The Cytoplasmic Shuttling and Subsequent Degradation of p27^Kip1 Mediated by Jab1/CSN5 and the COP9 Signalosome Complex*

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Mammalian cell proliferation is strictly controlled during the G1 phase of the cell cycle. During the last decade, many factors were found and characterized, each of which positively or negatively participates in regulation of the G1 progression (1). Among them, the cyclin-dependent kinase (Cdk) inhibitor p27 (p27^Kip1) is induced or activated by a variety of anti-mitogenic stimuli and is involved in developmental regulation and tumor suppression as well as in cell proliferation in vitro and in vivo (2–4). The expression of p27 is controlled both at the level of transcription and by multiple post-translational mechanisms, among which cell cycle-dependent and substrate-specific proteolysis seems to play an important role (3, 4). Down-regulation of p27 has been reported to involve the following: (i) phosphorylation of the Thr-187 residue by cyclin E-Cdk2 complex (5, 6), (ii) transportation from the nucleus to the cytoplasm (7–9), (iii) ubiquitination mediated by the ubiquitin ligase SCF^{CSN} complex (10–12), and (iv) proteolysis by the 26 S proteasome. However, the precise biochemical link between these events remains to be clarified.

Nuclear exportation and subsequent degradation of p27 is induced by ectopic expression of Jab1/CSN5 (7). Jab1/CSN5 was originally identified as a co-activator of c-Jun transcription factor (13), and recent findings (14–17) indicate that Jab1/CSN5 is the fifth component of the COP9 signalosome (CSN) complex (originally known as the COP9 complex or the Jab1-containing signalosome; the nomenclature of the 8 subunits is now unified as CSN1–8). Jab1/CSN5 can directly interact with p27 in vitro as well as in vivo, and in cells overexpressing ectopic Jab1, p27 was exported from the nucleus to the cytoplasm and induced to be degraded in a manner sensitive to chemical inhibitors of CRM1-dependent nuclear export and the 26 S proteasome. Jab1/CSN5-induced p27 down-regulation does not seem to be mediated through activation of c-Jun, because Jab1/CSN5 overexpression alone does not significantly increase AP-1 activity, and enhancement of AP-1 activity by c-Jun overexpression does not result in p27 down-regulation. Jab1/CSN5 overexpression enables murine fibroblasts to progress from G0 to S phase in low serum, indicating that Jab1/CSN5 plays an important role in G1 progression and cell proliferation (7).

Transportation of many proteins from the nucleus to the cytoplasm is mediated by a nuclear export signal (NES) sequence (19), which often contains a conserved stretch of characteristic spaced leucine residues. Mutation of these leucines in the NES core domain disrupts the ability of the protein to locate exclusively in the cytoplasm. CRM1, which

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The fifth component of the COP9 signalosome complex, Jab1/CSN5, directly binds to and induces specific down-regulation of the cyclin-dependent kinase inhibitor p27 (p27^Kip1). Nuclear-cytoplasmic translocation plays an important role because leptomycin B (LMB), a chemical inhibitor of CRM1-dependent nuclear export, prevents p27 degradation mediated by Jab1/CSN5. Here we show that Jab1/CSN5 functions as an adaptor between p27 and CRM1 to induce nuclear export and subsequent degradation. Jab1/CSN5, but not p27, contains a typical leucine-rich nuclear export signal (NES) sequence conserved among different species, through which CRM1 binding to Jab1/CSN5-NES abolished the interaction with CRM1 in vitro and impaired LMB-sensitive nuclear export and the ability to induce p27 breakdown in cultured cells. A Jab1/CSN5 truncation mutant lacking NES reversed p27 down-regulation induced by the full-length Jab1/CSN5, indicating that this mutant functions as a dominant negative (DN-Jab1). Introduction of DN-Jab1 into proliferating fibroblasts increased the level of p27 protein, thereby inducing growth arrest of the cells. Random mutagenesis analysis revealed that specific aspartic acid, leucine, and asparagine residues contained in the Jab1/CSN5-binding domain of p27 were required for interaction with Jab1/CSN5 and for down-regulation of p27. Glycerol gradient and cell fractionation experiments showed that at least two different forms of Jab1/CSN5-containing complexes existed within the cell. One is the conventional 450-kDa COP9 signalosome (CSN) complex located in the nucleus, and the other is much smaller (around 100-kDa), containing only a subset of CSN components (CSN4–8 but not CSN1–3), and mainly located in the cytoplasm. Treatment of cells with LMB greatly reduced the level of the smaller complex, suggesting that it originated from the CSN complex by nuclear export. Besides Jab1/CSN5, CSN3, −6, −7, and −8 were capable of inducing p27 down-regulation, when ectopically expressed. These results indicate that cytoplasmic shuttling regulated by Jab1/CSN5 and other CSN components may be a new pathway to control the intracellular abundance of the key cell cycle regulator.
belongs to the family of importin β-related nuclear transport receptors, directly and specifically associates with NES and mediates nuclear export of the protein (19, 20). The cytotoxin leptomycin B (LMB) disrupts the interaction between CRM1 and NES by directly binding to CRM1 and thereby inhibiting the action of CRM1 (21). The energy dependence of nucleocytoplasmic transport can be partly explained by the requirement of the Ran GTase protein, which is involved in the loading and unloading of the cargo proteins by the exportin (19, 22, 23).

