The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF^{Skp2}

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p130 is a tumor suppressor of the pocket protein family whose expression is posttranscriptionally regulated and largely G0 restricted. The mechanism of down-regulation of p130 expression in proliferating cells was investigated. Our results indicate that the decline of p130 expression as G0 cells reenter the cell cycle is due to a decrease in protein stability. The enhancement of p130 turnover in late G1 and S phase compared with G0 and early G1 phase was dependent on Cdk4/6-specific phosphorylation of p130 on Serine 672, and independent of Cdk2 activity. The activity of the ubiquitin ligase complex Skp1-Cul1/Cdc53-F-box protein Skp2 (SCF^{Skp2}) and the proteasome were necessary for p130 degradation. In vitro, recombinant Skp2 was able to bind hyperphosphorylated but not dephosphorylated p130. Furthermore, in vitro polyubiquitination of p130 by SCF^{Skp2} was specifically dependent on phosphorylation of p130 on Serine 672. Thus, like the Cdk inhibitor p27^{Kip1}, p130 turnover is regulated by Cdk-dependent G1 phosphorylation leading to ubiquitin-dependent proteolysis.

[**Keywords:** p130; Skp2; Cdk4; Cks1; ubiquitin; proteasome]

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p130 and its cognates, pRb and p107, constitute the "pocket protein" family. These proteins are key negative regulators of cell cycle progression and modulators of cellular differentiation processes in mammals. Although structurally related, their expression and biological activity are differentially regulated during the cell cycle, and their relevance to cell proliferation/differentiation tasks is also dependent on cell type and development stage (Garra et al. 1998; Stiegler et al. 1998b; Lipinski and Jacks 1999). pRb serves as a critical regulator of the G1/S transition, because the functional inactivation of pRb is essential in most cell types for the progression beyond the G1/S boundary. The role of p107, which normally accumulates at the G1/S boundary, is not well understood. p130 appears to be involved in the maintenance of the G0 state, although it can also have a primary role in the control of G1/S transition, depending on cell type (Grana et al. 1998; Hoshikawa et al. 1998; Mulligan and Jacks 1998; Stiegler and Giordano 1999). The antiproliferative function common to the three pocket proteins is transcription factor E2F (E2F)-depen-

dent inhibition of transcription through the formation of suppressor complexes with E2F factors and histone deacetylase (HDAC) enzymes and/or switch/sucrose-nonfermenting chromatin remodeling (SWI/SNF) complexes (Trouche et al. 1997; Ferreira et al. 1998; Stiegler et al. 1998a; Takahashi et al. 2000; O'Connor et al. 2001; Singh et al. 2001; Rayman et al. 2002). Furthermore, p130 and p107 share the ability to bind to and directly inhibit the activity of Cdk2/cyclin E and A complexes (De Luca et al. 1997; Lacy and White 1997; Woo et al. 1997; Castano et al. 1998). Consistent with this function, cyclin E/Cdk2 activity is efficiently inhibited by p130 in serum-starved *p27^{Kip1}-/- p21^{Cip1}-/-* double knockout mouse embryonal fibroblasts (MEFs; Coats et al. 1999).

Even though the ability to negatively control cell cycle progression is a common feature of all three members of the pocket protein family, only Rb meets all the requirements to be a bona fide tumor suppressor. Homozygous Rb mutations are very frequently found in tumors and reintroduction of a wild-type *RB* gene into *RB*-deficient tumor-derived cell lines often reverts the malignant phenotype. Most significantly, heterozygous *RB* null mutations in mice predispose to cancer (Weinberg 1995; Paggi et al. 1996; Mulligan and Jacks 1998). Targeted disruption of the gene for p130 or its cognate p107, on the other

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hand, gives rise to no evident phenotype. Yet it is clear that p130 has a role in cell cycle control in the context of development because mice of the mixed genotypes *p130*^{-/-} *p107*^{-/-} and *p130*^{-/-} *RB*^{+/+} exhibit severe developmental defects (Classon and Dyson 2001). In addition, the *p130*^{-/-} genotype by itself confers developmental defects in the Balb/c background, which is mutated at the *INK4a* (p16) locus (LeCouter et al. 1998; Zhang et al. 1998). Although p130 disruption does not cause predisposition to cancer in mice, p130 is found to be mutated in some human cancers; furthermore, p130 levels inversely correlate with tumor grade (Baldi et al. 1996, 1997; Susini et al. 1998; Massaro-Giordano et al. 1999; Tanaka et al. 1999). Thus, whereas p130 functions appear to be limited to control of cell proliferation in the context of development and differentiation in the mouse, p130 has an additional, although not well-defined, role in suppression of cancer in humans (Yee et al. 1998; Classon and Dyson 2001; Paggi and Giordano 2001).

The expression of p107 and, to a lesser extent, pRb is transcriptionally regulated via E2F sites, consistent with their levels peaking at the G1/S boundary (Nevins 1998). pRb is also expressed at high levels in some terminally differentiated G0 cell types, by an E2F-independent transcriptional mechanism (Martelli et al. 1994). On the other hand, accumulation of p130 is posttranscriptionally regulated and largely restricted to G0. Although neither the p130 mRNA level nor its promoter activity fluctuate as a function of proliferation or cell cycle progression, p130 protein is abundant in G0 and early G1 and then progressively disappears as cells progress through the cell cycle (Richon et al. 1997; Grana et al. 1998; Nevins 1998; Smith et al. 1998; Fajas et al. 2000).

