SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27

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Degradation of the mammalian cyclin-dependent kinase (CDK) inhibitor p27 is required for the cellular transition from quiescence to the proliferative state. The ubiquitination and subsequent degradation of p27 depend on its phosphorylation by cyclin–CDK complexes. However, the ubiquitin–protein ligase necessary for p27 ubiquitination has not been identified. Here we show that the F-box protein SKP2 specifically recognizes p27 in a phosphorylation-dependent manner that is characteristic of an F-box-protein–substrate interaction. Furthermore, both *in vivo* and *in vitro*, SKP2 is a rate-limiting component of the machinery that ubiquitinates and degrades phosphorylated p27. Thus, p27 degradation is subject to dual control by the accumulation of both SKP2 and cyclins following mitogenic stimulation.

The cyclin-dependent kinase (CDK) inhibitor p27 is important in the control of mammalian cell proliferation (reviewed in refs 1–3). In fact, p27 negatively regulates the action of CDKs that are necessary for DNA replication. Levels of p27 are high in quiescent cells, but following growth stimulation by mitogenic stimuli p27 is degraded, allowing CDKs to drive cells into S phase (for reviews see refs 4–6). Consistent with the negative role of p27 in cell-cycle progression, p27 is destabilized in many types of human cancer, and this destabilization correlates with tumour aggressiveness and poor prognosis[7–13].

p27 is ubiquitinated (a prelude to proteasome-mediated degradation) *in vivo*[14–16]. CDK-dependent phosphorylation of p27 on threonine residue 187 (T187) is required for its degradation *in vivo*[17–19], and the *in vivo* ubiquitination of p27 correlates with its state of phosphorylation on T187, being present in proliferating cells but not in G1-phase cells[15]. *In vitro* ubiquitination and degradation of p27 also require CDK-dependent phosphorylation of p27 on T187, as well as association of p27 with a cyclin–CDK complex[15]. However, the enzymes necessary for the ubiquitination of phosphorylated p27 have not been identified. Of specific interest is the identity of the ubiquitin–protein ligase (E3) for p27, as E3s allow the transfer of activated ubiquitin from a ubiquitin-conjugating

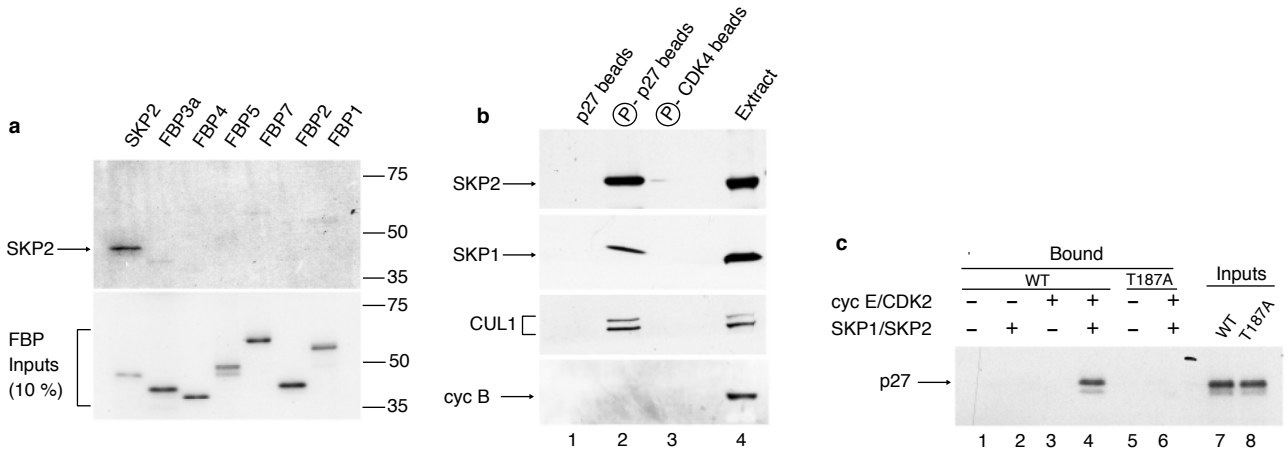

Figure 1 **Binding of phosphorylated p27 to SKP2. a**, *In vitro*-translated [^{35}S]FBPs were used in binding reactions with beads coupled to the phosphopeptide NAGSVEQT*PKKPGLRRRQT, which corresponds to the C terminus of human p27 with a phosphothreonine at position 187 (T*). Beads were washed with RIPA buffer and bound proteins were eluted and subjected to electrophoresis and autoradiography (top). The bottom panel shows 10% of the *in vitro*-translated [^{35}S]FBP inputs. Markers to the right indicate relative molecular mass (in thousands). **b**, HeLa cell extracts were incubated with beads coupled to the phosphorylated p27 peptide (lane 2), to unphosphorylated p27 peptide (lane 1) or to the control phosphopeptide AEIGVGAY*GTVYKARDPHS, which corresponds to an N-terminal peptide of human CDK4 with a phosphotyrosine at position 17 (Y*) (lane 3). Beads were washed with RIPA buffer and bound proteins were immunoblotted with antibodies to the proteins indicated on the left of each panel. A portion of the HeLa extract (25 μg) was used as a control (lane 4). The more slowly migrating band in the CUL-1 panel is probably generated by the covalent attachment of a ubiquitin-like molecule, as described for other cullins[48]. **c**, 1 μl *in vitro*-translated ^{35}S-labelled wild-type p27 (WT, lanes 1–4) or p27(T187A) mutant (T187A, lanes 5, 6) was incubated for 30 min at 30 °C in 10 μl kinase buffer. Where indicated, ~2.5 pmol recombinant purified cyclin E–CDK2 or ~1 pmol SKP2 (in SKP1–SKP2 complex) was added. Samples were then incubated with 6 μl protein-A beads to which antibodies to SKP2 had been covalently linked. Beads were washed with RIPA buffer and bound proteins subjected to electrophoresis and autoradiography. Lanes 1–6, SKP2-bound proteins; lanes 7, 8, 7.5% of the *in vitro*-translated [^{35}S]protein inputs.

enzyme (E2) to the target protein and ensure a high level of substrate specificity in the ubiquitination pathway (reviewed in ref. 20).