The majority of endogenous Jab1/CSN5 molecules are included in the COP9 signalosome complex in living cells. The COP9 signalosome was originally identified in Arabidopsis as a negative regulator of photomorphogenesis (15). Successful purification of the COP9 signalosome complex consisting of the identical 8 subunits from mammalian cells revealed that the function of the complex is not necessarily restricted to light/dark-mediated signal transduction in plants. Disruption of CSN5 leads to lethality at the late larva stage during Drosophila development (24). Detailed analysis of the mutant strains revealed that Arabidopsis lacking the COP9 signalosome complex not only fails to respond to darkness but also eventually fails to grow at a relatively early stage during development. These observations show that the COP9 signalosome complex plays a pivotal role in developmental regulation. Although no CSN genes were found in the Saccharomyces cerevisiae genome except for a Jab1/CSN5 homologue, Schizosaccharomyces pombe contains at least some CSN genes (CSN1 = Caa1, CSN2, -4, and -5) whose products function as a part of the checkpoint control mechanism (25). Although the precise biochemical functions of the COP9 signalosome complex except for its associated kinase activity have not been clarified yet, each subunit has been independently characterized. CSN1 (also called GPS1) inhibits JNK1 (Jun N-terminal kinase) and represses Jun-dependent promoter activity (26). CSN2 (also called TRIP15; thyroid hormone receptor-interacting protein 15) interacts with the ligand-binding domain of receptors for thyroid hormone and retinoic acid (27), and alien, a Drosophila homologue of CSN2, is shown to function as a co-repressor of nuclear receptors (28). The CSN3 gene is hemizygenously deleted in patients with Smith-Magenis syndrome (29). CSN6 is identical to human immunodeficiency virus, type 1, Vpr-interacting nuclear export and p27 regulation.

In the present study, we investigated the mechanism of other components of the COP9 signalosome complex. In anergic T-cells, p27 and Jab1/CSN5 participate in antigen-specific T-cell unresponsiveness (34). The next obvious question is whether the functions of Jab1/CSN5 mentioned above are mediated by the Jab1/CSN5 monomer or by the COP9 signalosome complex. In the present study, we investigated the mechanism of nuclear export mediated by Jab1/CSN5, the physiological importance of Jab1/CSN5 in p27 regulation, and the involvement of other components of the COP9 signalosome complex in nuclear export and p27 regulation.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Cycle Analysis, and High Efficiency Transfection—NIH3T3, Swiss 3T3, and COS7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. In some experiments, cells were treated with LMB (2 ng/ml, purified and prepared by M. Yoshida, see Ref. 21), MG132 (50 μM, Biomol), butyrolactone I (40 μM, Funakoshi Co., Ltd.), and LLM (20 μM, Biomol) for 5 h. For cell cycle experiments, cells were fixed in 3:1 methanol/acetone at 0°C, rinsed with 70% ethanol, and stained with 0.1% propidium iodide for 45 h, and the cell cycle was re-activated by transferring the cells to 10% serum or 10% confluence. The cell cycle profile was monitored by flow cytometric analysis of DNA content or by BrdUrd incorporation as described (7, 35). cDNAs were cloned into the expression vectors, pCGL (designed by Dr. M. Tanaka and modified by Dr. J. Fujisawa, see Ref. 36) and pFLAG-CMV-2 (Takara Bio Industries, Ltd.), and used to transfect COS7 cells, which stably expressed HA-tagged and FLAG-tagged proteins, respectively. Expression vectors were transfected into cells by the modified calcium phosphate-DNA precipitation method, which was optimized for efficiency as described (7).

Immunofluorescent Staining—Cells were stained with rabbit polyclonal and mouse monoclonal antibodies and incubated with fluorescein isothiocyanate- and Texas Red (TR)-linked goat-antirabbit IgG (Amersham Biosciences) as described previously (7, 35). For the evaluation of BrdUrd incorporation, cells stained with rabbit polyclonal antibody followed by TR-linked anti-rabbit IgG were treated with 1.5 M HCl and stained with anti-BrdUrd mouse monoclonal antibody (Amersham Biosciences) and fluorescein isothiocyanate-linked anti-mouse IgG. The cell samples were viewed by phase contrast or fluorescence microscopy. More than 500 cells were enumerated for each sample.

In Vitro Protein Binding Assay—cDNA fragments corresponding to amino acid residues 1–334, 1–198, and 199–334 of mouse Jab1/CSN5 were amplified by PCR and sequenced to confirm sequence integrity. cDNA fragments were inserted into pGEX (Amersham Biosciences) in-frame with GST. GST fusion proteins were expressed in bacteria and purified as described (36). Mammalian cell extracts were isolated from proliferating mouse NIH3T3 fibroblasts in Tewen 20 buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.1% Tewen 20, and 1 mM dithiothreitol) supplemented with 1 mM sodium orthovanadate, 1 mM NaF, 1% aprotinin, and 0.5% phenylmethylsulfonyl fluoride. Binding was performed by incubating GST fusion proteins (10 μg) immobilized on the beads with cell lysates (corresponding to 5 × 10^6 cells) supplemented with 0.5% bovine serum albumin at 37°C for 1 h, and the protein complexes were washed in the same buffer. Bound CRM1 was detected by immunoblotting using a CRM1-specific antibody. To examine interaction between p27 mutants and Jab1/CSN5, the same amount of GST fusion proteins containing p27 variants was mixed with recombinant Jab1/CSN5 or cyclin D1-Cdk4 proteins in Tewen 20 buffer as described (7). Bound proteins were measured by immunoblotting followed by densitometry. The amount of proteins bound to wild type p27 was set to 100%.