Exit from G0 and progression through G1 into S phase requires both the activity of Cdks and E2F-dependent transcription, both of which are regulated by pocket proteins (Grana and Reddy 1995; Helin 1998). The control of Cdk activity is carried out at multiple levels, one of which is binding to kinase inhibitors such as p130 (Morgan 1995; Arellano and Moreno 1997; Obaya and Sedivy 2002). E2F-dependent transcription is controlled by the modulation of the relative amounts of free and pocket protein-bound E2F factors, leading to either transcriptional activation or repression (Dyson 1998; Black and Azizkhan-Clifford 1999). Furthermore, the expression of E2F1–2–3 is transcriptionally repressed specifically by E2F4/p130 repressor complexes, which are the most abundant E2F species in G0 (Furukawa et al. 1999; Takahashi et al. 2000). The disappearance of such complexes occurs after the transition from G0 to G1 in cells reentering the cell cycle, following Cdk-dependent hyperphosphorylation of p130 and dissociation of the complexes (Smith et al. 1996). The activities of Cdk4/6 and Cdk2 appear to be redundant in this context (Cheng et al. 2000; Hansen et al. 2001). On the other hand, p130 binding to and inhibition of Cdk2 is not controlled by phosphorylation, but only by the relative abundance of p130, which progressively decreases as G0 cells reenter the cell cycle and approach S phase (Grana et al. 1998; Smith et

al. 1998; Hansen et al. 2001). Therefore, the mechanism underlying p130 down-regulation is particularly relevant for p130 functions involving negative regulation of Cdk2 kinase activity.

Previous studies have suggested that posttranscriptional regulation of p130 levels is at the level of proteasome-mediated protein turnover (Smith et al. 1996; Studdal et al. 1996; Prince et al. 2002). Indeed, ubiquitin-dependent proteolysis by the proteasome is a common regulatory motif for a growing number of proteins involved in cell cycle control. Depending on either the substrate or the specific protein–ubiquitin ligase (or E3 enzyme) used, the signals triggering the polyubiquitination cascade can rely on different events such as (1) covalent or conformational modification of the target protein, (2) changes in its association with other proteins, or (3) direct activation of the relevant protein–ubiquitin ligase (Hershko and Ciechanover 1998; Peters 1998; Laney and Hochstrasser 1999; DeSalle and Pagano 2001; Yew 2001). A class of E3 enzymes, known as Skp1–Cul1/Cdc53–F-box protein (SCF) complexes, recognizes and polyubiquitinates substrates phosphorylated at specific sites. Roc1, Cul1, and Skp1 are the invariant core components of SCF complexes, and one of several F-box proteins is responsible for substrate recognition and specificity (Deshaies 1999; Krek 1998; Jackson et al. 2000). Specific SCF complexes polyubiquitinate phosphorylated IKB α , p27^{Kip1} and cyclin E, respectively, and target them to proteasome-mediated degradation (Clurman et al. 1996; Won and Reed 1996; Diehl et al. 1997; Vlach et al. 1997; Montagnoli et al. 1999; Winston et al. 1999; Koepf et al. 2001; Strohmaier et al. 2001). Correlation between p130 phosphorylation and down-regulation suggested that p130 might also be a target of SCF and subsequent proteasome-mediated degradation. In the present study, we show that this is indeed the case, with SCF^{Skp2} being the relevant protein–ubiquitin ligase. We show that recognition by SCF^{Skp2} is possible only after p130 is specifically phosphorylated at Ser 672 by Cdk4 and/or Cdk6. Cdk4 is already known to have a role in activation of Cdk2 by sequestration of the Cdk inhibitor p27 (Sherr and Roberts 1995, 1999; Cheng et al. 1998). Now we reveal an additional mechanism whereby Cdk4/6 activates Cdk2, triggering the degradation of the Cdk2 inhibitor p130.

Results

p130 is regulated through the cell cycle at the level of turnover

p130 levels are regulated in response to the proliferative state of cells. Serum-starved G0 human diploid fibroblasts contain high levels of p130 in a hypophosphorylated state (Fig. 1A). Subsequent to cell cycle entry in response to serum stimulation, mid-G1 cells exhibit an increase in p130 phosphorylation, which correlates with a decline in p130 steady state levels (Mayol et al. 1995; Grana et al. 1998; Fig. 1A.). Because p130 mRNA levels do not fluctuate through the cell cycle (Richon et al. 1997; Smith et al. 1998), we sought to determine

Figure 1. p130 expression and stability in human fibroblasts after release from G0 block. (A) G0-arrested human fibroblasts were released into the cell cycle and samples were harvested for Western blot analysis at the indicated time points after release. For each sample, the percentage of cells in the G0/G1, S, and G2 phases of the cell cycle was calculated by FACS analysis. The last sample on the right is from cells synchronized in early S phase by thymidine block. (B) G0-arrested human fibroblasts were released into the cell cycle and samples were harvested for Western blot analysis at the indicated time points after release. Samples treated with cycloheximide (CHX) received the drug at the time at which the corresponding untreated samples were harvested, and were then harvested 2 h later.

