Molecular Dissection of the Interaction between p27 and Kip1 Ubiquitylation-promoting Complex, the Ubiquitin Ligase That Regulates Proteolysis of p27 in G1 Phase

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The cyclin-dependent kinase (CDK) inhibitor p27 is degraded at the G0-G1 transition of the cell cycle by the ubiquitin-proteasome pathway in a Skp2-independent manner. We recently identified a novel ubiquitin ligase, KPC (Kip1 ubiquitylation-promoting complex), consisting of KPC1 and KPC2, which regulates the ubiquitin-dependent degradation of p27 at G1 phase. We have now investigated the structural requirements for the interactions of KPC1 with KPC2 and p27. The NH2-terminal region of KPC1 was found to be responsible for binding to KPC2 and to p27. KPC1 mutants that lack this region failed to mediate polyubiquitylation of p27 in vitro and expression of one such mutant delayed p27 degradation in vivo. We also generated a series of deletion mutants of p27 and found that KPC failed to polyubiquitylate a p27 mutant that lacks the CDK inhibitory domain. Interestingly, the cyclin E-CDK2 complex prevented both the interaction of KPC with p27 as well as KPC-mediated polyubiquitylation of p27. A complex of cyclin E with a kinase-negative mutant of CDK2 also exhibited these inhibitory effects, suggesting that cyclin E-CDK2 competes with KPC1 for access to the CDK inhibitory domain of p27. These results suggest that free p27 is recognized by the NH2-terminal region of KPC1, which also associates with KPC2, and that p27 is then polyubiquitylated by the COOH-terminal RING-finger domain of KPC1.

Progression of the cell cycle in eukaryotic cells is controlled by a series of protein complexes composed of cyclins and cyclin-dependent kinases (CDKs)1 (1). The association of CDK inhibitors with cyclin-CDK complexes is triggered by a variety of antimitogenic signals and results in inhibition of the catalytic activity of these complexes and consequent restraint of cell cycle progression (1, 2). Among the various CDK inhibitors identified, p27 plays a pivotal role in the control of cell proliferation (3, 4). Mice homozygous for deletion of the p27 gene are larger than normal mice and exhibit multiple organ hyperplasia as well as a predisposition to both spontaneous and radiation- or chemical-induced tumors (5–8).

The ubiquitin-proteasome pathway mediates the degradation of short-lived regulatory proteins, including those that participate in the cell cycle, cellular signaling in response to stress or other extracellular stimuli, morphogenesis, the secretory pathway, DNA repair, and organelle biogenesis (9). The concentration of p27 is thought to be regulated predominantly by the proteolytic pathway (10). Degradation of p27 is triggered by its phosphorylation on Thr187 by the cyclin E-CDK2 complex (11, 12). The phosphorylation of Thr187 is required for the binding of p27 to Skp2, the F-box protein component of an SCF ubiquitin ligase (E3) complex, and such binding in turn results in the polyubiquitylation and degradation of p27 (13–19).

In normal cells, the amount of p27 is high during G1 phase of the cell cycle but decreases rapidly upon re-entry of cells into G1 phase (20, 21). However, Skp2 is not expressed until the G1-S transition of the cell cycle, unequivocally later than the degradation of p27 apparent at G0-G1 (22). Moreover, p27 is exported from the nucleus to the cytoplasm at G1-S (23–25), whereas Skp2 is restricted to the nucleus. The discrepancies between the temporal and spatial patterns of p27 expression and those of Skp2 expression suggested the existence of a Skp2-independent pathway for the degradation of p27. Consistent with this notion, p27 appears to be degraded normally at the G0-G1 transition in Skp2−/− mice (22). Biochemical analysis of crude extracts of Skp2−/− cells revealed the presence in the cytosolic fraction of a Skp2-independent E3 activity that mediates the polyubiquitylation of p27 (22). This ubiquitylation is not dependent on the phosphorylation of p27 on Thr187, which is a prerequisite for Skp2-mediated ubiquitylation. Skp2 seems to play an important role in ubiquitylation and degradation of p27 during S and G2 phases (26, 27). Lack of Skp2 resulted in the abnormal accumulation of p27 in S and G2 phases, which inhibits Cdc2 kinase and blocks the progression into mitosis (26). The factor to induce p27 ubiquitylation at G1 phase had been elusive until recently.