Antibodies—Entire coding sequences of mouse and human cDNA were amplified by PCR (CSN1 and -8 from human K562 erythroleukemia cDNA library and CSN2–4, -6, -7a, and -7b from mouse T-cell lymphoma cDNA library) and were inserted into pGEX (Amersham Biosciences). pGEX and pGEX vectors (Qiagen). Polyclonal rabbit antibodies were produced against His6-tagged polypeptides expressed in bacteria. The generation of antibodies to Jab1/CSN5 and CRM1 was described previously (7, 37). All anti-CSN antibodies were tested for specificity and found not to cross-react with other CSN subunits but to recognize both human and mouse proteins. Rabbit polyclonal antibodies were affinity-purified using purified GST-tagged recombinant proteins immobilized on CNBr-activated Sepharose beads. Elution of bound IgG was performed at pH 2.5 and 3.5. Rabbit polyclonal antibodies against GST (Z-5), p27 (C-19), Cdk2 (M2), FLAG (D-8), eUN (D), Jun B (210), Jun D (329), Cdk4 (C-22), and Grb2 (C-23) and mouse monoclonal antibody to cyclin D1 (72–13G) were purchased from Santa Cruz Bio-technology. Mouse monoclonal antibodies to mouse retinoblastoma protein (X281, G3–245), p27 (clone 57), and an HA peptide epitope (23CA5) were obtained from Pharmingen, Transduction Laboratories, and Sigma, respectively. Anti-HA rabbit polyclonal antibody (HA.11) was obtained from Babco.

Protein Analyses—Cell lysis, immunoprecipitation, gel electrophoresis, immunoblotting, in vitro kinase assay, and biosynthetic experiments were performed as described (7, 35, 38, 39). For glycerol gradient centrifugation analysis, 1 ml of cell lysate was layered onto 10 ml of a 10%–40% linear glycerol gradient containing 10 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol. After centrifugation for 24 h at 27,000 rpm in a Beckman Ti40 rotor at 4°C, 0.5-ml fractions were collected from the bottom and analyzed by immunoblotting. To perform nuclear and cytoplasmic fractionation, cells were harvested in medium containing 0.1% serum or kept contact-inhibited for 2 min on ice, and nuclear fractions were collected.

For selective immunoprecipitation of mini-CSN by our antibody against Jab1/CSN5, incubation of Tewen 20 cell lysates with antibody was performed for less than 5 h. An overnight reaction allowed the

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antibody to recognize certain amounts of the COP9 signalosome complex. This is presumably because the minor population of polyclonal antibody can slowly recognize the 450-kDa COP9 signalosome complex or alternatively because incubation at 4 °C for an extended period alters the conformation of the COP9 signalosome complex such that Jab1/CSN5 epitopes become accessible to the antibody.

Random Mutagenesis by PCR and Screening of Mutants by the Yeast Two-hybrid Method—cDNA corresponding to C-terminal amino acid residues 80–197 of p27 was amplified by PCR (35 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min) in a low fidelity PCR buffer (40) containing 0.5 mM MnCl₂, twice as much Taq polymerase (5 units/100 μl) with increased concentration of MgCl₂ (final concentration 7 mM) and extra dCTP and dTTP (final concentration 1 mM) in addition to the standard PCR mixture (preliminary experiments using the lacZ gene as a target showed that the mutation rate under these conditions was more than 80%), and cloned into the pAS2 vector. The size of this mini-p27 library was about 10⁶. All six clones randomly picked from the mini-library contained 1–6 point mutations that contribute to amino acid substitutions. The p27 mini-library was transformed into Y190 yeast cells together with the pACT-Jab1/CSN5 plasmid. To select the mutants unable to interact with Jab1/CSN5, transfected colonies grown on Trp mutants unable to interact with Jab1/CSN5, transfectant colonies yeast cells together with the pACT-Jab1/CSN5 plasmid. To select the mutants unable to interact with Jab1/CSN5, transfected colonies grown on Trp-Leu− selection medium were tested for β-galactosidase activity, and the white colonies were chosen. Plasmids containing mutated p27 cDNA were recovered from yeast cells and were again tested as to whether they either confer the His requirement or induce the β-galactosidase activity in Y190 cells when transformed with pACT-Jab1/CSN5. The relative β-galactosidase activities contained in lysates isolated from yeast cells were evaluated using β-Galactosidase Reporter Gene Assay kits (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