whether p130 protein levels are regulated at the level of proteolysis. We first determined the stability of p130 at different time points after release from G0. Serum-starved human fibroblasts were released into the cell cycle by addition of serum, and duplicate samples were harvested at different time points after the release, ranging from early G1 to S/G2. For each time point, one sample was harvested immediately, and the other was cultured in the presence of a protein synthesis inhibitor, cycloheximide, for two additional hours prior to harvest (Fig. 1B). Hyperphosphorylated p130 present from late G1 on was substantially less stable than the hypophosphorylated p130 associated with early G1. Although the related protein pRb was phosphorylated with similar cell cycle kinetics, no change in stability was observed (Fig. 1B). Thus, p130 levels are indeed regulated through controlled proteolysis, possibly in a phosphorylation-dependent manner.

p130 turnover is regulated by phosphorylation

To establish a causal relationship between p130 hyperphosphorylation and its degradation, we investigated the effect of the protein kinase inhibitor flavopiridol. Flavopiridol targets Cdk2, Cdk4, and Cdk6, all of which can phosphorylate p130 (Hansen et al. 2001; D. Tedesco and S.I. Reed, unpubl.). To exclude cell cycle effects of the Cdk inhibitor, we first arrested cells in early S phase by treatment with thymidine, prior to treatment with the

inhibitor. A similar approach was used with all subsequent manipulations that might affect the cell cycle distribution of cells under analysis. After the first 4 h of incubation with flavopiridol, cells were treated with cycloheximide for various lengths of time before harvesting, and p130 stability was determined. Cdk inhibition by flavopiridol abolished both the hyperphosphorylation and the degradation of p130, as shown in Figure 2A. This confirms that p130 phosphorylation is potentially a signal for its degradation. Similar experiments were then carried out with the Cdk1/Cdk2-specific inhibitor roscovitine. Even though roscovitine can potentially affect Cdk4/6 activity indirectly, by inhibiting MAP kinases if administered in early G1, no effect on Cdk4/6 activity was detectable in thymidine-arrested cells, as judged by pocket proteins phosphorylation state (data not shown). After roscovitine treatment, p130 remained hyperphosphorylated and unstable, indicating that neither phosphorylation nor turnover depend on Cdk2 activity (Fig. 2B; Cdk1 is not active in S-phase cells). Furthermore, inhibition of Cdk4 and Cdk6 by adenovirus-mediated overexpression of the Cdk4/6 inhibitor p16^{INK4} in S-phase (thymidine)-arrested cells led to hypophosphorylation and accumulation of p130 similarly to p27, which when greatly overexpressed inhibits Cdk4/6 as well as Cdk2 (Fig. 2C). Thus, the response of p130 levels and stability to general and specific Cdk inhibitors suggests that Cdk4 and/or Cdk6, but not Cdk2 activity, is required for p130 degradation, possibly through site-specific phosphorylation.

Figure 2. Effects of kinase inhibitors on p130 stability. Thymidine-arrested human fibroblasts were treated with either Flavopiridol (A) or Roscovitine (B). Protein-synthesis inhibitor cycloheximide (CHX) was added after the first 4 h of treatment, and cells were harvested for Western blot analysis after the indicated time. DMSO, dimethyl sulfoxide. (C) Thymidine-arrested human fibroblasts were infected with the indicated recombinant adenoviruses, maintained under thymidine arrest, and harvested 18 h later for Western blot analysis.

p130 turnover depends on proteasome function

We next determined whether proteasomal function is required for p130 proteolysis. In an experiment analogous to those just described for Cdk inhibitors, S-phase-arrested human fibroblasts were pretreated with the proteasome-specific inhibitor MG-132, followed by treatment with cycloheximide to measure the stability of p130 (Fig. 3A). Proteasome inhibition resulted in dramatic stabilization of p130, but also in an inhibition of p130 hyperphosphorylation. The latter phenomenon was likely attributable to the induced accumulation of the Cdk inhibitors p21 and p27 (data not shown), both substrates of the proteasome (Pagano et al. 1995; Sheaff et al. 2000). Therefore, it was not possible to determine whether p130 stabilization was a direct effect of proteasome inhibition or an indirect effect of inhibition of p130 phosphorylation. However, in a similar experiment in which both MG-132 and cycloheximide were administered simultaneously so that kinase inhibitors could not

accumulate, it was found that proteasome inhibition stabilized p130 without an effect on p130 phosphorylation (Fig. 3B).