In yeast, SCF complexes formed by Skp1, CulA (Cdc53) and an F-box protein act as ubiquitin–protein ligases in which different F-box protein (FBP) subunits recruit specific substrates for ubiquitination (reviewed in refs 4–6, 21). While the CulA component of the SCF ubiquitin–protein ligase interacts with a specific ubiquitin-conjugating enzyme (Ubc3), the FBP subunit recruits specific phosphorylated substrates. As in yeast, the human SKP1–CUL1 complex forms a scaffold for several human FBPs, including SKP2 (reviewed in ref. 6), FBP1 (also called β-Trcp or Slimb)[22,23], FBP2, FBP3a and FBP4 (C. Cenciarelli, D. Chiaur, M. Vidal and M.P., unpublished observations). These different SCF complexes potentially target different phosphorylated substrates for ubiquitin-mediated degradation. The best-characterized example is SCF^{FBP1}, which specifically targets IκBα and β-catenin (reviewed in ref. 24).

Human SKP1 and SKP2 were originally identified as two proteins associated with cyclin A, hence their designation as S-phase-kinase-associated proteins[25]. Although there is evidence that SKP2 is a substrate of cyclin A–CDK2, the significance of the interaction of SKP2 with cyclin A[25] is still not understood.

The structural similarities between ubiquitinating enzymes from yeast and human indicate that the human enzymes may have conserved a function similar to that of the yeast proteins. It is possible that human SCF complexes play a part in the ubiquitination of G1-phase regulatory proteins, as do their yeast homologues. This idea, together with the fact that phosphorylation of p27 triggers its ubiquitination[15], prompted us to test the hypothesis that a specific human FBP targets p27 for ligation to ubiquitin. Here we show that the FBP SKP2 is essential in the ubiquitin-mediated degradation of p27.

Results

SKP2 interacts physically with phosphorylated p27 *in vitro* and *in vivo*. The recruitment of specific substrates by yeast and human FBPs to SKP1–cullin complexes is phosphorylation dependent. Accordingly, peptides derived from IκBα and β-catenin bind to FBP1 specifically and in a phosphorylation-dependent manner[23,26]. We tested whether a p27 phosphopeptide, with a phosphothreonine at position 187, would bind to human FBPs, including SKP2 and six new FBPs whose complementary DNA we cloned recently in a two-hybrid screen using SKP1 as bait (C. Cenciarelli, D. Chiaur, M. Vidal and M.P., unpublished observations). Four of these FBPs contain potential substrate-interaction domains, such as WD40 domains in FBP1 and FBP2 and leucine-rich repeats in SKP2 and FBP3a. The p27 phosphopeptide was immobilized on Sepharose beads and incubated with these seven *in vitro*-translated FBPs (Fig. 1a). Only one FBP, SKP2, was able to bind to the phosphopeptide. We then incubated beads linked to either p27 peptides (in either phosphorylated or unphosphorylated forms) or an unrelated phosphopeptide with HeLa cell extracts. We studied proteins that stably associated with the beads by immunoblotting. SKP2 and its associated proteins, SKP1 and CUL-1, were readily detected as proteins bound to the p27 phosphopeptide but did not bind to control peptides (Fig. 1b).

To study p27 association with SKP2 further, we incubated *in vitro*-translated p27 with SKP1–SKP2 complex, cyclin E–CDK2 complex, or the combination of both complexes under conditions in which p27 is phosphorylated on T187 by cyclin E–CDK2 (ref. 15). Samples were then immunoprecipitated with an anti-SKP2 antibody. p27 co-immunoprecipitated with SKP2 only in the presence of cyclin E–CDK2 (Fig. 1c). Notably, under the same conditions, a threonine-to-alanine p27 mutant, p27(T187A), was not co-immunoprecipitated by the anti-SKP2 antibody.

Finally, we studied the association of SKP2 and p27 *in vivo*. Extracts from HeLa cells and IMR90 human diploid fibroblasts were subjected to immunoprecipitation with two different antibodies to SKP2 and then immunoblotted. p27 and CUL-1, but not cyclin D1 and cyclin B1, were specifically detected in SKP2 immunoprecipitates (Fig. 2). Using an antibody against the phosphorylated T187 site of p27 (ref. 15), we showed that SKP2-bound p27 was phosphorylated on T187 (Fig. 2, lane 2, bottom panel). Furthermore, an anti-p27-peptide antibody specifically co-immunoprecipitated SKP2 with p27 (data not shown). We conclude that the stable interaction of p27 with SKP2 is highly specific and dependent upon phosphorylation of p27 on T187.

SKP2 and cyclin E–CDK2 are rate limiting for p27 ubiquitination. We have developed a cell-free assay of p27 ubiquitination that faith-