RESULTS

Jab1/CSN5 Contains the Nuclear Export Signal—Nuclear export plays an important role in the down-regulation of p27 in cells ectopically overexpressing Jab1/CSN5 because LMB prevents p27 breakdown (7), but we did not find the typical leucine-rich type NES sequence in p27, which prompted us to hypothesize that it is not p27 but Jab1/CSN5 that is the direct target for CRM1-dependent nuclear export. To examine this possibility, we fused the entire coding sequence of Jab1/CSN5 to GST, and we employed an in vitro binding assay using GST fusion proteins immobilized on beads and cell lysates from proliferating mouse fibroblasts. As expected, the GST-fused Jab1/CSN5 recombinant protein, but not GST alone, was able to interact with CRM1 contained in the lysates in vitro (Fig. 1A). Under these conditions, we did not detect interaction between GST-tagged p27 and CRM1 (negative data not shown), indicating that p27 does not contain functional CRM1-binding motifs. Addition of the strong ionic detergent (0.1% SDS) to the reaction mixture inhibited genuine interaction, but the presence of 0.1% non-ionic detergents such as Nonidet P-40 and Tween 20 did not disrupt the Jab1/CSN5-CRM1 complex and reduced the nonspecific binding. Tween 20 had less inhibitory effect on the complex formation than Nonidet P-40. Incubation at 4 °C prevented interaction between the two components (negative data not shown), suggesting that certain enzymatic activities are required for efficient binding of CRM1 to Jab1/CSN5. In fact, it is reported that the efficient and specific association of CRM1 with the NES sequence depends upon the GTP-bound form of the Ran GTPase (19, 22, 23). Based upon these results, we performed in vitro binding assays in a buffer containing Tween 20 at 37 °C. In addition to the full-length Jab1/CSN5 protein, the C-terminal truncated mutant (Jab1(C)) containing amino acids 199–334, but not the N terminus mutant (amino acids 1–198, Jab1(N)), bound to CRM1 under these in vitro conditions (Fig. 1A). Careful observation of the C-terminal amino acid sequence of Jab1/CSN5 revealed that this region contains the leucine-rich NES-like sequence at amino acids 233–242 (Fig. 1B), which is conserved among Jab1/CSN5 proteins from various species. The minimum region (amino acids 233–242) consisting only of this motif was sufficient to interact with CRM1 in vitro, and conversion of leucine residues at 237, 240, and 241 to alanine disrupted the binding (Fig. 1C). Furthermore, addition of LMB to the binding reaction buffer completely blocked the interaction (Fig. 1D). Thus, Jab1/CSN5 contains the leucine-rich NES-like sequence in the C-terminal region, through which Jab1/CSN5 can interact with CRM1.

Ectopically expressed Jab1/CSN5 is located both in the nucleus and in the cytoplasm and induces a reduction in the nuclear p27 signals. Treatment of cells with 2 ng/ml LMB for 5 h markedly increased the amount of Jab1/CSN5 in the nucleus and prevented down-regulation of p27 (Fig. 1, E and F, and see Ref. 7). The Jab1/CSN5 molecule containing a mutant NES (alanine from leucine at 237, 240, and 241) was located mostly in the nucleus, although some signal was still observed in the cytoplasm. However, these cytoplasmic signals did not disappear in the presence of LMB, indicating that the NES...
mutant of Jab1/CSN5 is refractory to the action of LMB (Fig. 1E). Importantly, mutations in the NES sequence markedly impaired the ability of Jab1/CSN5 to mislocalize and down-regulate the endogenous p27 protein (Fig. 1, E and F). Thus, Jab1/CSN5 contains the LMB-sensitive NES sequence in the C-terminal region, and interaction of CRM1 with this motif is required for nuclear export of Jab1/CSN5 and subsequent down-regulation of p27.

Although we did not find the typical leucine-rich type NES sequence in other regions of Jab1/CSN5, the C terminus region containing amino acids 242–334 was still capable of interacting with CRM1 in vitro (data not shown), suggesting that there is an additional NES in this region. In fact, the effect of the mutation in NES (amino acids 233–242) was partial (Fig. 1F), and removal of all C-terminal amino acids (the Jab1(N) mutant) completely disrupted the interaction with CRM1 (Fig. 1A) and the ability to down-regulate p27 (data not shown, but see below). However, we do not rule out the possibility that the C terminus of Jab1/CSN5 contains unidentified functional domains other than NES, which play an essential role in the function of Jab1/CSN5.

**Jab1/CSN5 Is Required for Down-regulation of p27 in Proliferating Fibroblasts**—To investigate whether Jab1/CSN5 participates in regulation of p27 in proliferating cells, we utilized a dominant negative form of Jab1/CSN5. As we showed above that the NES function plays an important role in p27 down-regulation by Jab1/CSN5, we focused on the mutant, Jab1(N). This mutant lacks all, including the putative NES sequences and was unable to interact with CRM1 at least in vitro, but it retains the N-terminal half containing the entire MPN domain (41) (Fig. 1A), which is highly conserved among the Mov34 protein family (42). Jab1(N) was shown to function in a dominant negative fashion because co-expression of Jab1(N) inhibited down-regulation of p27 induced by the full-length Jab1/CSN5 molecule (Fig. 2A). The point mutant of NES (Jab1(NES Mut)) was less effective in this assay (data not shown).