SCF^{Skp2} is required for the phosphorylation-dependent degradation of p130

The cell cycle-dependent expression profile of p130 closely resembles that of the Cdk inhibitor p27, and the levels of both proteins are controlled through phosphorylation-triggered proteasome-mediated degradation. We investigated whether hyperphosphorylated p130 might be targeted to the proteasome by the SCF^{Skp2} protein-ubiquitin ligase, which is responsible for the polyubiquitination and subsequent degradation of p27 (Carrano et al. 1999). To this end, we compared the regulation of p130 in *SKP2*^{+/-} and *SKP2*^{-/-} mouse fibroblasts (Nakayama et al. 2000). In *SKP2*^{+/-} and *SKP2*^{-/-} fibroblasts arrested in G₀, p130 was present at comparably high levels. In contrast, p130 levels in thymidine-arrested *SKP2*^{-/-} cells were much higher than in corresponding *SKP2*^{+/-} cells (Fig. 4A,B). To confirm that both *SKP2*^{-/-} and *SKP2*^{+/-} cells had exited G₀, traversed G₁, and similarly arrested in S phase, parallel immunoblots were performed for cyclin A, a marker for entry into S phase. As can be seen, cyclin A signals were comparable for both *SKP2*^{-/-} and *SKP2*^{+/-} fibroblasts (Fig. 4A,B). In Figure 4A, there is an apparent decrease in p130 levels as cells

Figure 3. Effect of proteasome inhibition on p130 stability. (A) Thymidine-arrested human fibroblasts were treated with proteasome inhibitor MG-132. Protein-synthesis inhibitor cycloheximide (CHX) was added after the first 4 h of treatment, and cells were harvested for Western blot analysis after the indicated time. DMSO, dimethyl sulfoxide. (B) Thymidine-arrested human fibroblasts were treated either with CHX alone, or with CHX and MG-132, and cells were harvested for Western blot analysis after the indicated time.

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Figure 4. Effects of Skp2 and Cks1 on p130 expression. (A) G0-arrested and thymidine-arrested *SKP2*^{+/-} and *SKP2*^{-/-} primary MEFs were analyzed for p130, p27, and cyclin A levels by Western blot. Gel loading was normalized to protein concentration. Signals were quantitated by ImageQuant. (B) p130 and cyclin A levels were analyzed as in A, except that all loading was normalized to cell number. (C) Thymidine-arrested *p27*^{+/+} and *p27*^{-/-} MEFs were infected with either empty, Skp2-expressing, or Skp2(Δ Fbox)-expressing recombinant adenoviruses, maintained under thymidine arrest, and harvested 18 h after infection for Western blot analysis. (D) G0-arrested, asynchronous, and thymidine-arrested *CKS1*^{+/+} and *CKS1*^{-/-} MEFs were analyzed for p130 expression by Western blot. Thymidine-arrested *CKS1*^{-/-} MEFs were also infected with either empty or FLAG-tagged Cks1-expressing recombinant adenoviruses, maintained under thymidine arrest, and analyzed 18 h after infection. Loading was normalized to protein concentration.

progress from G0 to S phase, suggesting that Skp2-independent mechanisms may also regulate p130 levels. However, a similar reduction was observed for p27, for which turnover is thought to be largely Skp2-dependent (Malek et al. 2001). Because loading of gels in Figure 4A was normalized to protein concentration, we hypothesized that the lower protein content of G0 cells compared with cycling cells might account for the apparent relative reduction in p130 levels in S-phase-arrested cells. Therefore another gel was run, in which loading

was normalized to cell number (Fig. 4B). Using these assay conditions, it is clear that the amount of p130 per cell decreases minimally as *SKP2*^{-/-} fibroblasts progress from G0 to S phase. Furthermore, in S-phase-arrested wild-type (wt) fibroblasts, adenovirus-mediated overexpression of wt Skp2 resulted in down-regulation of p130, whereas overexpression of dominant-negative, F-box-deleted Skp2 led to the accumulation of p130 in a hyperphosphorylated state (Fig. 4C). Similar results were obtained using *p27*^{Kip1}^{-/-} fibroblasts, thus ruling out the possibility that the effects of Skp2 expression on p130 levels might be mediated indirectly via changes in the level of *p27*^{Kip1}, leading to changes in p130 phosphorylation (Fig. 4C).

Cks1 is required for SCF^{Skp2}-mediated degradation of p130

Cks1 is an essential cofactor for Skp2-dependent degradation of phosphorylated p27 (Ganoth et al. 2001; Spruck et al. 2001), and thus might be required for ubiquitination of other SCF^{Skp2} substrates. In wt and *CKS1*^{-/-} fibroblasts arrested in G0, p130 was present at comparably high levels (Fig. 4D). However, as with *SKP2*^{-/-} fibroblasts, p130 levels in asynchronous and thymidine-arrested *CKS1*^{-/-} cells were much higher than in corresponding wt cells, consistent with a role for Cks1 in p130 degradation. Transduction of *CKS1*^{-/-} fibroblasts with a Cks1-expressing recombinant adenovirus restored p130 to wt levels, confirming that the defect in p130 regulation is due specifically to loss of Cks1 function (Fig. 4D; data not shown).