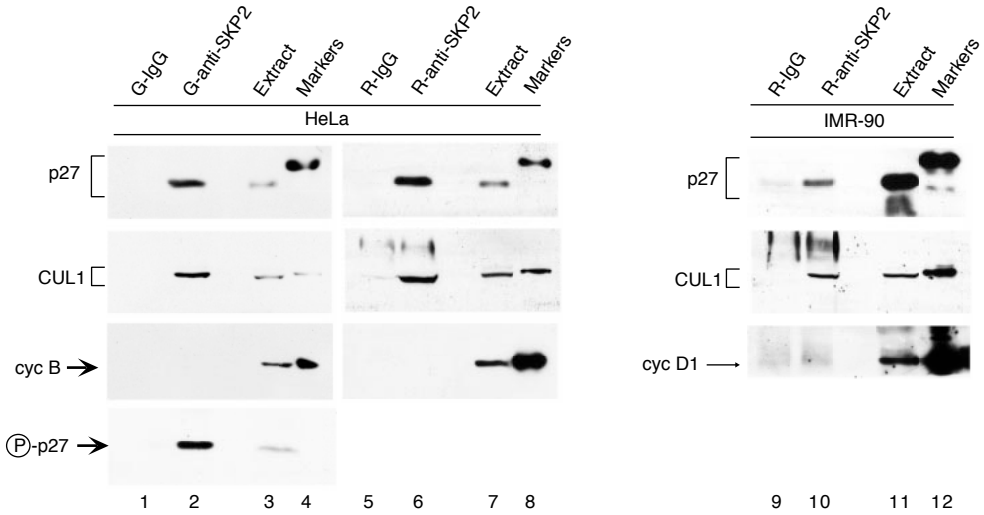

Figure 2 ***In vivo* binding of SKP2 to p27.** Extracts from HeLa cells (lanes 1, 2, 5, 6) or IMR90 fibroblasts (lanes 9, 10) were immunoprecipitated with different affinity-purified antibodies to SKP2 or with purified control IgG fractions. Extracts were immunoprecipitated with: lane 1, a goat IgG (G-IgG); lane 2, an affinity-purified goat antibody to an N-terminal SKP2 peptide (G-anti-SKP2,); lanes 5, 9, a rabbit IgG (R-IgG); lanes 6, 10, an affinity-purified rabbit antibody to SKP2 (R-anti-SKP2). Immunoprecipitates were immunoblotted with antibodies to the proteins indicated on the left of each panel. Lanes 1–4 in the bottom panel were immunoblotted with an antibody against phosphorylated residue T187 of p27 C-terminal peptide. Lanes 3, 7 and 11 contain 25 μg cell extracts; lanes 4, 8 and 12 contain the relevant recombinant proteins used as markers. The altered migration of some markers is due to the presence of tags on the recombinant proteins.

fully reproduces the cell-cycle-stage-specific ubiquitination and degradation of p27 (ref. 15). Using this assay, we found that p27–ubiquitin ligation activity is higher in extracts from asynchronously growing cells than in those from G1-arrested cells (Fig. 3a, lanes 2, 4; ref. 15). In agreement with our previous findings[15], the addition of cyclin E–CDK2 stimulated the ubiquitination of p27 in both types of extract (Fig. 3a, lanes 3, 5). However, this stimulation was much lower in extracts from G1-arrested cells than in those from growing cells, indicating that, in addition to cyclin E–CDK2, some other component of the p27–ubiquitin ligation system is rate limiting in G1. We reasoned that this component could be SKP2 because, in contrast to other SCF subunits, levels of this protein are lower in extracts from G1 cells than in those from asynchronous cells and are inversely correlated with levels of p27 (Fig. 3b and Fig. 7; refs 25, 27, 28). We therefore tested the hypothesis that SKP2 is a rate-limiting component of a p27–ubiquitin ligase activity. The addition of recombinant purified SKP1–SKP2 complex alone to G1 extracts did not stimulate p27 ubiquitination significantly (Fig. 3a, lane 6). In contrast, the combined addition of SKP1–SKP2 and cyclin E–CDK2 complexes markedly stimulated p27 ubiquitination in G1 extracts (Fig. 3a, lane 7). Similarly, the combined addition of SKP1–SKP2 and cyclin E–CDK2 markedly stimulated p27 proteolysis, as measured by a degradation assay (Fig. 3a, lanes 13–16).

As the SKP1–SKP2 complex used for these experiments was isolated from insect cells co-expressing baculovirus His-tagged SKP1 and untagged SKP2 (co-purified by nickel–agarose chromatography), it was possible that an insect-derived F-box protein co-purified with His-tagged SKP1 and was responsible for the stimulation of p27 ubiquitination in G1 extracts. We eliminated this possibility by showing that the addition of a similar amount of His-tagged SKP1, expressed in the absence of SKP2 in insect cells and purified by the same procedure, did not stimulate p27 ubiquitination in the presence of cyclin E–CDK2 (Fig. 3a, lane 8). Furthermore, neither FBP1 nor FBP3a could replace SKP2 in the stimulation of p27–ubiquitin ligation in G1 extracts (Fig. 3a, lanes 9–12). Stimulation of p27 ubiquitination in G1 extracts by the combined addition of SKP1–SKP2 and cyclin E–CDK2 was observed only with wild-type p27, but not with the p27(T187A) mutant (Fig. 3a, lanes 17–20), indicating that phosphorylation of p27 on T187 is required for SKP2-mediated ubiquitination of p27. These findings indicate that both cyclin E–CDK2 and SKP1–SKP2 complexes are rate limiting for p27 ubiquitination and degradation in G1 phase.

SKP2 is essential for *in vitro* ubiquitination of p27. To further investigate the requirement of SKP2 for p27–ubiquitin ligation, we specifically removed SKP2 from extracts of asynchronously growing cells by immunodepletion with an antibody against SKP2. The immunodepletion procedure efficiently removed most SKP2 from these extracts (data not shown) and caused a dramatic reduction of p27–ubiquitin ligation activity (Fig. 4a, lane 4) as well as of p27-degradation activity (data not shown). This effect was specific, as shown by the following observations: first, similar treatment with preimmune serum did not inhibit p27 ubiquitination (Fig. 4a, lane 3); second, preincubation of anti-SKP2 antibody with a recombinant fusion protein consisting of glutathione-*S*-transferase (GST) and SKP2 (Fig. 4a, lane 5), but not with a control protein (lane 4), prevented the immunodepletion of p27-ubiquitination activity from extracts; and third, p27-ubiquitinating activity could be restored in SKP2-depleted extracts by the addition of a His-tagged SKP1–SKP2 complex (Fig. 4b, lane 3) but not of His-tagged SKP1 (lane 2), His-tagged SKP1–CUL1 (lane 4) or His-tagged SKP1–FBP1 (data not shown).

We then immunoprecipitated SKP2 from HeLa extracts and tested whether this immunoprecipitate contained a p27-ubiquitinating activity. The SKP2 immunoprecipitate, but not an immunoprecipitate made with a preimmune serum, induced p27 ubiquitination in the presence of cyclin E–CDK2 (Fig. 4c, lanes 2, 3). The addition of purified recombinant ubiquitin-activating enzyme (E1) and purified recombinant Ubc3 did not greatly increase the ability of the SKP2 immunoprecipitate to sustain p27 ubiquitination (Fig. 4c, lane 5), probably because both proteins are already present in the rabbit reticulocyte lysate used for p27 *in vitro* translation (data not shown).