Introduction of Jab1(N) into proliferating NIH3T3 mouse fibroblasts increased total expression levels of the endogenous p27 protein in a dose-dependent manner (Fig. 2B). Jab1(N) was preferentially localized in the nucleus, and up-regulated immunofluorescent signals specific to p27 were observed in the Jab1(N)-expressing nuclei (Fig. 2C, lower panels). In contrast, full-length Jab1/CSN5 was located both in the nucleus and in the cytoplasm, and cells expressing ectopic Jab1/CSN5 exhibited much less p27 signal (Fig. 2C, upper panels). Approximately 25% of Jab1(N)-expressing cells showed markedly enhanced p27 signals (Fig. 2D), and a 100% increase of Jab1(N) expression resulted in 50% up-regulation of nuclear p27. Fig. 2E shows that the introduction of Jab1(N) into proliferating fibroblasts reduced the percentage of BrdUrd-positive cells by ~44% (from 80 to 35%), indicating that Jab1/CSN5 function is required for G1 progression, presumably for p27 down-regulation, but we do not exclude the possibility that Jab1/CSN5 may have target molecules other than p27, which play an important role in cell cycle progression.

Full-length Jab1/CSN5 is not found in a complex with wild type p27 in proliferating cells unless mutations that increase the stability of p27 are generated (7). In Jab1(N)-transfected cells, however, we detected a complex between wild type p27 and truncated Jab1/CSN5 mutant (Fig. 2F). This is presumably because the dominant negative blockade mediated by Jab1(N) allowed the accumulation of intermediates of the nuclear export reaction. This result also indicates that the N-terminal region of Jab1/CSN5 including the MPN domain is sufficient for interaction with a target molecule. Therefore, although the interaction between p27 and Jab1/CSN5 is transient in the cell,
positions 108, 130, and 140 (D108G/L130M/N140K), which exhibited the least ability to bind Jab1/CSN5 in vitro among the three mutants (Fig. 3B). In mouse fibroblasts, ectopically expressed p27(DLN) was as stable as the wild type protein. However, in cells co-transfected with Jab1/CSN5, although the wild type protein was degraded, p27(DLN) was much more stable (Fig. 3C). p27(DLN) was located within the nucleus, and the localization remained the same in cells co-expressing Jab1/CSN5 (Fig. 3D).

To elucidate whether the integrity of Asp-108, Leu-130, and Asn-140 in p27 is important for the association between c-Jun and Jab1/CSN5, we carefully examined the amino acid sequence of c-Jun and found that aspartic acid and leucine were conserved within the Jab1-binding domain (Asp-12 and Leu-34) (Fig. 3A). c-Jun contains aspartic acid at codon 44 instead of asparagine, but we assume that a basic amino acid at this position disrupts the conformation required for interaction with Jab1/CSN5. Therefore, we generated a triple mutant of c-Jun (D112G/L34M/D44K) and tested it for binding to Jab1/CSN5. The two-hybrid assay in yeast, by which the interaction between c-Jun and Jab1/CSN5 was originally identified (13), revealed that wild type c-Jun and p27, but not the c-Jun triple mutant, efficiently interacted with Jab1/CSN5 under these conditions (Fig. 3E). Careful examination of the amino acid sequences of other Jab1/CSN5 interactants revealed that in another Jab1/CSN5 binding domain, the C-terminal cytoplasmic region of the LFA-1 β2 subunit (33), Asp, Leu, and Asn are conserved at positions 731, 753, and 763, respectively (Fig. 3A). Thus, several amino acids are conserved within the Jab1/CSN5 binding domain from different proteins and play an essential role in interaction with Jab1/CSN5.

Characterization of Two Different Forms of Jab1/CSN5—To investigate further the regulatory and functional mechanisms of Jab1/CSN5, we generated N- and C-terminal-specific antibodies (anti-Jab1(N) and anti-Jab1(C), respectively) by affinity purification from antisera that contain both types of antibodies. Western blotting analysis using recombinant proteins containing different regions of Jab1/CSN5 confirmed the specificity of each antibody; anti-Jab1(N) recognized the GST fusion protein containing amino acids 1–198 of Jab1/CSN5 (GST-Jab1(N)) and full-length Jab1 (GST-Jab1) but not GST-Jab1(C), which contains amino acids 199–334 (Fig. 4A). In contrast, anti-Jab1(C) interacted with GST-Jab1(C) and GST-Jab1 but not with GST-Jab1(N) (Fig. 4B).

From mouse fibroblast cell lysates supplemented with a strong detergent such as SDS and deoxycholate (RIPA), all three antibodies (including the original antiserum containing both types of antibodies; anti-Jab1(W)) equivalently immunoprecipitated most Jab1/CSN5 protein. However, from lysates containing a weak detergent (0.1% Tween 20), only a small portion of the Jab1/CSN5 protein was recognized by anti-Jab1(W) and anti-Jab1(C) and no protein at all by anti-Jab1(N) (Fig. 4C). It does not seem likely that the different forms of Jab1 were extracted from cells in different conditions because equivalent amounts of Jab1/CSN5 protein were isolated from cells in both RIPA and Tween 20 buffers. These observations suggest that Jab1/CSN5 exists in at least two different forms; one form contains accessible C-terminal and masked N-terminal epitopes, and the other form has both N- and C-terminal epitopes blocked.

