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# Opposite Regulation of the Expression of Cyclin-Dependent Kinase Inhibitors during Contact Inhibition[1]

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**Contact inhibition is a well-known phenomenon, but the details of its mechanism are poorly understood. Recently, cyclin-dependent kinase inhibitors have been studied intensively with respect to their regulatory role in the cell cycle, and of them, $p27^{Kip1}$ is particularly involved in contact inhibition. $p27^{Kip1}$ is believed to be regulated primarily through posttranscriptional mechanisms. We now report that cyclin-dependent kinase inhibitors, including p27, are regulated differently at the mRNA level during contact inhibition in murine BALB/c-3T3 fibroblasts. The mRNA expression of p15, p16, and p27 was up-regulated as the cell density increased, but, on the contrary, the mRNA level of $p21^{Cip1/WAF1/Sdi1}$ markedly decreased when the cells became confluent. The protein levels of these genes were regulated in the same way as their mRNA levels, and cyclin-dependent kinase-2 activity was markedly inhibited on density-mediated growth arrest of the cells. These results indicate that the regulation of mRNA expression of cyclin-dependent kinase inhibitors appears to contribute to their protein levels and to the arrest of cell growth through contact inhibition.**

**Key words: BALB/c-3T3 fibroblasts, contact inhibition, cyclin-dependent kinase inhibitors, $G_1$ arrest, Rb protein.**

Contact inhibition is a characteristic feature of untransformed cells grown in monolayers, whereas transformed cells often do not show this regulation. The mechanism underlying contact inhibition is presumed to be initiated by cell-cell interaction (*1*), but the details of the cell surface molecules involved in contact inhibition and the following signal transduction pathway that inhibits cell growth remain unknown. Recently, $p27^{Kip1}$, one of the regulatory molecules for cell-cycle progression, was reported to be involved in contact inhibition (*2, 3*).

$p27^{Kip1}$ is a negative regulator of the cyclin-dependent kinases (Cdks) that trigger cell-cycle progression. The activities of Cdks are controlled by both positive regulators, cyclins, and negative regulators, cyclin-dependent kinase inhibitors (CKIs), as well as through phosphorylation of the Cdks themselves (*4, 5*). The mammalian CKIs fall into two families: the first family, which includes $p16^{Ink4A}$ (*6*), $p15^{Ink4B}$ (*7*), $p18^{Ink4C}$ (*8, 9*), and $p19^{Ink4D}$ (*9, 10*), specifically inhibits Cdk4 and Cdk6, and the second family, which includes $p21^{Cip1/WAF1/Sdi1}$ (*11-14*), $p27^{Kip1}$ (*15, 16*), and $p57^{Kip2}$ (*17, 18*), can inhibit a broad range of Cdk activities. Among the CKIs, $p27^{Kip1}$ was suggested to be involved in contact inhibition (*2, 3*), and the protein level of $p27^{Kip1}$ was reported to be regulated primarily through posttranscriptional mechanisms (*19-21*). CKIs have also been well studied in fibroblasts entering the $G_0/G_1$, S transitional state from the growth-arrested state caused by serum deprivation or high cell density (*3, 14, 22-25*), but contact inhibition itself has not been the focus of any CKI investigations.

In the course of studies on the $G_0/G_1$, S transitional state involving BALB/c-3T3 cells (*26, 27*), we found that the mRNA levels of CKIs are also controlled according to the growth state. We now report the differential expression of CKI gene products caused by contact inhibition, and its involvement in contact inhibition in BALB/c-3T3 cells.

## MATERIALS AND METHODS

*Cell Lines and Reagents*—BALB/c-3T3 cells (clone A31) were a gift from Dr. C. Stiles (Harvard Medical School). BALB/c-3T3 cells were grown to the $G_0$ state and stimulated with serum as described previously (*28*). To investigate the mechanism involved in contact inhibition, $1.5 \times 10^6$ BALB/c-3T3 cells were freshly cultivated in a 15-cm culture dish. Half of the medium was replaced with fresh medium every 24 h. Cells were also harvested every 24 h for further analysis. The antibodies used for immunoblotting and immunoprecipitation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

*Northern Blotting Analysis*—Total cellular RNA was

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Abbreviations: RT-PCR, reverse transcription-PCR; TGF-$\beta$, transforming growth factor-$\beta$.

extracted from BALB/c-3T3 cells at the indicated time points with Isogen (Nippon Gene, Tokyo) according to the manufacturer's instructions. Northern blotting analysis employing 25 $\mu$g of total RNA/lane was performed by the standard formaldehyde method using the following mouse cDNAs; p15 (kindly provided by Dr. C. Sherr, St. Jude Research Hospital, Memphis, TN), p21 (provided by Dr. S. Elledge, Baylor College of Medicine, Houston, TX), and p27 (provided by Dr. J. Massagué, Memorial Sloan-Kettering, New York). The cDNA fragment derived from mouse p16 exon 1, corresponding to nucleotides 13 to 204 (*29*), was amplified by RT-PCR, and used as the specific probe for mouse p16 mRNA, because it does not bind with $p19^{ARF}$ mRNA.

The mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fluctuated markedly in our system, as shown in Fig. 1, and this phenomenon was observed reproducibly. Therefore, 28S ribosomal RNA stained with methylene blue on a nylon membrane is shown in Figs. 1 and 3 as an internal standard.

*Cell-Cycle Analysis*—Cells were harvested on the stated days by trypsinization, fixed in 70% ethanol, and then treated with 0.1 mg/ml RNase A. Then cellular DNA was stained with 0.05 mg/ml propidium iodide in phosphate-buffered saline and then analyzed with a FACScan (Becton-Dickinson). Data acquisition and cell-cycle analysis were performed using CellQuest (Becton-Dickinson) and Modifit (Verity Software House, Topsham, ME), respectively.

*Immunoprecipitation, Western Blotting Analysis, and Kinase Assays*—BALB/c-3T3 cells were lysed in the lysis buffer [50 mM HEPES (pH 8.0), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween 20, 10% glycerol, 50 mM NaF, 0.1 mM $Na_3VO_4$, and Complete™ protease inhibitor cocktail (Boehringer Mannheim)] each day after splitting. Immunoprecipitation was carried out in the lysis buffer using anti-p16, p21, p27, Cdk2, Cdk4, cyclin D1, and retinoblastoma protein (pRB) antibodies for 1 h at 4°C, followed by the addition of protein A Sepharose (Pharmacia Biotech) and incubation for 1 h at 4°C. After three washes with the lysis buffer, the immunoprecipitated proteins were analyzed by Western blotting or *in-vitro* kinase assay as described previously (*3*) with some modifications.

In order to separate the phosphorylated and dephosphorylated pRB, the samples immunoprecipitated with anti-pRB antibody were separated by 7.5% SDS-PAGE (*30*). The samples were run until the lower molecular weight proteins ran out of the gel for better separation; prestained Rainbow markers (Amersham) were used for this purpose.

For the kinase assay, immunoprecipitated pellets were incubated for 30 min at 30°C in 30 $\mu$l of kinase buffer [50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM $\beta$-glycerophosphate, 1 mM NaF, and 0.1 mM $Na_3VO_4$] containing 0.83 $\mu$M ATP, 12 $\mu$g of purified glutathione S-transferase (GST)-pRB fusion protein, and 5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol) (Amersham). The reactions were stopped by boiling in the sample buffer for SDS-PAGE, and proteins were separated on a 5-20% polyacrylamide gel (Bio Craft, Tokyo). Phosphorylated proteins were analyzed using a BAS 2000 system (Fuji Film, Tokyo).

## RESULTS

*Northern Blotting Analysis of the Expression of CKIs in BALB/c-3T3 Cells Entering the $G_0/G_1$, S Transition*—We determined the mRNA expression levels of CKIs by Northern hybridization in growing, confluent, and serum-stimulated BALB/c-3T3 cells, and found that the expression of the genes involved is regulated at the mRNA level. As shown in Fig. 1, the mRNAs of p15 and p16 were induced as the cells became confluent, and then they were down-regulated on serum stimulation. The RNA expression level of p27 also changed, as for p15 and p16, but to a lesser degree. After serum stimulation, p27 mRNA decreased more rapidly and reached the nadir 2 h after stimulation, but recovered more distinctly than the mRNAs of p15 and p16. More interestingly, unlike other CKIs, the mRNA expression of p21 was depressed when the cells became confluent. It decreased further still when the cells were grown to the $G_0$ state by incubation in DMEM+5% platelet-poor plasma (designated as 0 in Fig. 1). Furthermore, p21 expression was induced on serum stimulation, as reported previously for human fibroblasts (*14, 24*).