SKP2 targets p27 for ubiquitin-mediated degradation *in vivo*. To test the significance of one mammalian F-box protein in the stability of its putative substrate *in vivo*, a dominant-negative F-box-mutant approach is routinely used. For instance, the F-box-deleted FBP1 mutant, (ΔF)FBP1, acts *in vivo* as a dominant-negative mutant, most likely because without the F-box it is unable to

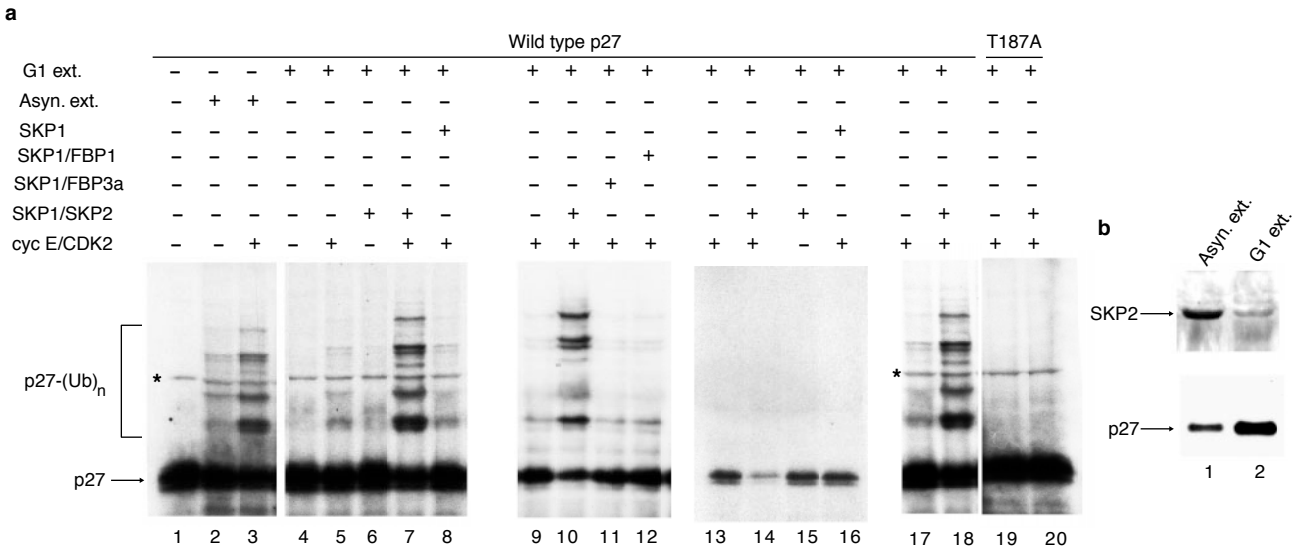

Figure 3 **SKP2 and cyclin E–CDK2 are rate limiting for p27 ubiquitination in G1 extracts. a**, *In vitro* ubiquitin ligation (lanes 1–12 and 17–20) and degradation (lanes 13–16) of p27 were carried out with extracts from asynchronously growing (Asyn. ext., lanes 2, 3) or G1-arrested (G1 ext., lanes 4–20) HeLa cells. Lane 1 contains no extract. Recombinant purified proteins were supplemented as indicated. Reactions were done using wild-type p27 (lanes 1–18) or the p27(T187A) mutant (T187A, lanes 19, 20). Lanes 1–8, 9–12 and 17–20 were obtained from three separate ubiquitination experiments.The bracket on the left side of the panels marks a ladder of bands of relative molecular mass >27,000, corresponding to polyubiquitinated p27. The asterisk indicates a nonspecific band present in most samples. **b**, Immunoblot analysis of levels of SKP2 and p27 in extracts from asynchronous (lane 1) or G1-arrested (lane 2) HeLa cells.