Skp2 binds to and targets phosphorylated but not unphosphorylated p130

Because p130 degradation was shown to be phosphorylation-dependent, *in vitro* binding assays were carried out to determine whether Skp2 could bind hyperphosphorylated, but not dephosphorylated p130, as is the case for other SCF substrates such as *p27*^{Kip1} (Carrano et al. 1999). Thymidine-arrested *CKS1*^{-/-} fibroblasts were exploited as a source of native hyperphosphorylated p130 (see Fig. 4D). Anti-p130 immunoprecipitates were then challenged with purified recombinant glutathione-S transferase (GST)-Skp2/Skp1/Cks1 complexes. Figures 5A and 5B show that Skp2 bound untreated, but not protein phosphatase-treated, p130 immunoprecipitates. Recombinant adenoviruses were then used to show that the effect of Skp2 on p130 turnover was phosphorylation dependent. U2OS cells were chosen for these experiments because Skp2 activity was found to be limiting and p130, therefore, intrinsically stable in these cells (data not shown). Adenoviral transduction of wt Skp2 dramatically down-regulated endogenous p130, whereas neither the F-box-deleted Skp2 nor the Cdk4/6 inhibitor p16 caused accumulation of the already stable p130 (Fig. 6A). However, when Skp2 was expressed in conjunction with p16, the down-regulation of p130 was substantially re-

Figure 5. In vitro Skp2 binding to hyperphosphorylated p130. (A) Nonimmune, anti-p130, and anti-p27^{Kip1} immunoprecipitates were immobilized on beads from thymidine-arrested *CKS1*^{-/-} MEFs, and probed for binding to soluble GST-Skp2/Skp1/Cks1 complexes. After binding, beads were extensively washed and retained proteins were analyzed by Western blot. IP, immunoprecipitating antibody; NI, nonimmune serum. (B) Nonimmune and anti-p130 immunoprecipitates prepared as in A were either treated or not treated with λ protein phosphatase, and then assayed for binding, as in A.

duced (Fig. 6A), indicating that in vivo, p130 requires phosphorylation by Cdk4/6 in order to be targeted by Skp2.

Phosphorylation of Ser 672 is essential for Skp2 binding and p130 degradation

In order to confirm that the Skp2-induced degradation of p130 depends on site-specific phosphorylation of p130, p130 phosphorylation site mutants were analyzed for their susceptibility to Skp2 overexpression. Because p130 degradation is blocked by the inhibition of Cdk4/6, but not of Cdk2, at least one of the relevant phosphorylation sites in p130 has to be Cdk4/6 specific. By means of retroviral transduction, we obtained U2OS-derived populations expressing either hemagglutinin epitope (HA)-tagged p130 wt, HA-tagged p130 lacking the three previously identified specific Cdk4/6 phosphorylation sites (p130ΔCdk4; Hansen et al. 2001), or HA-tagged p130 lacking all Cdk phosphorylation sites (p130PM19A; Hansen et al. 2001). The effect of Skp2 activity was tested by adenoviral transduction of Skp2 in thymidine-arrested cultures. The Western blot in Figure 6B shows that mutation of the three Cdk4/6-specific phosphorylation sites sufficed to render p130 insensitive to Skp2 overexpression. To further refine the phosphorylation requirements for p130 degradation, we constructed single-residue mutants corresponding to individual Cdk4/6-specific phosphorylation sites and analyzed as described earlier. Of the three sites (T401, S672, and S1035), only the C-terminal two were considered in this analysis, because the N-terminal site is not conserved. It was found that a single substitution, S672A, rendered p130 as refractory to Skp2 activity as the triple mutant, whereas S1035A had no effect (data not shown), indicating that

phosphorylation of Ser 672 is specifically required for p130 degradation (Fig. 6B). To ensure that the differences observed in steady-state level unambiguously reflect changes in turnover rate, we measured the p130 turnover directly by pulse-chase analysis. U2OS cells expressing either HA-wt-p130 or HA-S672A-p130 were transduced with either Skp2 or control adenoviruses, and the decay of the HA-tagged proteins was analyzed. As illustrated in Figure 6C, the results of two independent pulse-chase experiments confirmed that Skp2 overexpression induced a significant shortening of the p130 half-life that

Figure 6. Phosphorylation requirements for Skp2 binding and destabilization of p130. (A) Thymidine-arrested U2OS cells were infected with either empty, Skp2-expressing, Skp2(ΔFbox)-expressing, or p16^{INK4}-expressing recombinant adenoviruses, maintained under thymidine arrest, and harvested 18 h after infection for Western blot analysis. (B) Thymidine-arrested U2OS cells expressing the indicated HA-tagged recombinant alleles of p130 were infected with either empty or Skp2-expressing recombinant adenoviruses, maintained under thymidine arrest, and harvested 18 h after infection for Western blot analysis. (C) Pulse-chase analysis of p130 decay in thymidine-arrested U2OS cells expressing either β-galactosidase, HA-tagged wt p130, or HA-tagged S672A p130 after infection with either empty or Skp2-expressing recombinant adenoviruses.

depended on the ability of p130 to be phosphorylated on Ser 672.

p130 is a target of ubiquitination by SCF^{Skp2}

In vitro assays were performed to determine whether the effect of Skp2 on the stability of p130 is mediated by polyubiquitination. Soluble FLAG-tagged SCF^{Skp2} complexes were immunoaffinity purified from transfected 293T cells, and assayed for the ability to attach polyubiquitin chains to p130 molecules. Native endogenous p130 was purified from *CKS1*^{-/-} fibroblasts, and HA-tagged wt and S672A p130 were prepared from retrovirally transduced U2OS cells. In vitro ubiquitination reactions were performed as previously described (Spruck et al. 2001; Strohmaier et al. 2001), and the reaction products analyzed by SDS-PAGE and Western blot. As illustrated in Figure 7A, SCF^{Skp2} complexes were capable of polyubiquitinating wt p130 but not p130 S672A, confirming that p130 phosphorylated on Ser 672 is a direct ubiquitination target of SCF^{Skp2}. On the other hand, SCF complexes containing a different F-box protein, hCdc4, were incapable of ubiquitinating p130, although they were capable of ubiquitinating cyclin E (Fig. 7B). To eliminate the possibility that p130 was being monoubiquitinated at multiple sites rather than polyubiquitinated, reactions were performed using methylated ubiquitin rather than ubiquitin. Methylated ubiquitin can form monoubiquitin conjugates but not polyubiquitin conjugates. In vitro reactions using methylated ubiquitin produced a distinct monoubiquitinated p130 band, but no more slowly migrating species were observed (Fig. 7C).