*Cell-Cycle Analysis of the Course of Contact Inhibition*—The above data prompted us to investigate the details of the regulation of CKI expression during contact inhibition. Many previous studies as well as the experiment shown in Fig. 1 involved both density-arrest and serum depletion to obtain quiescent fibroblasts. To eliminate the influence of serum depletion, we changed half of the culture medium every 24 h after $1.5 \times 10^6$ cells had been spread on

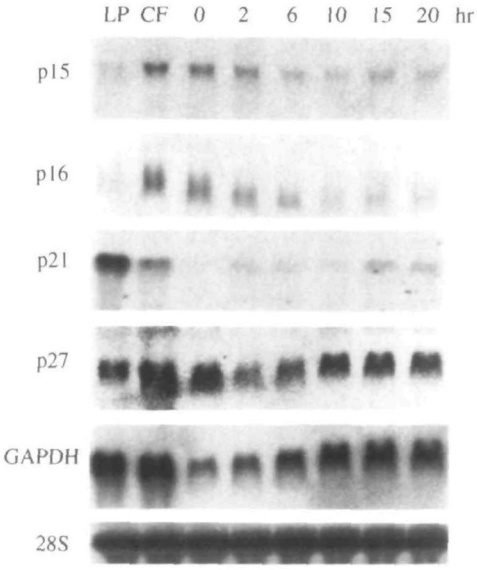


Fig. 1. **Northern blotting analysis of the expression of CKIs in serum-stimulated BALB/c-3T3 cells.** RNA was extracted from cells 1 day after splitting (LP; log phase), and 3 days after splitting (CF; confluent). The confluent cells were further cultivated for 48 h in DMEM+5% platelet-poor plasma (*28*), and then RNA was extracted (designated as 0), or the cells were stimulated with serum and RNA was extracted at the indicated time, h (2-20). Equal amounts of RNA (25 $\mu$g) were loaded on a 1% agarose gel, and Northern blotting analysis was performed as described under "MATERIALS AND METHODS."

a 15-cm dish. The cell density at each time point is shown in Fig. 2A, which reveals that the cells reached confluency 3 days after spreading under these conditions. The cell cycle was monitored by flow cytometry, and the relative number of cells in the S phase was markedly reduced when the cells became confluent, whereas the relative number of cells in $G_1$ increased, as shown in Fig. 2B.

*Northern Blotting Analysis of the Expression of CKIs during Contact Inhibition*—Cells were harvested every 24 h and then analyzed by Northern blotting (Fig. 3). The mRNA levels of p15, p16, and p27 increased as the cell density increased while the cells were still growing exponentially, and reached their maxima on day 3. On the other hand, the mRNA level of p21 did not change for 2 days, and decreased markedly on day 3 when the cells became confluent.

*The Protein Levels of CKIs and Related Proteins*—The CKI protein level was determined by Western blotting or immunoprecipitation (IP)-Western blotting. The p16 and

