells were transfected and treated with LLnL 4 hours before harvesting. Next, cells were washed in PBS, lysed at 95°C for 15 minutes in NP40 buffer supplemented with 5% SDS and 10 mM iodoacetamide, and diluted in NP40 buffer supplemented with 10 mM iodoacetamide. Proteins were separated by SDS-PAGE, and gels were electroblotted onto nitrocellulose membranes and probed with anti-HA and anti-GFP antibodies.

Small interfering RNA (siRNA) assays

We used pSUPER-Cdh1 and pSUPER-Cdc20 to suppress the expression of the endogenous *CDH1* and *CDC20* genes (Brummelkamp et al., 2002). HeLa cells were transiently transfected by electroporation, and harvested 48 hours post-transfection. Reduction of protein levels was confirmed by western blotting.

To inhibit *CDC27/APC3*, we used synthetic siRNA oligonucleotides. In this case, transient transfection of HeLa cells was carried out with 100 nM *CDC27* siRNAs or a nonspecific control pool (Santa Cruz Biotech), using the Oligofectamine method as described by the manufacturer for adherent cells (Invitrogen). 48 hours after transfection, cells were irradiated or left untreated, collected 1 hour 30 minutes later, and analyzed for CDC27/APC3 and securin expression.

Immunofluorescence microscopy

HeLa cells were grown on coverslips, biochemically arrested in S and G2 phases, and irradiated or not with 100 J/m^2. Control nonirradiated cells were harvested at the point of UV irradiation, and irradiated cells were harvested 1 hour 30 minutes after treatment. Cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Immunostaining was carried out according to standard protocols. Epifluorescence microscopy was performed using a Leica microscope.

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