Direct Binding of the Signal-transducing Adaptor Grb2 Facilitates Down-regulation of the Cyclin-dependent Kinase Inhibitor p27^Kip1*

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Ectopic expression of Jab1/CSN5 induces specific down-regulation of the cyclin-dependent kinase (Cdk) inhibitor p27 (p27^Kip1) in a manner dependent upon transportation from the nucleus to the cytoplasm. Here we show that Grb2 and Grb3-3, the molecules functioning as an adaptor in the signal transduction pathway, specifically and directly bind to p27 in the cytoplasm and participate in the regulation of p27. The interaction requires the C-terminal SH3-domain of Grb2/3-3 and the proline-rich sequence contained in p27 immediately downstream of the Cdk binding domain. In living cells, enforcement of the cytoplasmic localization of p27, either by artificial manipulation of the nuclear/cytoplasmic transport signal sequence or by coexpression of ectopic Jab1/CSN5, markedly enhances the stable interaction between p27 and Grb2. Overexpression of Grb2 accelerates Jab1/CSN5-mediated degradation of p27, while Grb3-3 expression suppresses it. A p27 mutant unable to bind to Grb2 is transported into the cytoplasm in cells ectopically expressing Jab1/CSN5 but is refractory to the subsequent degradation. These findings indicate that Grb2 participates in a negative regulation of p27 and may directly link the signal transduction pathway with the cell cycle regulatory machinery.

The proliferation of mammalian cells is strictly regulated by extracellular signals, which largely exert their effects during the G1 phase of the cell cycle. Among G1 regulatory factors, the Cdk inhibitor p27 (p27^Kip1), which negatively regulates cell proliferation, is the downstream target of mitogen-stimulated signal transduction, and modulation of p27 activity is one of the most important steps not only in the control of mammalian cell proliferation but also in the regulation of normal tissue development and in the suppression of malignant transformation (1, 2). The expression level of p27 is regulated in several ways, thereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Grb2 and its alternatively spliced form, Grb3-3, bind to p27 in the cytoplasm and that the interaction between Grb2 and p27 is required for efficient degradation of p27. Our findings may, at least in part, explain why nuclear p27 is translocated to the cytoplasm before degradation and provide a novel pathway from the signal transduction machinery directly to the cell cycle regulator.

EXPERIMENTAL PROCEDURES

Recombinant Proteins and in Vitro Protein Binding Assay—To construct the p27(PA) mutant, overlapping fragments of p27, introducing the relevant nucleotide changes, were made. The 3′-fragment was made by PCR using wild-type p27 cDNA as a template and the PA mutation primer (5′-C TAC TAC AAG GCC GCG CGC GCC GCC AAG AGC GCC TGC-3′) and 3′-p27 primer (5′-AAGCTT CGT CGT TCG AAG G-3′). The 5′-fragment was made using the same template but with the reverse PA mutation primer (5′-GCA GGC CCT GTA GTA G-3′) and 5′-p27 primer (5′-GGATCC ATG GA-3′) respectively; GST, glutathione S-transferase.

Results

Identification of Grb2 and Grb3-3 as New Interactors Specific to p27—Using a yeast two-hybrid screen, we identified three kinds of DNAs (5), all of which encode polypeptides capable of specifically interacting with the C-terminal domain of p27(9-91). We previously showed that one of them encodes Jab1/CSN5, which negatively regulates p27 by translocating it from the nucleus to the cytoplasm and subsequently inducing its degradation (5). The second class of DNA contained the C-terminal SH3 domain of Grb2, which is well known to function as an adaptor molecule in the signal transduction pathway (14). To determine the specificity of the interaction between p27 and Grb3, we analyzed the capability of p27 to bind to other SH3-containing proteins. We selected from the data base several genes that encode proteins containing a SH3 domain closely related to the C-terminal SH3 domain of Grb2 (more than 40% identity), including STAM, SH3P5, SH3P13, and mNck-a (accession numbers are U43900, U58885, U58887, and AF043259, respectively) in addition to Grb2 and its alternatively spliced form, Grb3-3 (21).