We recently identified an E3 complex, designated KPC (Kip1 ubiquitylation-promoting complex), consisting of KPC1 and KPC2 subunits, as a potential mediator of p27 ubiquitylation in G1 phase (28). KPC1 contains a RING-finger domain, and KPC2 contains a ubiquitin-like (UBL) domain and two ubiquitin-associated (UBA) domains. The nuclear export of p27...
mediated by CRM1 appears to be necessary for its KPC-mediated ubiquitylation and proteolysis. KPC is thus localized in the cytoplasm, where it interacts with and ubiquitylates p27 that is exported from the nucleus. Depletion of KPC1 by RNA interference inhibited p27 degradation. KPC thus likely regulates degradation of p27 in G1.

We have now investigated the molecular interactions among p27, KPC1, and KPC2. We determined the domains of KPC1 that are required for its interactions with p27 and KPC2 as well as for its ability to ubiquitylate p27 and to promote p27 degradation. Detailed structural and functional analyses of KPC2 will be described elsewhere. Furthermore, we also identified the region of p27 that is necessary for binding to KPC. KPC1 and the cyclin ECDK2 complex were found to bind to p27 in a competitive manner. Our data suggest that KPC targets free p27 that is exported from the nucleus to the cytoplasm.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit polyclonal antibodies to KPC1 and to KPC2 were described previously (28). Monoclonal antibodies to p27, to glutathione S-transferase (GST), and to HSP90 were obtained from BD Biosciences. Monoclonal antibodies (M2 and M5) to FLAG were from Sigma, a monoclonal antibody to T7 was from Novagen, and a monoclonal antibody (9E10) to Myc was from Roche Applied Science. Rabbit polyclonal antibodies (Y11) to the hemagglutinin epitope (HA) were from Santa Cruz Biotechnology.

Expression of Recombinant Proteins in HEK293T Cells—Complementary DNAs encoding wild-type or mutant (ΔS, Δ1, Δ2, Δ3, Δ4, and ΔR) forms of human KPC1, each tagged at their NH2 termini with hexahistidine (His6) and FLAG, and a cDNA for human KPC2 containing COOH-terminal His6 and HA tags were subcloned into pCI-neo (Promega). Complementary DNAs encoding wild-type or mutant (1-
Expression and purification of recombinant KPC1-KPC2, SCF

FIG. 2. Identification of the region of KPC1 responsible for recognition of p27. A, lysates of SF21 cells expressing the FLAG-tagged KPC1 derivatives described in Fig. 1B were incubated with recombinant mouse p27 and then subjected to immunoprecipitation with anti-FLAG (M2). The resulting precipitates, as well as a portion of the original cell lysates, were subjected to immunoblot analysis with antibodies to FLAG (M2) or to p27. B, lysates of SF21 cells coexpressing the FLAG-tagged KPC1 derivatives and HA-tagged KPC2 were subjected to immunoprecipitation with anti-FLAG (M2), and a portion of the resulting precipitates was subjected to immunoblot analysis with antibodies to FLAG (M2) or to HA. A portion of the original cell lysates was also subjected directly to immunoblot analysis with the same antibodies. C, the remainder of the immunoprecipitates prepared in B were assayed for E3 activity in an in vitro assay of p27 polyubiquitylation with GST-ubiquitin (Ub). The reaction products were subjected to immunoblot analysis with antibodies to p27 or to GST. IgH, immunoglobulin heavy chain.

RESULTS

The NH2-terminal Region of KPC1 Is Required for Interaction with KPC2—We previously showed that KPC1 is an authentic RING-finger-type E3 (Fig. 1A) and that a deletion mutant, KPC1ΔR, that lacks the RING-finger domain retains the ability to interact with KPC2, suggesting that this domain is dispensable for binding to KPC2 (28). To identify the region of KPC1 responsible for binding to KPC2, we expressed HA-tagged KPC2 together with FLAG epitope-tagged wild-type (W) KPC1 or a series of internal deletion mutants thereof in HEK293T cells (Fig. 1B). Cell lysates were subjected to immunoprecipitation with antibodies to FLAG or to HA, and the resulting precipitates were subjected to immunoblot analysis with these same antibodies. Whereas W4, Δ2, Δ3, Δ4, and ΔR forms of KPC1 were associated with KPC2, lysates of SF21 insect cells that had been infected with baculoviruses encoding the KPC1 derivatives and KPC2 were subjected to immunoprecipitation with antibodies to FLAG (M2) and protein A-Sepharose beads (Amersham Biosciences). The beads were mixed with 50 ng of UbEA, 100 ng of Ubch5A, 3 μg of GST-ubiquitin, and 50 ng of recombinant p27 in a final volume of 15 μl containing 40 mM Hepes-NaOH (pH 7.6), 60 mM potassium acetate, 2 mM diithiothreitol, 5 mM MgCl2, 0.5 mM EDTA, 10% glycerol, and 1.5 mM ATP. Reaction mixtures were incubated for 30 min at 26 °C. For assay of the ability of purified recombinant KPC1-KPC2 or SCF\(^{ΔR}\) complexes to mediate polyubiquitylation of p27, each complex (200 ng) was mixed with 50 ng of recombinant p27 in the absence or presence of 100 ng of recombinant cyclin E-CDK2 and was then subjected to the in vitro ubiquitylation assay as described above.