Therefore, the slowly migrating species observed in Figure 7A must correspond to polyubiquitin conjugates of p130.

Discussion

Both cell cycle inhibitory functions of p130 are antagonized by phosphorylation-dependent mechanisms

The ability of p130 to confer a sustained G1 block when ectopically expressed has been ascribed to both phosphorylation-sensitive and phosphorylation-insensitive functions (Hansen et al. 2001). Here we show that, ultimately, all cell cycle inhibitory functions of p130 are phosphorylation-sensitive in that the steady-state level of p130 itself is regulated by phosphorylation-dependent proteolysis.

Our initial observation of a strong correlation between p130 hyperphosphorylation and instability prompted us to investigate the possibility of a causal relationship between the two phenomena. Using the kinase inhibitors flavopiridol and roscovitine, both active against Cdk2, but only the former active against Cdk4/6, we could show that Cdk2 activity was not necessary for triggering p130 degradation, whereas Cdk4/6 was. Additional experiments using adenovirus-mediated overexpression of the Cdk4/6 specific inhibitor p16 confirmed the requirement for Cdk4/6 in the regulation of p130 degradation. A caveat concerning the use of p16 as a Cdk4/6 specific inhibitor is the ability of INK4 family inhibitors to displace p27 from Cdk4/6 to Cdk2, thereby leading to inhi-

Figure 7. In vitro ubiquitination of p130 by SCF^{Skp2}. (A) Anti-p130 immunoprecipitates were immobilized on beads from thymidine-arrested *CKS1*^{-/-} MEFs, or thymidine-arrested U2OS cells expressing either wt or S672A HA-tagged p130. Ubiquitination reactions by soluble immunopurified SCF^{FLAG-Skp2} were performed, and polyubiquitination of the immobilized immunoprecipitates was assayed by Western blot analysis. (B) Anti-cyclin E and anti-p130 immunoprecipitates were immobilized on beads from thymidine-arrested U2OS cells. Ubiquitination reactions by soluble immunopurified SCF^{FLAG-hCdc4} were performed, and polyubiquitination of the immobilized immunoprecipitates was assayed by Western blot analysis. (C) Anti-p130 immunoprecipitates were immobilized on beads from thymidine-arrested U2OS cells. Ubiquitination reactions by soluble immunopurified SCF^{FLAG-Skp2} were performed in the presence of either native ubiquitin (Ub) or methylated ubiquitin (mUb), and mono/polyubiquitination of the immobilized immunoprecipitates was assayed by Western blot analysis.

bition of both Cdk4/6 and Cdk2 (Sherr and Roberts 1995; Cheng et al. 1998). However, an indirect inhibitory effect of p16 on Cdk2 activity requires a large pool of p27 to be released from Cdk4/6 containing complexes, which is the case in early or mid-G1 cells but not in thymidine-arrested S-phase cells used in our study. Thus, as cells emerge from G0 and enter the cell cycle with concomitant accumulation of D-type cyclins and formation of active cyclin D/Cdk4/6 complexes, p130 is simultaneously dissociated from E2F4 relieving transcriptional repression of E2F-dependent genes and targeted for proteasome-mediated degradation, relieving inhibition of cyclin E/Cdk2 complexes. In this manner, p130 serves as a link between positive proliferative signals shown to promote accumulation of D-type cyclins and the core cell cycle machinery driven by cyclin E/Cdk2 and E2F-dependent transcription.

The relationship between p130 and p27

The observation that Cdk4/6 activity is the functional trigger for p130 degradation and, as a consequence, the release of Cdk2 inhibition, suggests a parallelism between p130 and p27 in the regulation of Cdk2 activity. Both are Cdk2 inhibitors targeted for ubiquitin-dependent proteasome-mediated degradation at the G1/S boundary by Cdk-dependent phosphorylation. However, there are also key differences. First, p27 down-regulation in cells emerging from G0 begins early in G1 prior to p27 phosphorylation. At least in some cell types, this is likely to occur through a cytoplasmic mechanism that is dependent on polyubiquitination and proteasome-mediated degradation but independent of Skp2 (Hara et al. 2001). Second, Cdk2 inhibition by p27 is relieved not only by degradation, but also by the equilibration of p27 away from Cdk2 to cyclin D/Cdk4/6 complexes as these accumulate (Sherr and Roberts 1999). In cells exiting from G0, it is therefore conceivable that the release from p27-mediated inhibition of Cdk2 is achieved more rapidly than p130-mediated inhibition, because p27 degradation begins in early G1 and only the physical accumulation of cyclin D/Cdk4/6 complexes is needed to titrate p27 away from Cdk2. On the contrary, p130 is stable in early G1 and cannot be sequestered by Cdk4/6, and it is only through phosphorylation by Cdk4/6 and subsequent degradation of p130 that Cdk2 is released from inhibition. Although it is likely that the increase of p130 turnover following hyperphosphorylation is a rapid event, phosphorylation of the bulk of p130 that has accumulated in G0 cells appears to be rate limiting and is not complete until cells are in late G1 (Mayol et al. 1995; D. Tedesco and S.I. Reed, unpubl.). Furthermore, because the half-life of hyperphosphorylated p130 is not dramatically short (at least 1 h), p130 expression does not drop to minimal levels until cells are at the G1/S boundary. In light of these observations and considerations, the roles of p27 and p130 in regulation of Cdk2 activity, although overlapping, are probably not completely redundant. We suggest a sequential inhibition, first primarily by p27, and then when p27 pools have been reequilibrated to