Among a variety of GST fusion proteins containing a portion of the p27 molecule (Fig. 1B), all that contained amino acids 89–96 bound to 35S-labeled, in vitro-transcribed/translated Grb2 and Grb3-3 proteins in a test tube (Fig. 1C). The N terminus deletion mutant, p27(89–197), associated with Grb2/3-3 slightly weaker than other mutants, suggesting that amino acids adjacent to 89 are required for efficient interaction. The region of amino acids 89–96 is rich in proline residues and contains two overlapping SH3-binding motifs (PXXP) (14). Alteration of all of these 4 proline residues to alanine (RPPRPK) completely abolished the binding activity for Grb2/3-3, while this mutant, designated as p27PA, retained the capability of interacting with cyclin D1-Cdk4 complexes (Fig. 1D), inhibiting the kinase activity of cyclin-Cdk complexes and inducing G1 arrest when overexpressed in mouse fibroblasts (data not shown, but see Fig. 4C). This proline-rich motif is unique to p27 and not found in sequences of other similar Cdk inhibitors, p21 and p57. In fact, we did not detect interaction between p21 and Grb2/Grb3-3 under these conditions in vitro (negative data not shown).

Grb3-3 consisting of two functional SH3 domains but lacking half of the SH2 domain (21) is able to bind to p27, indicating that the SH3 but not SH2 domain is involved in interaction with p27. To determine more precisely the binding domain within Grb proteins, we generated truncated mutants of Grb3-3, one containing the N terminus SH3 domain (SH3N) and the other the C terminus SH3 (SH3C) (Fig. 1E), and then tested them for activity to bind to 35S-labeled full length p27 proteins in vitro (Fig. 1F). We found that the GST fusion protein containing the C terminus SH3 but not the N terminus SH3 interacted with p27. Thus, these results clearly indicate that p27 and Grb2/3-3 specifically interact with each other at least in vitro through the proline-rich motif and a specific SH3 domain.

Grb2 and Grb3-3 Binding to p27 in the Cytoplasm In Vivo—In

Cell Culture and High Efficiency Transfection—NIH3T3 and COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Grb2/Grb3-3 cDNA was inserted into the pFLAG-CMV-2 expression vector (Eastman Kodak Co.) in frame with a FLAG epitope. The expression vectors for p27 variants and Jab1 were previously described (5). Cells were transfected with vectors by a modified calcium phosphate-DNA precipitation method (20). The highest efficiency was obtained as described (5). Consistently, 50–80% of the transfected cells expressed exogenous proteins coted in the plasmid. Antisense oligonucleotides and controls directed to Grb2 have been designed and manufactured by Biognostik, Germany, and were directly purified, mixed, and used as the template for the second PCR. The 9 long and 3′-p27 primers introducing a 5′ BamHI and a 3′ HindIII site, respectively. cDNA fragments encoding p27 variants, Grb2, Grb3-3, and a variety of SH3-containing proteins were amplified by PCR using a pair of primers specific to each of them. The resulting PCR fragments were sequenced to confirm sequence integrity, and inserted into pGEX (Amersham Pharmacia Biotech) in frame with glutathione S-transferase (GST) and pBluescript. GST fusion proteins were expressed in bacteria and purified as described (18). Crude cell extracts containing recombinant mammalian cyclin D1-Cdk4 complex expressed in Sf9 cells by infection with baculovirus expression vectors were prepared as previously described (19). pBluescript plasmids containing cDNA were transcribed and translated in vitro in the presence of [35S]methionine, and used these 35S-labeled proteins to assay in vitro the binding to GST and GST-fused p27 recombinant proteins preabsorbed onto glutathione beads. Fig. 1A shows that 35S-labeled Grb2 and Grb3-3 but not the others associated with GST-p27 fusion proteins in a test tube. The amounts of bound Grb2 and Grb3-3 were almost the same, and neither molecule bound to GST alone, indicating that the interaction between p27 and Grb proteins is specific and that Grb2 and Grb3-3 have equivalent binding capabilities at least in vitro.

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Grb2 and Grb3-3 Bind to p27 in the Cytoplasm In Vivo—In