In Vitro Assay of Ubiquitylation Activity—For assay of the ability of immunoprecipitated wild-type or mutant (ΔS, Δ1, Δ2, Δ3, Δ4, and ΔR) forms of KPC1 to mediate polyubiquitylation of p27, lysates of SF21 insect cells that had been infected with baculoviruses encoding the KPC1 derivatives were mixed with 500 ng of recombinant mouse wild-type p27, rotated for 2 h at 4 °C, and then subjected to immunoprecipitation with antibodies to FLAG (M2) and protein G-Sepharose beads (Amersham Biosciences).

For assay of the ability of the purified recombinant KPC1-KPC2 complex to bind to p27, the complex (1 μg) was mixed with 500 ng of recombinant T7-tagged human wild-type p27 in the absence or presence of 750 ng of recombinant cyclin E-CDK2, rotated for 2 h at 4 °C, and then subjected to immunoprecipitation with antibodies to FLAG (M2) immobilized on NHS-activated Sepharose (Amersham Biosciences).
KPC1-(1–766) in addition to the Wt protein, consistent with the results obtained with the internal deletion mutants of KPC1 (Fig. 1B). We then tested additional deletion mutants derived from KPC1-(1–507) for the ability to interact with KPC2 in HEK293T cells (Fig. 1D). Immunoprecipitation analysis revealed that the 3xFLAG-tagged KPC1 derivatives 1–507, 1–450, and 1–399 interacted with endogenous KPC2, whereas the derivatives 1–350 and 132–507 did not. These results thus indicate that the NH2-terminal region of KPC1 comprising residues 1–399 and including the SPRY domain is sufficient for binding to KPC2.

The NH2-terminal Region of KPC1 Is Responsible for Recognition of p27—We previously showed that KPC1 directly interacts with p27 in vivo and in vitro (28). To map the region of KPC1 required for interaction with p27, we performed an in vitro binding assay with KPC1 deletion constructs and p27. Lysates of SF21 insect cells that had been infected with baculoviruses encoding FLAG-tagged KPC1 derivatives were mixed with recombinant mouse p27 and then subjected to immunoprecipitation with antibodies to FLAG. Immunoblot analysis of the resulting precipitates revealed that p27 coprecipitated with Wt, ΔS, ΔA, and ΔR forms of KPC1 (Fig. 2A; the data are also summarized in Fig. 1B). These data indicate that the NH2-terminal portion of KPC1 also interacts with p27, although the region required for association with p27 extends more COOH-terminally than does that required for interaction with KPC2.

We next evaluated the ability of the KPC1 derivatives to mediate the polyubiquitylation of p27. Lysates of SF21 insect cells that had been infected with baculoviruses encoding FLAG-tagged KPC1 derivatives and HA-tagged KPC2 were subjected to immunoprecipitation with antibodies to FLAG. Immunoblot analysis of the resulting precipitates (Fig. 2B) yielded results for the binding of KPC2 to the KPC1 derivatives that were identical to those obtained with HEK293T cells (Fig. 1B). The immunoprecipitates were also assayed for ubiquitylation activity with p27 in vitro (Fig. 2C; the data are also summarized in Fig. 1B). As previously demonstrated, KPC1(ΔR) did not mediate polyubiquitylation of p27 (28). In addition, the ΔS, ΔA, and Δ2 mutants of KPC1, which did not bind to p27, also did not catalyze polyubiquitylation of p27. In contrast, KPC1(Δ3) and KPC1(Δ4) retained the ability both to associate with p27 and to mediate its polyubiquitylation, although the catalytic efficiency of the mutants appeared reduced compared with that of wild-type KPC1. Given that KPC1(Δ2) interacted with KPC2 but not with p27, the inability of this mutant to polyubiquitylate p27 was not attributable to an inability to associate with KPC2.

These data indicate that an NH2-terminal region (residues 1–766) of KPC1, which includes the KPC2 binding domain, is responsible for recognition of p27.