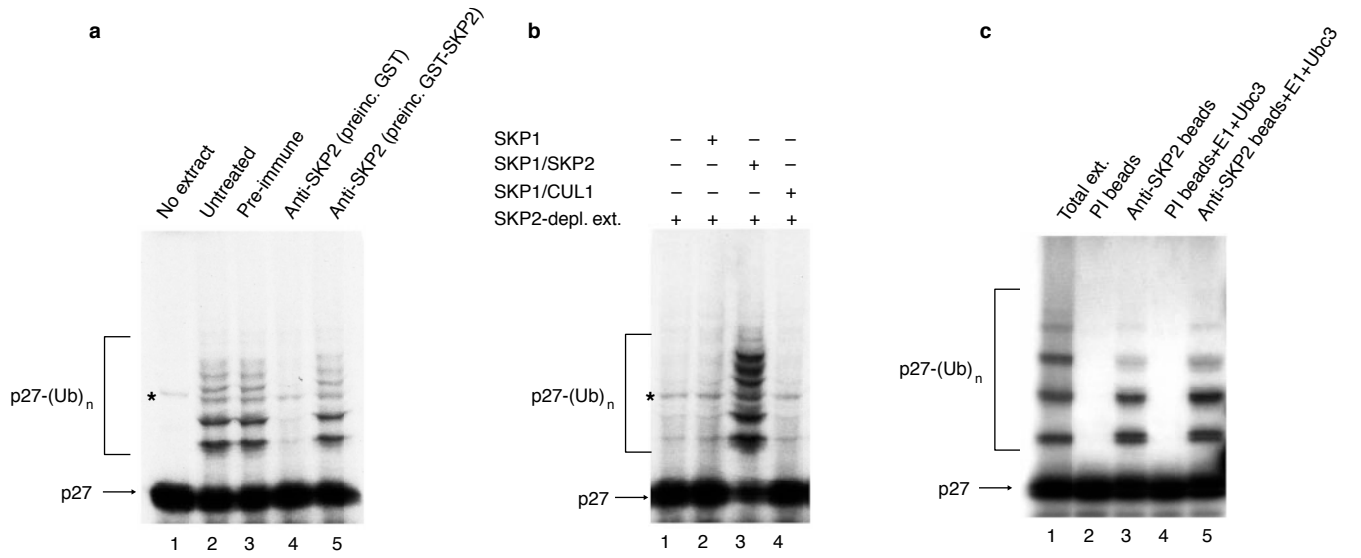

Figure 4 **SKP2 is required for p27–ubiquitin ligation activity. a**, Immunodepletion. Extracts from asynchronous HeLa cells were untreated (lane 2) or immunodepleted with preimmune serum (lane 3), anti-SKP2 antibody preincubated with 2 μg purified GST (lane 4), or anti-SKP2 antibody preincubated with 2 μg purified GST–SKP2 (lane 5). Lane 1 contains no extract. Samples (30 μg protein) were assayed for p27 ubiquitination in the presence of cyclin E–CDK2. The bracket on the left side of the panels marks a ladder of bands of relative molecular mass >27,000, corresponding to polyubiquitinated p27. The asterisk indicates a nonspecific band present in all samples. **b**, Reconstitution. The restoration of p27-ubiquitination activity in SKP2-immunodepleted extracts was tested by the addition of the indicated purified proteins. All samples contained 30 μg SKP2-depleted extract (SKP2-depl. ext.) and cyclin E–CDK2. **c**, Immunopurification. Extracts from asynchronous HeLa cells were immunoprecipitated with a rabbit anti-SKP2 antibody (lanes 3, 5) or preimmune serum (PI, lanes 2, 4). Total extract (lane 1) and immuno-beads (lanes 2–5) were added with p27, recombinant purified cyclin E–CDK2 and ubiquitination reaction mix. Samples in lanes 4, 5 were supplemented with recombinant purified E1 and Ubc3. All samples were then assayed for p27 ubiquitination.

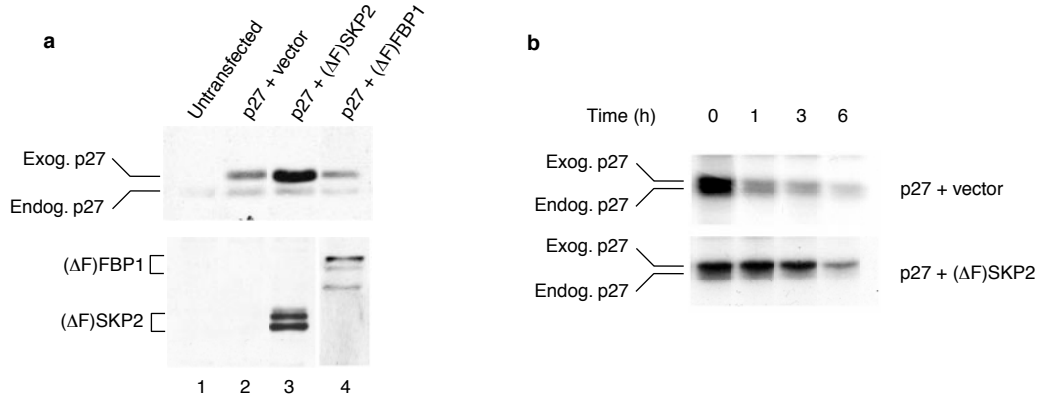

Figure 5 ***In vivo* role of SKP2 in p27 degradation. a**, Stabilization of p27 by (ΔF)SKP2 *in vivo*. NIH3T3 cells were transfected with mammalian expression vectors encoding human p27 alone (lane 2) or p27 in combination with either (ΔF)SKP2 (lane 3) or (ΔF)FBP1 (lane 4). Lane 1, untransfected cells. Cells were lysed and extracts were subjected to immunoblotting with antibodies to p27, SKP2 or FLAG (to detect FLAG-tagged (ΔF)FBP1). Exogenous human p27 protein migrates more slowly than the endogenous murine p27. **b**, Pulse–chase analysis of p27 turnover rate. Human p27 in combination with either an empty vector or (ΔF)SKP2 was transfected into NIH3T3 cells. 24 h later, cells were labelled with [^{35}S]methionine for 20 min and chased with medium for the indicated times. Extracts were then subjected to immunoprecipitation with a mouse anti-p27 antibody.

bind SKP1–CUL1 complex but retains the ability to bind its substrates. Therefore, once expressed in cells, (ΔF)FBP1 sequesters β-catenin and IκBα and causes their stabilization[22,26,29,30]. We constructed an F-box-deleted SKP2 mutant, (ΔF)SKP2, and then expressed p27 in murine cells either alone or in combination with (ΔF)SKP2 or (ΔF)FBP1. The presence of (ΔF)SKP2 (Fig. 5a, lane 3), but not of (ΔF)FBP1 (lane 4), led to the accumulation of higher quantities of p27. To determine whether this accumulation was due to an increase in p27 stability, we measured the half-life of p27 using pulse–chase analysis. Indeed, (ΔF)SKP2 prolonged the half-life of p27 from less than 1 hour to more than 3 hours (Fig. 5b).

Finally, we targeted SKP2 messenger RNA with antisense oligonucleotides to determine whether a decrease in SKP2 levels would influence the abundance of endogenous p27. Two different antisense oligonucleotides, but not control oligodeoxynucleotides, induced a decrease in SKP2 protein levels (Fig. 6). Concomitant with the decrease in SKP2 protein, there was a substantial increase in the level of endogenous p27 protein. Similar results were obtained with cells blocked at the G1-to-S-phase transition with hydroxyurea or aphidicolin treatment (Fig. 6, lanes 9–16). Thus, the effect of the SKP2 antisense oligonucleotides on p27 was not a secondary consequence of a possible block in G1 resulting from the decrease in SKP2 levels.