The abbreviations used are: PCR, polymerase chain reaction; HA, hemagglutinin; SH2 and SH3, Src homology domain 2 and 3, respectively; GST, glutathione S-transferase.
mouse fibroblasts, which express p27 and Grb2 but not Grb3-3 (data not shown), we were unable to detect a complex between endogenous p27 and Grb2; nor did we see any interaction between the two in COS cells overexpressing exogenous HA-tagged full-length p27 and FLAG-tagged Grb2 proteins (data not shown). This could be because the intracellular localization of the two proteins is different (p27 is in the nucleus and Grb2 is in the cytoplasm) and because the intermediate p27-Grb2 complex exists only for a very limited time. We examined these possibilities by using p27 mutants. When we ectopically expressed FLAG-tagged Grb proteins together with the p27 mutant (p27(NES)) that localizes mainly in the cytoplasm because of the artificially fused NES sequence (5), we found that p27(NES) efficiently formed a complex with Grb2 and Grb3-3 in vivo (Fig. 2A). In addition, we detected very stable interaction between Grb2/Grb3-3 and another p27 mutant, p27-(1–151), which lacks the nuclear localization signal and remains in the cytoplasm (5) (data not shown). In another experiment, we observed a p27/Grb association when we used degradation-resistant p27 mutants (e.g., p27(T187A), p27-(1–186)) (3–5) (data not shown, but see Fig. 2C and below). With wild-type p27, Grb3-3, but not Grb2, formed a detectable amount of complex (Fig. 2B, seventh lane from the left). Thus, we conclude that (i) p27 binds to Grb proteins when it localizes in the cytoplasm, (ii) the p27/Grb2 interaction is very transient, and (iii) inhibition of Thr187 phosphorylation in p27 or disruption of SH2 function in Grb2 increases the stability of the complex (see below). In control experiments, we tested for p21 in lieu of p27 in normal NIH3T3 mouse fibroblasts and transfected COS cells and found that the interaction with Grb2/Grb3-3 was specific to p27 (negative data not shown).

As for the factor that causes the cytoplasmic localization of p27, we focused on Jab1/CSN5, which directly binds to p27 and down-regulates the protein by translocating it from the nucleus to the cytoplasm (5). Since ectopic coexpression of HA-Jab1 (CSN5) induces down-regulation of wild-type p27 and its effect is prominent 48–72 h after transfection, we assayed for the complex formation 24 h after transfection, at which time p27 is not markedly down-regulated. Fig. 2B shows that coexpression of HA-Jab1 significantly increased the amount of complex formed between ectopic p27 and Grb3-3 proteins (seventh and eighth lanes from the left). Interestingly, upon immunoprecipitation with antibody specifically recognizing the C-terminal half of the p27 protein, Grb3-3 proteins were not coprecipi-
**FIG. 2.** Specific interaction of Grb proteins with cytoplasmic p27 in vitro. COS cells were transfected with the expression vectors shown at the top of the panels. Lysates from cells harvested 24 h post-transfection were directly analyzed by immunoblot with antibodies to HA and FLAG epitopes or subjected to immunoprecipitation (IP)/immunoblot analysis using the same antibodies. A, Grb2/3-3 form stable complexes with a p27 mutant containing artificial NES. B, binding of Grb3-3 with p27 was enhanced by ectopic expression of Jab1. C, both binding sites for Grb2 and Jab1 contained in p27 are required for p27/Grb2 interaction.

**FIG. 3.** Effect of Grb proteins on the stability of p27. A, NIH3T3 cells were transfected with expression vectors (open circle, p27 alone; closed circle, p27 and Jab1; open triangle, p27, Jab1, and Grb2; closed triangle, p27, Jab1, and Grb3-3), pulse-labeled with [35S]methionine for 30 min at 48 h post-transfection and chased with excess cold methionine. At the indicated times, cells were collected, and the relative 35S in HA-p27 was measured. B, activation of the Ras signaling pathway is dispensable for accelerated degradation of p27 by Grb2. NIH3T3 cells were transfected with GFP together with p27, Jab1/CSN5, and Grb2 as indicated at the top, incubated for 6 h in the presence of chemical inhibitors (10 μM PD98059 (PD) and 2 μM wortmannin (WT)) at 48 h post-transfection, and harvested. Cell lysates containing the same amount of protein were analyzed by immunoblotting with antibodies specifically recognizing p27, Grb2, Jab1/CSN5, and GFP, respectively. We confirmed that PD98059 suppressed the activation of mitogen-activated protein kinase under these conditions. Wortmannin was used as described (16). DMSO, dimethyl sulfoxide.
shown. Since we did not detect Grb3-3 in anti-HA-immunoprecipitates from cells expressing HA-Jab1 and FLAG-Grb3-3, it seems likely that this anti-p27 antibody interfered with the formation of complex between p27 and Grb proteins, or, alternatively, HA-Jab1 may assist in increasing the stability of the p27-Grb protein complex. We detected interaction between wild-type p27 and Grb2 in the presence of ectopic HA-Jab1, but not in its absence (Fig. 2C). Mutation of the C terminus phosphorylation site (T187A), which renders p27 more stable (3–5), enhanced the complex formation, while mutation in the Grb2-binding site (p27(Del 97–151)) abolished the interaction. The p27/Grb2/ Jab1 interaction was completely disrupted in the presence of leptomycin B, a specific inhibitor of the NER/CRM1-dependent nuclear export (22) (data not shown). Ectopic Jab1/CSN5 markedly reduced its stability (closed circle) as previously reported (5). Additional coexpression of Grb2 further reduced the stability of p27 (open triangle), while coexpression of Grb3-3 blocked Jab1/CSN5-mediated down-regulation of p27 (closed triangle). Therefore, although only the SH3 domain is required for direct binding to p27, the integrity of the SH2 domain in Grb2 is important for induction of p27 down-regulation. The simplest interpretation is that the recruitment of a certain cellular protein, which most likely contains phosphorylated tyrosine residues, into the Grb2-p27-Jab1 complex would facilitate down-regulation of p27. However, Grb2 is well known to function in the signal transduction pathway (14), especially upstream of the Ras/Raf/mitogen-activated protein kinase pathway, that activation of which is reported to induce degradation of p27 (15–17). To analyze the possible involvement of the Ras signaling pathway in down-regulation of p27, we examined the effect of chemical inhibitors to mitogen-activated protein kinase pathway (PD98059 (PD); see Ref. 17) and phosphatidylinositol 3-kinase (wortmannin (WT); see Ref. 16) on the level of p27 in NIH3T3 cells transfected with HA-p27 together with Jab1/CSN5 and Grb2 (Fig. 3B). We found that Grb2 accelerated the degradation of p27 in the presence and absence of these inhibitors. These results strongly support our interpretation that Grb2 plays an important role in the regulation of p27 by the direct binding but not by activating the Ras signaling pathway.