Cdk4/6 complexes, by p130. The resistance of p130 to rapid inactivation by titration would promote the accumulation of inactive cyclin E/Cdk2/p130 complexes until a threshold level of cyclin D/Cdk4/6 activity triggers p130 degradation and concomitant rapid activation of Cdk2. However, the ability of Skp2 to target both cell cycle inhibitors may explain its potency, when overexpressed, in driving quiescent cells into S phase (Sutterluty et al. 1999).

p27 and p130 are both targets of SCF^{Skp2}

Many cell cycle regulatory proteins are targeted for proteasome-mediated degradation by polyubiquitination following site-specific phosphorylation and recognition by specific SCF ubiquitin ligases (DeSalle and Pagano 2001; Yew 2001). The degradation of the Cdk inhibitor p27 depends on polyubiquitination by SCF^{Skp2} following phosphorylation on Thr187 (Carrano et al. 1999). SCF-mediated ubiquitination of p27 depends on a physical interaction between Skp2 and the small Cdk interacting protein, Cks1 (Ganoth et al. 2001; Spruck et al. 2001). Consistent with this requirement, in both *SKP2*^{-/-} and *CKS1*^{-/-} cells, p27 is stable even when phosphorylated, and as a consequence, is maintained at a relatively high level even at the G1/S transition, where it is normally down-regulated. p130 exhibits a similar pattern of hyperaccumulation in *SKP2*^{-/-} and *CKS1*^{-/-} fibroblasts. In principle, p130 accumulation under these circumstances could be an indirect effect of p27 accumulation and concomitant inhibition of p130 phosphorylation. However, the fact the p130 accumulates in its hyperphosphorylated forms renders this mechanism unlikely, and prompted us to perform the series of experiments that led to the conclusion that Skp2 is the F-box protein responsible for the ubiquitination and degradation of p130. Thus, p27 and p130 are regulated in a similar fashion, the primary difference being the specific Cdk activity conferring affinity for Skp2 and hence triggering degradation: cyclin D/Cdk4/6 for p130 and cyclin E/Cdk2 for p27. Although it is not surprising that two proteins with partially redundant functions are regulated by parallel mechanisms, the apparent lack of homology between p130 and p27 in the regions surrounding the relevant phosphorylation sites raises some interesting questions concerning the nature of the Skp2 "degron," the immediate protein structure actually recognized by Skp2. It has recently been reported that another F-box protein, Cdc4, recognizes a specific, although somewhat degenerate, consensus, surrounding the substrate phosphorylation site, designated the Cdc4 degron (Nash et al. 2001; Strohmaier et al. 2001). In contrast, it seems, based on sequence comparisons of p27 and p130, that a consensus degron for Skp2 might not exist, raising the possibility that Skp2 might interact with different substrates through different subregions in its large leucine-rich repeat domain (Kobe and Kajava 2001). In this context, the fact that Skp2 requires Cks1 for ubiquitination of both p27 and p130 suggests a general Skp2 activation function for Cks1.

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Materials and methods

Cell lines

hTERT-immortalized human fibroblasts were a kind gift of J.W. Shay (The University of Texas Southwestern Medical Center, Dallas, TX). *CKS1*^{+/+} and *CKS1*^{-/-} MEF lines were previously described (Spruck et al. 2001). *p27*^{+/+} and *p27*^{-/-} MEF lines were a kind gift of J. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA). Human osteosarcoma cell line U2OS is ATCC no. HTB-96. *SKP2*^{+/+} and *SKP2*^{-/-} MEFs were a kind gift of K.-I. and K. Nakayama (Kyushu University, Fukuoka, Japan).

Cell culture and synchronization

All cells were routinely cultured in 10% FBS DMEM. Synchronization in G0 was achieved by 48-h serum starvation of confluent cultures (0.1% FBS). Re-entry into the cell cycle of G0-arrested cells was achieved by trypsinization, sparse reseeding, and cultivation in 10% FBS DMEM. Synchronization in early S phase was achieved by restimulating G0-synchronized cells in the continuous presence of 2 mM thymidine for 24 h.