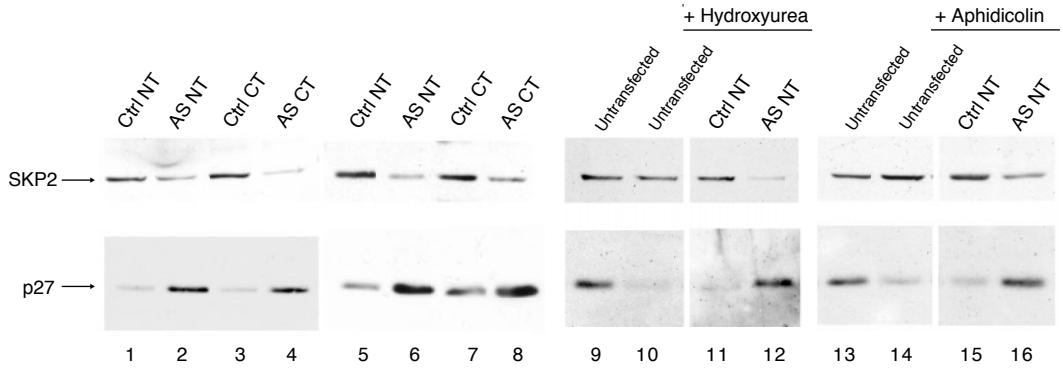

Figure 6 **Stabilization of cellular p27 by antisense oligonucleotides targeting SKP2 mRNA.** HeLa cells were treated for 16–18 h with two different antisense oligodeoxynucleotides (AS) targeting two different regions of SKP2 mRNA: lanes 2, 6, 12, 16, AS targeting the N-terminal SKP2 mRNA region (NT); lanes 4, 8, AS targeting the C-terminal SKP2 mRNA region (CT); lanes 1, 3, 5, 7, 11, 15, control oligodeoxynucleotide pairs (Ctrl). Lanes 1–4 and 5–8 are results of two separate experiments. Lanes 11, 12, 15, 16, HeLa cells were blocked in G1/S by either hydroxyurea or aphidicolin treatment, respectively, for 24 h. Cells were then transfected with oligodeoxynucleotides, lysed after 12 h (before cells had re-entered G1) and immunoblotted with antibodies to SKP2 (top) and p27 (bottom). Lanes 9,13, untransfected HeLa cells; lanes 10, 14, untransfected HeLa cells treated with drugs as for transfected cells.

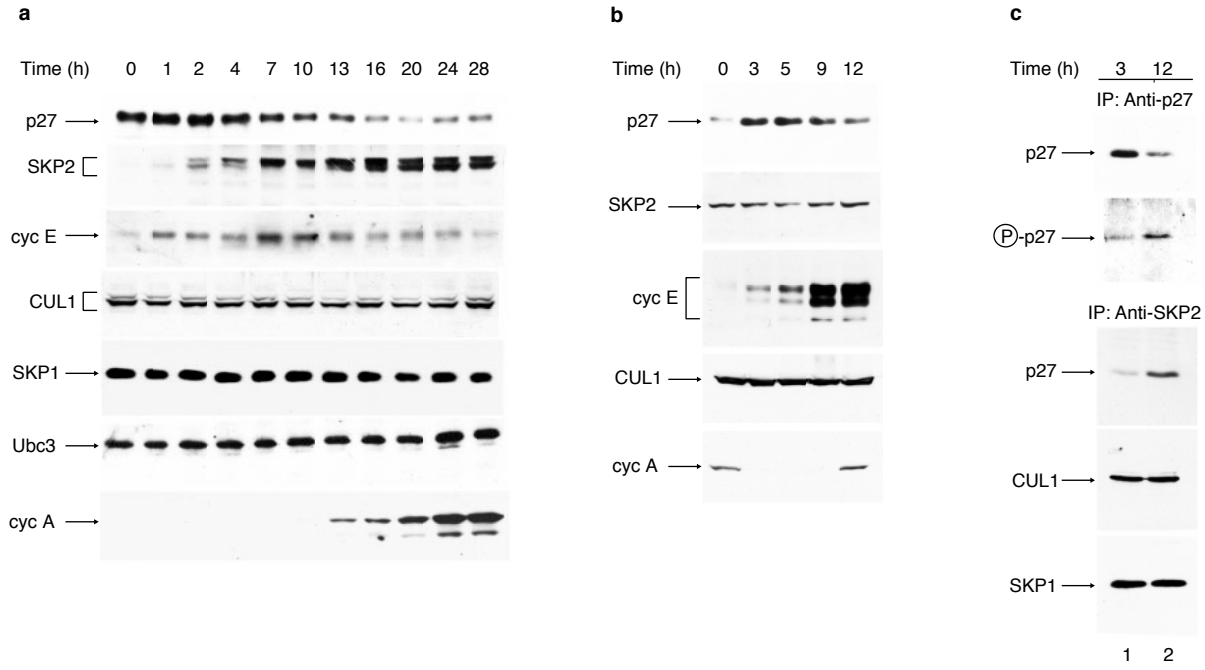

Figure 7 **Timing of SKP2 action in the process of p27 degradation. a**, IMR90 fibroblasts were synchronized in G0/G1 by serum deprivation, reactivated with serum, and sampled at the indicated intervals. Protein extracts were analysed by immunoblot with the antibodies to the indicated proteins. The SKP2 doublet is probably generated by phosphorylation, as it was consistently observed (when using a 12.5% SDS–PAGE gel) only when cell lysis was performed in the presence of okadaic acid. **b**, HeLa cells blocked in mitosis with nocodazole were shaken off, released from the mitotic block in fresh medium and sampled at the indicated intervals. Protein extracts were analysed by immunoblotting with the antibodies to the indicated proteins. **c**, Extracts from G1-phase (3 h after release from nocodazole block; lane 1) and S-phase (12 h after release from the nocodazole block; lane 2) HeLa cells were immunoprecipitated (IP) with either an anti-p27 antibody (top two panels) or an anti-SKP2 antibody (bottom three panels) and then immunoblotted with the antibodies to the proteins indicated at the left.

To document the timing of SKP2 action in the process of p27 degradation, we analysed levels of p27, SKP2 and other cell-cycle regulators during cell-cycle progression in IMR90 fibroblasts (Fig. 7a) and HeLa cells (Fig. 7b). We monitored synchronization by immunodetection of an S-phase protein, cyclin A. Following re-entry into the cell cycle, the overall amount of p27 gradually decreased. This decrease was inversely proportional to the increase in levels of both SKP2 and cyclin E. In contrast, levels of CUL-1, SKP1 (as already reported[27,28]) and Ubc3 were constant during cell-cycle progression. We have observed previously that the state of p27 phosphorylation on T187 and the ubiquitination of p27 correlate in synchronized human cells[15]. We have now extended these findings by showing that p27 phosphorylation on T187 and the association of p27 with SKP2 also correlate, both processes occurring to a great extent in S-phase cells and to a low extent in G1 cells (Fig. 7c). Together with the other findings presented here, these results indicate that, in late G1, SKP2 recognizes phosphorylated p27, targeting it for ubiquitination.