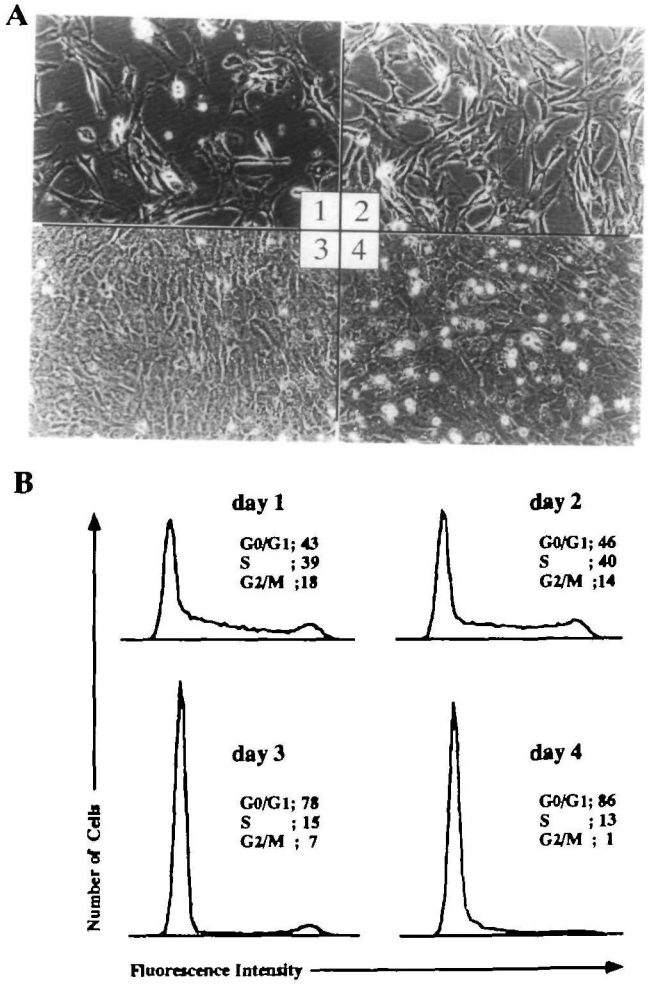


Fig. 2. **Contact inhibition study with BALB/c-3T3 cells and cell-cycle analysis.** A: BALB/c-3T3 cells were cultivated for the contact-inhibition study as described under "MATERIALS AND METHODS"; photomicrographs were taken every day and are designated as 1-4. B: Cells were harvested on the stated days and analyzed by flow cytometry as described under "MATERIALS AND METHODS." Data are representative of three independent experiments.

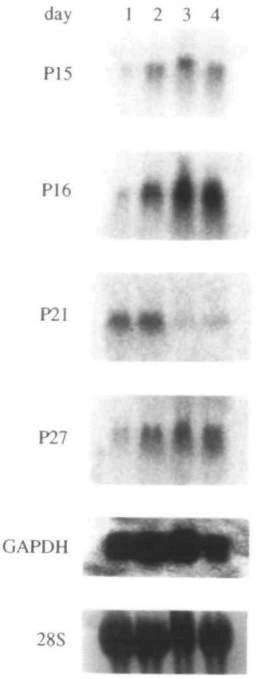


Fig. 3. **CKI mRNA expression during contact inhibition.** BALB/c-3T3 cells were cultivated as described in Fig. 2, and total RNA was isolated every day (days 1-4) from the cells, and Northern blotting analysis of the CKI expression was performed as described under "MATERIALS AND METHODS."

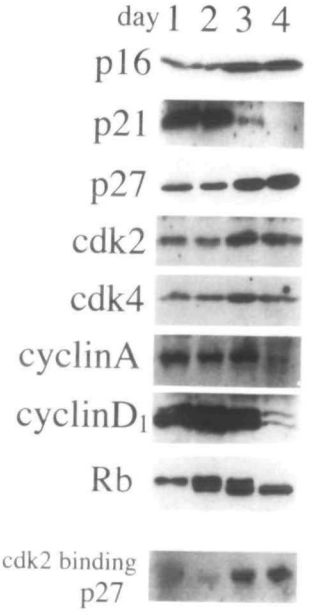


Fig. 4. **Immunoprecipitation and Western blotting analysis of CKIs and the proteins involved.** BALB/c-3T3 cells were grown as described in Fig. 2 and lysed each day (days 1-4), and then analyzed directly by Western blotting analysis (Cdk2, 4, cyclin A) or immunoprecipitated and then detected by Western blotting [p16, p21, p27, cyclin D1, and pRB(Rb)]. Cdk2 binding p27 was immunoprecipitated with anti-Cdk2 antibody and then detected by Western blotting using anti-p27 antibody.

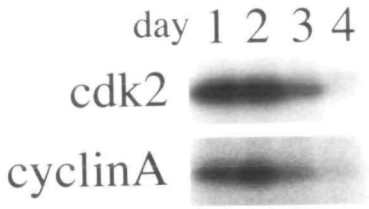


Fig. 5. **Cdk kinase assay during contact inhibition.** The cell lysate on each day (1-4) was immunoprecipitated with anti-Cdk2 or cyclin A antibodies, and then the kinase activity towards the GST-pRB protein was analyzed as described under "MATERIALS AND METHODS."

p27 proteins increased as the cell density increased up to day 4 after splitting (Fig. 4), whereas their mRNAs showed slight decreases on day 4 (Fig. 3). The amount of the p27 protein that was co-precipitated with Cdk2 was also significantly increased on days 3 and 4. In contrast, the increase in cell density induced a marked reduction of the p21 protein (Fig. 4) as well as of the mRNA of p21 (Fig. 3). The protein levels of Cdk2 and Cdk4 increased slightly on day 3, but the cyclin A and cyclin D1 proteins were markedly reduced on day 4. Most of the pRB was in the phosphorylated form (upper band) on day 1; however, the dephosphorylated form (lower band) was produced as the cell density increased, and on day 4, almost all of the pRB was dephosphorylated (Fig. 4).

*Cdk Activities during Contact Inhibition*—To examine the Cdk activities during contact inhibition, the Cdk2 and cyclin A proteins were immunoprecipitated and the associated kinase activity was examined using the GST-pRB fusion protein as the substrate. The activities precipitated with antibodies against Cdk2 and cyclin A were down-regulated on day 3, and hardly detected on day 4 (Fig. 5). Although the predominant Cdk activity has been reported to be associated with cyclin A in BALB/c-3T3 cells (*22*), a smaller amount of kinase activity was recovered with anti-cyclin A antibody than with anti-Cdk2 antibody.