Expression of an NH2-terminal Deletion Mutant of KPC1 Inhibits p27 Degradation—A KPC1 mutant that lacks the RING-finger domain acts in a dominant negative manner to delay the degradation of p27 at the G0-G1 transition in NIH 3T3 cells (28). We therefore next examined the effect of expression of the mutant KPC1-(508–1314), which lacks the NH2-terminal region but retains the RING-finger domain (Fig. 3A), on p27 degradation at G0-G1 in NIH 3T3 cells. The cells were infected with retroviral vectors encoding KPC2 and either wild-type KPC1 or KPC1-(508–1314). Expression of wild-type KPC1 and KPC2 markedly promoted the degradation of p27 compared with that apparent in control cells, as described previously, whereas expression of KPC1-(508–1314) with KPC2 resulted in a substantial delay in p27 degradation (Fig. 3B). These data suggest that KPC1-(508–1314) functions as a dominant negative mutant, possibly as a result of competition with the endogenous wild-type protein for the E2 enzyme or other unidentified molecule(s) that interacts with KPC1-(508–1314).

The CDK Inhibitory Domain of p27 Is Necessary for Ubiquitylation by KPC—Previous studies have indicated that Skp2 specifically interacts with the COOH terminus of p27 only when Thr187 has been phosphorylated by cyclin E-CDK2 (11, 12). This association of Skp2 with p27 results in recruitment of the latter to the SCF core complex, thereby promoting its ubiquitylation and degradation (13–19). However, KPC-mediated polyubiquitylation of p27 appears to be independent of Thr187 phosphorylation (28). To identify the region of p27 re-
required for polyubiquitylation by KPC, we performed the in vitro ubiquitylation assay with various T7-tagged deletion mutants of human p27 (Fig. 4A) and with the recombinant KPC1-KPC2 complex generated with a baculoviral expression system. Polyubiquitylation of wild-type p27 and of the mutants MD2 and CD3 was readily detected in the presence of E1 (Uba1), E2 (UbcH5A), GST-ubiquitin, and ATP. Reaction products were subjected to immunoblot analysis with antibodies to T7 or to GST.

We next investigated whether the KPC1-KPC2 complex interacts with and mediates the polyubiquitylation of free p27. The recombinant cyclin E/CDK2 complex generated with a baculoviral expression system was assayed for the ability to mediate polyubiquitylation of T7-tagged p27 derivatives in the presence of the combinations of E1 (Uba1), E2 (UbcH5A), GST-ubiquitin, and ATP. Reaction products were subjected to immunoblot analysis with antibodies to T7 or to GST.

FIG. 4. In vitro ubiquitylation of p27 derivatives mediated by KPC. A, schematic representation of human p27 derivatives. NLS, nuclear localization sequence. B, the recombinant KPC1-KPC2 complex was assayed for the ability to mediate polyubiquitylation of free p27 in the presence of a complex of cyclin E with a catalytically inactive mutant of CDK2 (CDK2[D145N]) (33), suggesting that p27 phosphorylation by cyclin E-CDK2 is not responsible for inhibition of the action of KPC. Rather, cyclin E-CDK2 might physically interfere with the association of KPC with p27 in a competitive manner. Consistent with previous observations, the recombinant cyclin E-CDK2 complex markedly enhanced the polyubiquitylation of p27 mediated by the recombinant SCF<sup>Skp<sub>2</sub></sup> complex (Fig. 5B) (14, 29). The cyclin E-CDK2[D145N] complex was much less effective in this regard, suggesting that phosphorylation of p27 by cyclin E-CDK2 is largely responsible for promotion of its polyubiquitylation by SCF<sup>Skp<sub>2</sub></sup> (14). The effects of cyclin E-CDK2 on polyubiquitylation of p27 are thus opposite for that mediated by KPC and that mediated by SCF<sup>Skp<sub>2</sub></sup>.

We finally examined whether the KPC1-KPC2 complex associates with p27 in the presence of cyclin E-CDK2. The recombinant KPC1-KPC2 complex was mixed with recombinant human p27 in the absence or presence of recombinant cyclin E-CDK2 and was then immunoprecipitated. Immunoblot analysis of the resulting precipitates revealed that p27 coprecipitated with the KPC1-KPC2 complex only in the absence of cyclin E-CDK2 (Fig. 5C). These observations suggest that the KPC1-KPC2 complex interacts with and mediates the polyubiquitylation of free p27.