**Direct Binding of Grb2 Is Required for Down-regulation of p27**—To investigate the requirement of direct Grb binding in p27 down-regulation, we utilized the PA mutant of p27 (p27(PA)), which is unable to bind to Grb proteins due to amino acid substitutions in the SH3-binding motif but retains the capability of binding to other p27 binding proteins such as cyclin-Cdk complexes and Jab1 (Fig. 1D and data not shown). p27(PA) was located in the nucleus and transported to the cytoplasm and by increasing the stability of the complex.

**Fig. 5.** Grb2 is required for down-regulation of p27 in proliferating fibroblasts. NIH3T3 cells (~10% confluence) were incubated in medium supplemented with 2 μM antisense oligonucleotides directed to Grb2 and control oligonucleotides and harvested after 3 days. Cell lysates containing the same amount of protein were analyzed by immunoblotting with antibodies specifically recognizing Grb2, p27, and p21.
cytoplasm in the presence of ectopic Jab1/CSN5 (Fig. 4A), indicating that the proline-to-alanine mutation did not affect nuclear import and Jab1/CSN5-mediated nuclear export of p27. The intensity of the nuclear staining was indistinguishable between the wild type and the PA mutant, but the signal of p27(PA) was much stronger than that of wild-type p27 in cells coexpressing Jab1/CSN5, implying that p27(PA) was not down-regulated in the presence of ectopic Jab1/CSN5. To directly examine this, we measured the half-life of these two p27 molecules in the presence and absence of ectopic Jab1 (Fig. 4B). Exogenous p27 was a relatively stable protein (open circle) and was induced to be degraded by coexpression of Jab1/CSN5 (closed circle). The PA mutant was kept stable whether Jab1/CSN5 was cotransfected or not (open and closed squares, respectively). In addition, although the ability of wild-type p27 to inhibit growth was partially rescued by Jab1/CSN5 coexpression, the PA mutant was quite resistant to the neutralizing effect of Jab1/CSN5 (Fig. 4C). These results, together with the observation that most of the cell lines we examined so far expressed Grb2 but not Grb3-3, indicate that the direct binding of Grb2 is required for cytoplasmic degradation of p27 but not for transportation of p27 from the nucleus to the cytoplasm.