Discussion

We have shown here that the FBP SKP2 is required for the ubiquitin-mediated degradation of the CDK inhibitor p27. First, p27 interacts with SKP2, but not with other FBPs, *in vitro*, and this interaction is dependent upon p27 phosphorylation (which is characteristic of an SCF–substrate interaction) (Fig. 1a–c). Second, SKP2 binds phosphorylated p27 *in vivo* (Fig. 2) and this binding is more predominant in S than in G1 phase (Fig. 7). Third, abundant ubiquitination of wild-type p27, but not of the mutant p27(T187A), can efficiently occur in G1 extracts only upon addition of SKP2 and cyclin E–CDK2 (Fig. 3a). Fourth, extracts depleted with an anti-SKP2 antibody are unable to sustain p27 ubiquitination unless reconstituted with recombinant purified SKP2 (Fig. 4a,b). Fifth, an anti-SKP2 antibody is able to precipitate a p27-ubiquitination activity (Fig. 4c). Finally, p27 is stabilized *in vivo* by the expression of a dominant-negative SKP2 and by antisense oligonucleotides targeting SKP2 mRNA (Fig. 5,Fig. 6).

The yeast CDK inhibitors Sic1 and Rum1 are targeted to a Skp1–cullin complex by the FBPs Cdc4 and Pop1/Pop2, respectively[31–36]. Similarly, our results strongly indicate that human SKP2 is the p27-recognition subunit of an ubiquitin ligase necessary for p27 ubiquitination. It is likely that such a ligase also contains SKP1 and CUL-1. However, the purified recombinant SKP1–CUL1–SKP2 complex (SCF^{SKP2}), supplemented with purified recombinant E1 and Ubc3, is not sufficient for the ubiquitination of p27 (our unpublished observations). These results show that this completely purified recombinant system still lacks component(s) that are essential for p27–ubiquitin ligation. Although the identity of every component of the ligase machinery required for p27 ubiquitination remains to be elucidated, our results clearly show that the SKP2 is a critical part of this machinery that provides substrate recognition and specificity.

Transfection studies have shown that a murine p27-interacting protein, Jab1, stimulates the translocation of p27 from the nucleus to the cytoplasm, where p27 is then degraded[37]. At the moment, the relationship between SKP2-dependent ubiquitin-mediated degradation and Jab1-dependent degradation of p27 is not clear. Results of an initial fractionation study indicate that Jab1 does not co-purify with p27-ubiquitinating activities (our unpublished observations). Thus, Jab1 may be necessary for the translocation and consequent degradation of ubiquitinated p27, but may not play a part in the enzymatic process of ubiquitination *per se*. Further studies will be required to clarify the function of Jab1 in p27 degradation.

In yeast, each SCF recruits more than one substrate for ubiquitination. For example, SCF^{Cdc4} recruits as substrates the CDK inhibitors Sic1 and Far1, the replication factor Cdc6, and the transcriptional activator Gcn4 (for a review, see ref. 6). Experiments with antisense oligonucleotides against human SKP2 have shown that a decrease in SKP2 levels correlates with an increase in the cellular abundance of p21 and cyclin D1, indicating a role for SKP2 in the regulation of the stability of these two CDK subunits[38]. However, a physical association of SKP2 with p21 (ref. 38) and cyclin D1 (Fig. 2 and ref. 38) has not been observed. Another SKP2-interacting protein, E2F-1, has recently been shown to be stabilized when lacking its SKP2-binding region[39]. However, at present there are no direct biochemical data, obtained using a reconstituted *in vitro* ubiquitination system, available to verify whether cyclin D1, p21 and E2F-1 are true SKP2 substrates. It is likely, however, that SKP2 targets other substrates, as well as p27, for ubiquitination.

We have shown that two different SKP2 antisense oligonucleotides decrease SKP2 expression and concomitantly increase the levels of endogenous p27 (Fig. 6). Initially, this result appears in conflict with those of Zhang and co-workers[38], who found no increase in p27 levels in the presence of SKP2 antisense oligonucleotides. One explanation for this discrepancy could be that, in the earlier experiment, higher p27 levels in the control precluded the detection of p27 stabilization by the SKP2 antisense oligonucleotide. Furthermore, Zhang and co-workers now have data on the function of SKP2 in p27 ubiquitination and degradation that are in agreement with our results (H. Zhang *et al.*, personal communication).

The requirement for SKP2 in p27 ubiquitination, together with the need for cyclin E–CDK2 or cyclin A–CDK2 (ref. 15), indicates the existence of a dual control mechanism for p27 degradation during cell-cycle progression. We propose the following model. In G0 or early G1 phase, p27 cannot be degraded, because of the low levels of both SKP2 and cyclin E. Following mitogenic stimulation, the rise in amounts of both SKP2 and cyclin E (Fig. 7 and refs 25, 27, 28) parallels and causes the rapid degradation of p27. During S and G2 phases, p27 levels are kept low by the high levels of SKP2 and cyclin A–CDK2. Of course, the involvment of other CDKs (such as CDK1 and CDK3) cannot be ruled out at present.

In many cancer cell lines amounts of SKP2 are high[25], which may account for the destabilization and resulting low levels of p27 that have been seen in many aggressive tumours[7,8,12,13]. A specific small-molecule inhibitor of SKP2 should lead to an increase in the cellular abundance of p27, and a consequent block in cellular proliferation and hence in disease progression. □

Methods

Extract preparation, immunoprecipitation, immunoblotting and cell synchronization.

Protein extraction was done as described[40] except that 1 μm okadaic acid was present in the lysis buffer. Immunoprecipitations and immunoblots were performed as described[14]. Human lung IMR90 fibroblasts were synchronized in G0/G1 by serum starvation for 48 h and then stimulated to re-enter the cell cycle by serum readdition. HeLa cells were synchronized by mitotic shake-off as described[41]. Synchronization was monitored by flow cytometry. For *in vitro* ubiquitination and degradation assays, G1 HeLa cells were obtained with 48-h lovastatin treatment[42] and protein extraction was done as described below.