To prove this point directly, we fractionated the cell lysate by glycerol gradient centrifugation. As expected, in lysates containing Tween 20, Jab1/CSN5 was found in two different fractions corresponding to molecular masses of ~450 and 100 kDa (Fig. 4D). We judged that the bigger complex is the COP9 signalosome complex (15) because its size is similar to that of
the COP9 signalosome complex and it contained all 8 subunits (see below). Each fraction was tested for immunoprecipitation with anti-Jab1(N) and anti-Jab1(C). Anti-Jab1(C) recognized the smaller form but not the COP9 signalosome complex. Anti-Jab1(N) recognized neither. Treatment with SDS and deoxycholate disrupted the complex, resulting in one peak with a molecular mass of 40 kDa, from which the majority of Jab1/CSN5 protein was efficiently immunoprecipitated by both anti-Jab1(N) and anti-Jab1(C) antibodies (Fig. 4E). These findings support the idea that Jab1/CSN5 exists in two different forms in proliferating mouse fibroblasts.

Ectopically expressed HA-Jab1/CSN5 was distributed between the big and small complexes, but more protein was repeatedly found in fractions corresponding to the small complex (Fig. 4F, 2nd panel), implying that the amount of ectopic Jab1/CSN5 entering into the COP9 signalosome complex may be limited. Interestingly, HA-Jab1(N) was found in fractions slightly bigger than the small complex (Fig. 4F, 3rd panel), suggesting that it formed a complex with other proteins. In fact, a portion of Jab1(N) was associated with p27 (Fig. 2F), but other proteins interacting with Jab1(N) are currently unknown. Importantly, in cells ectopically overexpressing Jab1(N), the amount as well as the size of the big complex was exactly the same as in non-transfected cells (Fig. 4F, bottom panel), which proves that Jab1(N) inhibits the function of wild type Jab1/CSN5 without disrupting the COP9 signalosome complex.

Endogenous p27 migrated between the two Jab1/CSN5-containing complexes and was in the same fraction as cyclin D1 and Cdk4 (Fig. 4F, 4th and 5th panels, and data not shown), suggesting that the majority of p27 in proliferating cells is not in a complex with Jab1/CSN5. In agreement with this idea, the
experiment using quantitative immunoblotting after highly efficient immunoprecipitation revealed that more than 90% of p27 protein was recovered in anti-cyclin D1 or anti-Cdk4, but not in anti-Jab1/CSN5, immunoprecipitates (negative data not shown). This could be because p27 is rapidly turned over after binding to Jab1/CSN5. Ectopically overexpressed p27 was co-fractionated with the Jab1/CSN5-containing small complex (Fig. 4F, 6th panel) and a portion of exogenous p27 was co-immunoprecipitated with Jab1/CSN5 (data not shown but see Ref. 7). c-Jun (as well as JunD and JunB) was found in the same fraction as the Jab1/CSN5-containing small complex (Fig. 4F, bottom panel), but no complexes of Jab1/CSN5 and Jun family transcription factors were detected using the immunoprecipitation methods. It could be that Jab1/CSN5 and Jun family proteins stably associate with each other only on chromosomal DNA, or alternatively, our antibody may not recognize Jab1/CSN5-Jun complexes.

To understand better the nature of the two Jab1/CSN5-containing complexes, we isolated the nuclear and cytoplasmic fractions (Fig. 4G). Although Jab1/CSN5 was found in both fractions, more Jab1/CSN5 protein was located in the nucleus in proliferating mouse fibroblasts. Glycerol gradient centrifugation analysis of both nuclear and cytoplasmic fractions clearly demonstrated that the COP9 signalosome complex and the small complex were exclusively located in the nucleus and the cytoplasm, respectively (Fig. 4H). More interestingly, treatment of the cells with LMB markedly reduced the amount of the small complex (Fig. 4I), whereas butyrolactone I (a chemical inhibitor specific to Cdk kinases, Ref. 44) had little effect on the small complex (Fig. 4J), whereas butyrolactone I (a chemical inhibitor specific to the 26 S proteasome, Ref. 45) had an intermediate effect. These results suggest that the cytoplasmic small Jab1/CSN5-containing complex comes from the nuclear COP9 signalosome complex by nuclear export.

**Involvement of the COP9 Signalosome Components in p27 Regulation**—The fact that the majority of Jab1/CSN5 is in the COP9 signalosome complex prompted us to investigate whether other components of the COP9 signalosome complex participate in the regulation of p27. We first amplified coding sequences of each CSN component from a cDNA library by PCR, and we generated polyclonal rabbit antibodies specific to each of them against bacterially produced polypeptides. Our antibody against CSN1 recognized the 450-kDa COP9 signalosome complex, and in fact, anti-CSN1 immunoprecipitates contained all other CSN components (Fig. 5A, left panel). In contrast, our anti-Jab1/CSN5 antibody, which recognized the small complex but not the 450-kDa COP9 signalosome complex (Fig. 4 and see above), co-immunoprecipitated only a subset of the CSN components (CSN4, -6, -7b, and -8). Glycerol gradient fractionation experiments showed that CSN4, -6, -7b, and -8 were distributed in two forms that corresponded to the same two fractions as Jab1/CSN5, whereas CSN1 was found only in a large complex (Fig. 5B). However, we do not yet know whether these components are in the same complex or form independent complexes with Jab1/CSN5.