**Grb2 Is Required for Maintenance of Low Expression of p27 in Proliferating Fibroblasts**—To investigate the physiological importance of Grb2 in the regulation of p27 in vivo, we manipulated the expression of the Grb2 protein by antisense technology and examined the effect on p27 expression. The addition of the antisense oligonucleotides to the medium did not have any apparent effect on NIH3T3 fibroblasts during the first 24 h; however, at 3 days post-treatment, the rate of proliferation gradually slowed. Importantly, most cells neither exhibited a rounded morphology nor detached from the solid support during this period, indicating that few cells lost their viability due to the treatment with Grb2-specific antisense oligonucleotides. In these cells, the expression of Grb2 proteins was reduced to 36% compared with that in control cells. In contrast, p27 expression was 5 times higher than that in cells untreated or treated with random oligonucleotides. Importantly, Grb2 antisense oligonucleotides did not significantly alter the level of endogenous p21 proteins, indicating that the effect of Grb2 was specific to p27 (Fig. 5). These results demonstrate that Grb2 specifically functions upstream of p27 and is required for maintaining the low level of p27 protein.

**DISCUSSION**

Proteolytic down-regulation specifically linked to transportation from the nucleus to the cytoplasm is occasionally observed in the control of key regulators of cell proliferation, such as cyclin D1, p53, p27, and β-catenin. Nuclear-cytoplasmic transportation and subsequent degradation of cyclin D1 is regulated by phosphorylation of a specific threonine residue (Thr286) by GSK-3β. The simplest interpretation is that a certain factor compartmentalized to a specific area in the cytoplasm is required for degradation of these proteins, although no such factor has been identified yet. In the present study, we have found that Grb proteins specifically bind to p27 in the cytoplasm, which is required for efficient down-regulation of p27. Because Grb proteins function as an adaptor in the signal transduction pathway, one can easily speculate that Grb proteins mediate interaction between p27 and some unknown factor that may contain activity for ubiquitination or proteolysis. Grb2 accelerates degradation of p27 and Grb3-3 exhibits an opposite effect, suggesting that the SH2 domain is important presumably for recruitment of such a factor. Although we have no clues as to the molecular identity of the factor, it is feasible that tyrosine phosphorylation of the factor by the growth factor receptor triggers association with the p27-Grb2 complex.

p27 is subject to multiple forms of regulation (1, 2). It is transcriptionally activated by the CBP coactivator in response to retinoic acid treatment (27) and by the Ah receptor (28). Translational control of p27 expression is also reported (29–31). However, because the level of p27 protein fluctuates during the cell cycle (high in G0/G1 and low in S, G2, and M), while the level of p27 mRNA is constant (29, 30), the main regulatory mechanism for p27 seems to be post-translational, mostly due to the activation and inactivation of substrate-specific and cell cycle-dependent proteolysis. Degradation of p27 has been reported to involve phosphorylation of Thr187 by cyclin E-Cdk2 complex (3, 4), nuclear export induced by Jab1/CSN5 (5), ubiquitination mediated by the ubiquitin ligase SCF<sub>Skp2</sub> complex (6–8), and proteolysis by the 26 S proteasome. However, the precise biochemical link between these events and the biochemical reaction that initiates p27 degradation remains to be clarified.

**Introduction of the dominant negative form of Jab1 increases levels of p27 expression in proliferating mouse fibroblasts.** Therefore, these two pathways seem to significantly participate in the regulation of p27 expression, and we have not obtained any evidence that p27 exported from the nucleus by the Jab1/CSN5 is ubiquitinated by SCF<sub>Skp2</sub> in the cytoplasm. This may suggest that these two pathways are independent. Although experiments using Skp2<sup>−/−</sup> cells need to be performed to clarify this issue, it is feasible that the down-regulation of p27 occurs in several steps. Jab1/CSN5 and Grb2 may be involved in the down-regulation during early to mid-G1, and the cyclin E-Cdk2-SCF<sub>Skp2</sub> pathway may govern the late G1 to S phase event.

Cancers with low p27 protein expression are reported to be well correlated with poor prognosis (33–35). This finding was originally made in breast and colorectal carcinomas and now is the case for a wide variety of human tumors. Since the p27 gene is rarely altered in human cancers, the genetic target for malignant transformation seems to be the gene functioning upstream of p27. Overexpression of the SKP2 gene is occasionally observed in transformed cells (36) but is not necessarily correlated with low expression of p27. So far, no alteration of Jab1/CSN5 expression has been reported, but other components of the COP9 signalosome complex are capable of down-regulating p27, it is necessary to investigate whether any other CSN is involved in human cancers. In addition, our results suggest that the possible role of Grb2 and its associated proteins in tumorigenesis has to be reevaluated in terms not only of activation of the signal transduction pathway leading to Ras activation but also of direct involvement in control of the key cell cycle regulator.

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2 K. Tomoda, Y. Arata, T. Tanaka, N. Yoneda-Kato, and J. Kato, unpublished observation.
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