Immunoreagents.

Rabbit polyclonal antibody to GST–SKP2 was generated, affinity-purified and characterized as described[43,44], in collaboration with Zymed Inc. Monoclonal antibodies to human CUL-1 (ref. 22) and cyclin E[45], affinity-purified rabbit antibodies to human p27 (ref. 15), SKP1 (ref. 22), CDK2 (ref. 46) and cyclin A[41], and the antibody against phosphorylated residue T187 of p27 C-terminal peptide (ref. 15) have been described previously. Monoclonal antibody to GST–Ubc3 was generated and characterized in collaboration with Zymed Inc. Monoclonal antibody to cyclin B was from Santa Cruz; monoclonal antibodies to p21 and p27 were from Transduction Laboratories; rabbit anti-FLAG antibody was from Zymed. Where indicated, an affinity-purified goat antibody to an amino-terminal SKP2 peptide (Santa Cruz) was used.

Protein extraction for *in vitro* ubiquitination assay.

Logarithmically growing HeLa-S3 cells were collected at a density of 6×10^5 cells per ml. About 4 ml HeLa-S3-cell pellet was suspended in 6 ml ice-cold buffer consisting of 20 mM Tris-HCl, pH 7.2, 2 mM dithiothreitol (DTT), 0.25 mM EDTA, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} pepstatin. The suspension was transferred to a cell nitrogen disruption bomb (Parr, Moline, IL) that had been rinsed thoroughly and chilled on ice before use. The bomb chamber was connected to a nitrogen tank and the pressure was brought slowly to 69 bars. The chamber was left on ice under the same pressure for 30 min and then the pressure was released slowly. The material was transferred to an Eppendorf tube and centrifuged in a microcentrifuge at 10,000*g* for 10 min. The supernatant (S-10) was divided into smaller samples and frozen at –80 °C.

In vitro ubiquitination and degradation assays.

The ubiquitination reaction mix (10 μl) contained 40 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1 mg ml^{-1} methylated ubiquitin, 1 μM ubiquitin aldehyde, 10 mM phosphocreatine, 100 μg ml^{-1} creatine phosphokinase, 0.5 mM ATP, 30 μg HeLa extract and 1 μl *in vitro*-translated [^{35}S]p27. Following incubation at 30 °C for 60 min, ubiquitination reactions were stopped with sample buffer and run on protein gels under denaturing conditions. Polyubiquitinated p27 forms were identified by autoradiography.

The p27-degradation assay was performed in a similar way, except that, first, methylated ubiquitin was omitted; second, the concentration of HeLa extract was 7 μg μl^{-1}; and third, extracts were prepared by hypotonic lysis[4], which preserves proteasome activity better than the 'nitrogen bomb' disruption procedure. Under these conditions, ubiquitinated p27 species are not visible because they are readily degraded by the proteasome.

Immunodepletion.

For immunodepletion studies, 3 μl SKP2 antiserum was adsorbed to 15 μl Affi-Prep protein-A beads (BioRad) at 4 °C for 90 min. The beads were washed and then mixed (4 °C, 2 h) with 40 μl HeLa extract (~400 μg protein). Beads were removed by centrifugation and supernatants were filtered through a 0.45-μm Microspin filter (Millipore).

Recombinant proteins.

Complementary DNA fragments encoding the entire coding region of human SKP2, SKP1 (His-tagged), FBP1 (FLAG-tagged), FBP3a (FLAG-tagged) and CUL-1 (haemagglutinin-tagged) were inserted into the baculoviral expression vector pBacpak-8 (Clonetech). Baculoviruses expressing human His-tagged cyc-

lin E and haemagglutinin-tagged CDK2 were supplied by D. Morgan[47]. All recombinant proteins were produced in 5B insect cells as described[15]. The different complexes were formed by coexpression of the appropriate baculoviruses and purified by nickel–agarose chromatography, using the His tag at the 5′ of SKP1 and cyclin E. Unless otherwise stated, recombinant proteins were added to incubations at the following amounts: cyclin E–CDK2, ~0.5 pmol; SKP1, ~0.5 pmol; SKP2, ~0.1 pmol; FBP1, ~0.1 pmol; FBP3a, ~0.1 pmol, CUL-1, ~0.1 pmol. The molar ratio of SKP1:SKP2, SKP1:FBP1, SKP1:FBP3a and SKP1:CUL-1 in the purified preparations was ~5.

Construction of ΔF mutants, transient transfections and pulse–chase analysis.

The (ΔF)SKP2 mutant was obtained by removing a DNA fragment (nucleotides 338–997) from the *SKP2* cDNA with *Bsp*EI and *Xba*I restriction enzymes, and replacing it with a polymerase chain reaction (PCR) fragment containing nucleotides 457–997. The final construct encoded a protein lacking residues 113–152. The construction of the (ΔF)FBP1 mutant has been described previously[22]. Cells were transfected with FuGENE transfection reagent (Boehringer) according to the manufacturer's instructions. Pulse–chase analysis was performed as described[14].

Antisense experiments.

Antisense experiments were performed as described[38]. Briefly, four oligodeoxynucleotides that contain a phosphorothioate backbone and C5 propyne pyrimidines were synthesized (Keck Biotechnology Resource Laboratory, Yale University) as follows: oligonucleotide 1, 5′-CCTGGGGGATGTTCTCA (the antisense direction of human SKP2 cDNA nucleotides 180–196); oligonucleotide 2, 5′-GGCTTCCG-GGCATTTAG (the scrambled control for oligonucleotide 1); oligonucleotide 3, 5′-CATCTGGCAC-GATTCCA (the antisense direction of SKP2 cDNA nucleotides 1,137–1,153); oligonucleotide 4, 5′-CCGCTCATCGTATGACA (the scrambled control for oligonucleotide 3). The oligonucleotides were delivered into HeLa cells using Cytofectin GS (Glen Research) according to the manufacturer's instructions. The cells were collected between 12 and 18 h post-transfection.

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