Expression vectors containing each of the CSN cDNA produced equivalent levels of CSN proteins in cultured mammalian cells (Fig. 5C). We then introduced these vectors into the proliferating mouse fibroblasts and examined whether ectopic CSN proteins down-regulate endogenous p27. In cells expressing exogenous CSN1, -2, and -4, we did not see any significant changes. However, introduction of CSN3, -6, -7a, -7b, and -8 in addition to Jab1/CSN5 resulted in a decrease in the number of cells exhibiting p27-positive staining (Fig. 5D), indicating that the ability to down-regulate p27 is not specific to Jab1/CSN5 but a function shared among a subset of the CSN components.

Because the CSN components found in a Jab1/CSN5-containing small complex (CSN4, -6, -7b, and 8) were not exactly the same set as those that were able to down-regulate endogenous p27 when overexpressed (CSN3, -6, -7a, -7b, and -8), the functions of each CSN component may be different and could be classified into several categories based on these observations. CSN5 (Jab1), -6, -7b, and -8 may function together because they were found in anti-Jab1/CSN5 immunoprecipitates and were able to down-regulate p27. The expression of CSN4 does not appear to be rate-limiting in p27 regulation. CSN3 and -7a may act on the COP9 signalosome complex and indirectly affect the activity of the small complex. CSN1 and -2 do not seem to be involved in the regulation of p27 abundance. Interestingly, ectopic expression of CSN7b altered the subcellular localization of endogenous Jab1/CSN5 (Fig. 5E). Endogenous Jab1/CSN5 was predominantly located in the nucleus, but in cells expressing HA-tagged CSN7b, Jab1/CSN5 was localized predominantly in the cytoplasm (Fig. 5E, upper panels). In these cells, the p27 signal disappeared (data not shown). Treatment of the cells with LMB induced a marked increase in the level of Jab1/CSN5 in the nucleus (Fig. 5E, bottom panels) as well as recovery of the p27 signal in the nucleus. These results suggest that the nuclear-cytoplasmic localization of Jab1/CSN5 is regulated by other CSN subunits and that the COP9 signalosome complex, but not the Jab1/CSN5 monomer, is involved in the regulation of p27.
DISCUSSION

Generally, proteins that are transported from the nucleus to the cytoplasm contain a leucine-rich NES (18), through which CRM1 interacts with a variety of substrates, but the amino acid sequence of p27 does not include the typical leucine-rich NES sequence. In this study, we showed that Jab1/CSN5, but not p27, contains a leucine-rich NES, which is conserved among Jab1/CSN5 proteins from different species and plays a pivotal role in Jab1/CSN5 activity. We have also identified the amino acids in the p27 molecule, which are required for the binding to Jab1/CSN5, and for subsequent nuclear export and degradation. The results indicate that Jab1/CSN5 is required to interact with both p27 and CRM1 during nuclear export of p27 and, therefore, suggest that one of the functions of Jab1/CSN5 is to serve as an adaptor between CRM1 and target molecules. Considering that a number of proteins containing the "Jab1/CSN5-binding motif" can be found in data bases and the number of proteins identified as a Jab1/CSN5-binding factor is rapidly increasing, it would be feasible for the subcellular localization of some of them to be regulated by Jab1/CSN5.

Phosphorylation by the cyclin E-Cdk2 kinase and ubiquitination by the SCFSkp2 ubiquitin ligase complex are proposed to play an important role in the regulation of p27 (2–4). So far, we have been unable to find a link between the cyclin E-Cdk2-SCFSkp2 pathway and the Jab1/CSN5 pathway. However, because the dominant negative Jab1/CSN5 increases the endogenous level of p27, and Skp2 knockout cells show elevated levels of p27 expression (46), both pathways seem to participate in p27 regulation in vivo. In addition, close examination of p27 down-regulation during G1 progression reveals that most p27 protein disappears long before the maximum activation of cyclin E-Cdk2 kinase. These results suggest that the cellular abundance of p27 is regulated in multiple steps. It is important to know in which pathway and at which time point Jab1/CSN5 is involved in p27 down-regulation during progression of the G1 phase.

By using different biochemical techniques, we have demonstrated that Jab1/CSN5 exists in two different complexes in mammalian cells; one is ~450 kDa in size, nuclear, and identical to the conventional COP9 signalosome complex, and the other is much smaller and located in the cytoplasm. Importantly, the small complex diminishes when nuclear export is blocked by LMB. These results are basically consistent with findings in Arabidopsis (47), except that the small complex was described as a Jab1 monomer, and we do not know yet whether this is because of a species difference or not. Most of the ectopically expressed Jab1/CSN5 protein is not incorporated into the COP9 signalosome complex (Ref. 48 and this work) but rather remains as a monomer or is included in the small complex. We found that the CSN composition of the ectopic Jab1/CSN5-containing small complex is the same as that of the endogenous Jab1/CSN5-containing small complex, suggesting that ectopic Jab1/CSN5-induced down-regulation of p27 is due to the increased amount or activity of the Jab1/CSN5-containing small complex. The COP9 signalosome complex may negatively regulate the nuclear export function of Jab1/CSN5, because the Jab1/CSN5-containing small complex is composed of the COP9 signalosome subunits, we proposed that this complex could be called the "mini-COP9 signalosome" or "mini-CSN."