Immunological reagents and procedures

Immunoprecipitations and Western blots were performed as described (Harlow and Lane 1988), unless otherwise specified. Gel loading was normalized to protein concentration, unless otherwise specified. Signals were quantitated by ImageQuant. Antibodies used included the following: p130 (C-20, Santa Cruz), p130 (C-20-G, Santa Cruz), p107 (C-18, Santa Cruz), pRb (G3-245, PharMingen), cyclin A (H-432, Santa Cruz), GST (B-14, Santa Cruz), HA (12CA5 ascitis), HA (HA.11 monoclonal, BAbCO), SKP2 (GP45, Zymed), p27 (cat no. K25020, Transduction), p27 (C-19, Santa Cruz), p16 (cat no. 3501-1, Clontech), p21 (C-19, Santa Cruz), and FLAG (M2, Sigma).

Retroviral production and infections

Recombinant retroviruses were generated and stable cell lines expressing HA-tagged p130 alleles were obtained by retroviral infection and selection, as described (Morgenstern and Land 1990). Viral stocks were prepared as described, using the 293T-derived packaging cell line Phoenix-Ampho, and the retroviral plasmid vectors of the pBABE series (Morgenstern and Land 1990; Kinsella and Nolan 1996).

Adenoviral production and infection

Recombinant adenoviruses expressing either wt or Δ Fbox Skp2 (Carrano et al. 1999) were generated as previously described (Bett et al. 1994; Strohmaier et al. 2001). The recombinant adenovirus for the expression of human p16 and human p27 were previously described (Niculescu et al. 1998; Schreiber et al. 1999). The recombinant adenovirus for the expression of human Cks1 was a kind gift of C. Spruck (The Scripps Research Institute, La Jolla, CA). Purified viral stocks were prepared by CsCl ultracentrifugation. U2OS cells and MEFs were infected at a multiplicity of infection of 400 and 1200 v.p./cell, respectively, by incubating thymidine-arrested cultures with CsCl-purified viral particles in media with no serum supplemented with 2 mM thymidine for 4 h. FBS to 10% final concentration was then added and cells were cultured in 2 mM thymidine 14 h further prior to harvesting.

In vitro binding assays

Baculovirus expressed GST-Skp2/6HIS-Skp1 complexes were purified on a Ni-NTA resin (QIAGEN) under nondenaturing conditions as previously described (Spruck et al. 2001). Cks1 was expressed in bacteria and purified as described (Bourne et al. 1996). Nonimmune, α -p130 and α -p27 immunoprecipitates immobilized on beads were prepared from *CKS1*^{-/-} thymidine-arrested fibroblasts, and probed for binding to the Cks1/Skp2/Skp1 soluble complex. The binding reaction was carried out at +4°C for 3 h in a buffer containing 10 mM Na Pyrophosphate, 10 mM Na β -glycerophosphate, 10 mM MgCl₂, 0.1% Tween-20, 5% glycerol, 1 mM DTT, 1% BSA, 2 μ g/mL aprotinin, 2 μ g/mL leupeptin, 2 μ g/mL pepstatin, 1 mM PMSF, and 50 mM TrisHCl (pH 7.5). Pellets were then washed five times in such buffer, boiled in SDS-sample buffer and analyzed by Western blot with a monoclonal anti-GST antibody. In the λ -phosphatase experiment, phosphatase inhibitors were not included in the washing buffer, and samples were dephosphorylated at +30°C for 1 h following manufacturer's specifications (Calbiochem) before the binding reaction.

In vitro ubiquitination assays

Recombinant SCF^{Skp2} and SCF^{hCdc4} complexes were isolated from transfected 293T cells, as previously described (Strohmaier et al. 2001), and then eluted in ubiquitination buffer containing 3 $\times$ -FLAG peptide (SIGMA) following manufacturer's specifications. Ubiquitination buffer contained 5mM NaF, 1 mM NaVO₄, 1.5 mM MgCl₂, 5 mM KCl, 1 mM DTT, 20 mM HEPES (pH 7.4), 2 μ g/mL aprotinin, 2 μ g/mL leupeptin, 2 μ g/mL pepstatin, and 1 mM PMSF. Equal amounts of SCF^{Skp2} complex were added to immunopurified substrates immobilized on beads. Ubiquitination was carried out in a 30- μ L volume at +30°C for 2 h with 2 μ g either Bovine Ubiquitin (SIGMA) or Methyl Ubiquitin (Boston Biochem), 1.3 μ g UbcH3/Cdc34 (Boston Biochem) and 0.65 μ g E1 enzyme (Boston Biochem), 0.1 μ g Cks1, 1 mM ATP, 20 mM creatine phosphate, and 5 μ g creatine kinase. Reactions were terminated by washing the pellets three times in RIPA buffer and boiling for 3 min in SDS sample buffer. Eluted substrates were then analyzed by Western blot.

Pulse-Chase experiments

Pulse-chase experiments were performed on thymidine-arrested cultures. Cells were incubated with starvation medium (without methionine, 2 mM thymidine, no serum) for 15 min and then labeled (150 μ Ci/mL Redivue 35S-methionine, Amersham Pharmacia) for 45 min. Cells were then chased with normal medium supplemented with 10% FBS, 2 mM thymidine, and 100 mg/mL methionine. Cells were then washed twice in cold PBS and lysed in RIPA buffer. Immunoprecipitations were performed with anti-HA 12CA5 monoclonal antibody.

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