## DISCUSSION

Cyclin-dependent kinases and their regulators should play important roles in cell-cycle arrest as well as cell-cycle progression. Growth arrest caused by TGF-$\beta$ has been reported with respect to CKIs (*2, 7*), and CKIs were also considered to be involved in contact inhibition (*2, 3, 14*).

In the course of studying the cell-cycle progression with BALB/c-3T3 cells, we found that the mRNA levels of CKIs are regulated in the $G_0/G_1$, S transition state (Fig. 1). The mRNA level of p27 changed in the same way as those of p15 and p16, but to a lesser extent, whereas it was reported not to change when Mv1Lu cells were released from contact inhibition (*15*), and the expression of p27 has been reported to be regulated primarily through posttranscriptional mechanisms (*19-21*). Unlike other CKIs, the mRNA of p21 was decreased in the $G_0$ state, while it has been reported to be increased in contact-inhibited or serum-starved normal human fibroblasts (*14, 24*). These discrepancies may be due to the difference between the cells used, or the state of p53 or the retinoblastoma tumor suppressor gene.

We thereafter focused on the regulation of CKIs during contact inhibition. The increases in the mRNA levels of p15, p16, and p27 even in growing cells as the cell density increased suggest that cell-cell interaction, by itself, produces signals that cause these changes (Fig. 3, day 2). On the other hand, the marked decrease in the mRNA level of p21 in the density-arrested cells suggests that the expression of p21 is differentially regulated from the expression of other CKIs, and confluent cells could produce signals suppressing the p21 mRNA level that are distinct from those produced through mere cell-cell contact. The p21 gene was originally cloned as a p53-induced gene (*11*), as well as identified independently by other groups with different strategies (*12-14*). However, in our case, it is unlikely that the p53 protein regulates the mRNA level of p21 in density-arrested fibroblasts, but this should be examined in the future.

The protein levels of p16 and p27 increased as the cell density increased up to day 4 after splitting (Fig. 4), while their mRNAs showed slight decreases on day 4 (Fig. 3). This may be due to the fact that the protein levels of p16 and p27 are also regulated posttranscriptionally, as reported previously for p27 (*19-21*), this regulation being in addition to the transcriptional control. Since pRB was demonstrated to be dephosphorylated as the cell density increased (Fig. 4), the increase in the p16 protein, whose function was reported to be dependent on the expression of wild-type pRB (*31*), would play an important role in the growth arrest caused by contact inhibition in BALB/c-3T3 cells.

In contrast, the increase in cell density induced a reduction of the p21 protein (Fig. 4) as well as of the mRNA of p21 (Fig. 3). The paradoxical increase in the p21 protein in growth-stimulated cells was reported previously (reviewed in Ref. *5*), but its decrease in growth-arrested cells has not been reported. The reverse regulation of p21 expression may suggest a unique function of the p21 protein other than as a cell-cycle inhibitor, as described previously (*32, 33*).

The cyclin A and cyclin D1 proteins were significantly decreased on day 4 (Fig. 4), which would be responsible for the marked depression of Cdk2 activity on day 4 (Fig. 5). The amount of the p27 protein that bound to Cdk2 was increased on days 3 and 4 (Fig. 4), and this is also considered to be a cause of the reduced Cdk2 activity due to contact inhibition, as described previously in the case of growth factor deprivation (*25*), and this could play a critical role in the $G_1$ arrest almost attained on day 3 (Fig. 2B).

Here we reported that the expression of CKIs is controlled at the mRNA level during contact inhibition in BALB/c-3T3 cells, as well as through posttranscriptional mechanisms. At present, it is not clear which regulation is more critical for contact inhibition. The mechanism that regulates the mRNA expression of these genes is presumed to be transcriptional regulation, although we have not excluded the possibility that the stability of the mRNA may have changed. In addition, whether or not contact inhibition requires the combined induction of the p15, P16, and p27 proteins, along with a reduction of p21 expression, is an intriguing question.

The results of our experiments suggest that there are certain steps in the course of contact inhibition: the induction of the mRNAs of p15, p16, and p27, and the accumulation of these proteins; the reduction of p21 expression and the amount of the p21 protein; and finally, the marked inhibition of Cdk2 kinase activity. The signals that are

induced by cell-cell contact and initiate the mechanism of contact inhibition remain to be determined.

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