Although a low expression level of p27 protein correlates well with the more malignant phenotype of human cancers (49–51), the molecular mechanisms of p27 down-regulation in tumors are ill-defined. Up-regulated expression of Skp2 is occasionally observed in transformed cells but is not necessarily correlated with weak expression of p27. Several gene products associated with human diseases have been reported to regulate the cellular abundance of the p27 protein, but the biochemical pathways between p27 and these proteins remain unknown. So far, identification of the human cancer-associated mutations within the Jab1/CSN5 gene locus has not been successful. However, because ectopic expression of CSN3, -6, -7a, -7b, and -8 as well as Jab1/CSN5 can induce down-regulation of p27, it will be necessary to investigate whether any of the CSN components are involved in human cancers.

The COP9 signalosome complex is involved in many important biological phenomena, such as photomorphogenesis, early development, apoptosis, cell cycle progression, and checkpoint control, but its biochemical functions and regulatory mechanisms are largely unknown except for its associated enzymatic activity of phosphorylation (14, 52) and deneddylation (53, 54). There is a particularly urgent need to identify the upstream signaling machinery that activates or inactivates the mammalian COP9 signalosome complex. Identification of the CSN interactor and biochemical examination of the CSN components after stimulation of the cells will be important to elucidate the nature of the complex that receives and sends "SIGNALs."

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REFERENCES

1. Sherr, C. J. (1996) Science 274, 1672–1677
2. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
3. Sagnabato, A., Cistadini, A., Faraglia, B., and Weinstein, J. B. (2000) J. Cell. Biol. 183, 18–27
4. Slingerland, J., and Pagano, M. (2000) J. Cell. Biol. 183, 10–17
5. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. (1999) Nat. Cell Biol. 1, 207–214
6. Tsvelev, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (1999) Curr. Biol. 9, 661–664
7. Clarret, P. X., Hibi, M., Dhum, S., Toda, T., and Karin, M. A. (1996) Nature 383, 453–457
8. Seeger, M., Kraft, R., Ferrell, K., Bech-Otscher, D., Remley, R., Schade, R., Gordon, C., Naumann, M., and Dubiel, W. (1998) FASEB J. 12, 469–478
9. Wei, N., and Deng, X. W. (1999) Trends Genet. 15, 98–103
10. Deng, X. W., Dubiel, W., Wei, N., Hofmann, K., Munkit, K., Cockieli, J., Kato, J.-Y., Naumann, M., Segal, D., Seeger, M., Carr, A., Gluckman, M., and Chamovitz, D. A. (2000) Trends Genet. 16, 202–203
11. Camovitz, D. A., Segal, D, (2001) EMBO Rep. 2, 96–101
12. Gerace, L. (1995) Cell 82, 341–344
13. Furerod, M., Ohno, M., Yoshida, M., and Mattai, I. W. (1997) Cell 90, 1051–1060
14. Ossareh-Nazari, B., Bachelet, E., and Di Gregorio, C. (1997) Science 278, 787–790
15. Yoshida, M., and Horinouchi, S. (1999) Ann. N. Y. Acad. Sci. 886, 21–36
16. Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997) Cell 90, 1041–1050
17. Aakjaer, P., Jensen, T. H., Nilsson, J., Englemeier, K., and Ljum, J. B. (1998) J. Biol. Chem. 273, 33414–33422
18. Freilich, S., Oror, E., Kapp, Y., Nevo-Caspi, Y., Orgad, S., Segal, D., and Chamovitz, D. A. (1999) Curr. Biol. 9, 1187–1190
19. Munkit, K., Porte, J., Murray, J. M., Brixon, C., Christensen, P. U., Caspari, T., Hagan, I. M., Millar, J. B., Simanis, V., Hofmann, K., and Carr, A. M. (1999) Curr. Biol. 9, 1427–1430
20. Spain, B. H., Bowdish, K. S., Fasal, A. R., Staub, S. B., Koo, D., Chang, C. Y., Xie, W., and Celicelj, S. (1996) Mol. Cell. Biol. 16, 6688–6706
21. Lee, J. W., Choi, H. S., Gyuris, J., Brent, R., and Moore, D. D. (1995) Mol. Cell. Biol. 15, 9833–9844
22. Elson, S. H., Mykytyn, K., Ferrell, K., Coulter, K. L., Das, P., Dubiel, W., Patel, P. I., and Metherell, J. E. (1999) Am. J. Med. Genet. 87, 342–348

2 K. Tomoda, N. Yoneda-Kato, and J.-Y. Kato, unpublished observations.
The Cytoplasmic Shuttling and Subsequent Degradation of p27Kip1 Mediated by Jab1/CSN5 and the COP9 Signalosome Complex
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