**DISCUSSION**

Degradation by the ubiquitin-proteasome pathway plays a fundamental role in determining the abundance of important regulatory proteins (9). The amount of p27 is high in quiescent (G<sub>0</sub>) cells but decreases rapidly, as a result of degradation by the ubiquitin-proteasome pathway, upon entry of cells into the cell cycle (10, 20–22). The degradation of p27 is regulated by two distinct RING-finger-type ubiquitin ligase complexes: KPC (28) and SCF<sup>Skp<sub>2</sub></sup> (13–19). The former mediates polyubiquitylation of free p27 in the cytoplasm during G<sub>1</sub> phase, whereas the latter is important for degradation of p27 in the nucleus during S and G<sub>2</sub> phases. Depletion of KPC1 or of Skp2 results in an increase in the stability of p27 in G<sub>1</sub> or S phase, respectively. We have now elucidated the structural requirements of KPC1 for interaction with KPC2 and p27. Both KPC2 and p27 bind to the NH<sub>2</sub>-terminal region of KPC1, and this region is also necessary for the degradation of p27 at the G<sub>0</sub>-G<sub>1</sub> transition in NIH 3T3 cells. The NH<sub>2</sub>-terminal region of KPC1 contains a SPRY domain, and the deletion of this domain abolished the interaction of KPC1 with KPC2 and with p27. The SPRY domain was originally identified as a motif of unknown function that is present in three copies in the mammalian ryanodine receptor (32). RanBP/RanBP9 was shown to interact through their SPRY domains with CDK11 and MET, a receptor tyrosine kinase for hepatocyte growth factor (scatter factor), respectively (34, 35). Together with our present results, these observations suggest that the SPRY domain functions in protein-protein interaction.

The polyubiquitylation of p27 mediated by KPC was found to differ from that mediated by SCF<sup>Skp<sub>2</sub></sup> in the effect of cyclin E-CDK2. Given that SCF<sup>Skp<sub>2</sub></sup> targets p27 for polyubiquitylation only after Thr<sup>187</sup> has been phosphorylated by cyclin E-CDK2 (11, 12), the addition of cyclin E-CDK2 to the in vitro ubiquitylation assay greatly enhanced the polyubiquitylation of p27 by this E3 (14, 29). In contrast, cyclin E-CDK2 inhibited KPC-mediated polyubiquitylation of p27 as well as the interaction of KPC with p27. These inhibitory effects were also observed with a complex composed of cyclin E and a kinase-inactive mutant of CDK2, suggesting that they were not attributable to phosphorylation of p27 but were rather due to physical interference with the p27-KPC1 interaction. The cyclin E-CDK2 complex
might thus function as a switch to change KPC-mediated degradation of p27 to SCFSkp2-mediated degradation. The timing of cyclin E\(\text{CDK2}\) expression appears consistent with that of the changeover in p27 degradation from that mediated by KPC to that mediated by the Skp2 pathway at the G1-S transition.

We have also studied the structural requirements of KPC2 for interaction with KPC1, the 26 S proteasome, and polyubiquitylated proteins.\(^\text{2}\) The UBL domain of KPC2 appears necessary for the interactions with KPC1 and the 26 S proteasome, whereas the UBA domains are important for recognition of polyubiquitylated proteins. The UBL-UBA-UBA structure of KPC2, like that of Rad23 (36) and Dsk2 (37), thus likely mediates interaction both with the proteasome and with polyubiquitylated proteins. On the basis of our findings with KPC1 (present study) and with KPC2,\(^\text{2}\) we propose a model for the recognition and ubiquitylation of p27 by KPC (Fig. 6). In the cytoplasm, free p27 is recognized by the NH\(_2\)-terminal domain of KPC1, which is also associated with KPC2, and p27 is then ubiquitylated by the COOH-terminal RING-finger domain of KPC1. KPC2 may function as an adapter protein that participates in delivery of polyubiquitylated p27 to the 26 S proteasome. The large complex of KPC and the proteasome likely mediate the rapid and efficient degradation of p27 during G1 phase.

Mutations in the p27 gene appear to be rare in human cancers (38–46). However, a reduced abundance of p27 has
been shown to correlate well with poor prognosis in many types of malignant tumors (47–53). Furthermore, the loss of p27 alleles increases the sensitivity of mice to cancer-inducing agents (5–8). Identification of the KPC pathway of p27 degradation may thus provide new insight into the altered expression of this protein in tumor cells as well as into whether such altered expression is a cause or a consequence of cell transformation. Indeed, Skp2 is overexpressed in many human cancer cells (54), suggesting that p27 degradation mediated by Skp2 may be related to carcinogenesis. It is thus possible that the KPC pathway of p27 degradation is also deregulated in cancer cells. Our present data might thus provide a molecular basis for the development of anticancer agents that prevent the interaction of KPC with p27.

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