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Loss of Cell Adhesion to Substratum Up-Regulates $p21^{Cip1/WAF1}$ Expression in BALB/c 3T3 Fibroblasts

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Cell adhesion to substratum is essential for the transition of G1 to S phase in mouse BALB/c 3T3 fibroblast cell cycle. Loss of cell adhesion in late G1 phase caused blockage of the G1/S phase transition and repression of cyclin E-associated cyclin-dependent kinase-2 (CDK2) activity. A CDK2 inhibitor abundant in quiescent cells, $p27^{Kip1}$, was down-regulated by growth factors in serum, and this down-regulation was partially prevented by loss of cell adhesion. Another CDK2 inhibitor, $p21^{Cip1/WAF1}$, which was undetectable in quiescent cells, was markedly induced by loss of cell adhesion. In exponentially growing cells, loss of cell adhesion also induced $p21^{Cip1/WAF1}$ expression but did not affect the abundance of $p27^{Kip1}$. These results suggest that loss of cell adhesion to substratum up-regulates $p21^{Cip1/WAF1}$ expression, which plays an essential role for arresting the BALB/c 3T3 fibroblast cell cycle.

Both growth factors in serum and cell adhesion to an extracellular substratum are essential factors for proliferation of fibroblasts in culture. The requirement of cell adhesion for growth of non-transformed cells in culture has been known as an anchorage dependence for normal cell proliferation (1). Loss of anchorage dependence is a hallmark of cellular transformation and closely correlates with tumorigenicity (2). Several lines of evidence indicate that cell adhesion to an extracellular substratum is essential for the transition of G1 to S phase in fibroblast cell cycle (3-5). One of the most crucial events for the transition of G1 to S phase is the activation of cyclin E-associated CDK2 (6). Recently, p27 and p21 have been identified as inhibitors of cyclin E / CDK2, cyclin A / CDK2, and cyclin D / CDK4 (6). Several reports have been shown the involvement of p27 and p21 in the regulation of cell growth by cell adhesion, but this involvement is various among different cell lines. In mouse NIH 3T3 and human fibroblast cell lines, loss of cell adhesion abrogates cyclin E / CDK2 activities by elevating both p21 and p27 levels (7, 8), or by elevating p27 level (9). However, in NRK fibroblasts and some rat fibroblast cell lines, it is reported that p27 and p21 are not involved in regulation of G1/S phase control by cell adhesion (7, 10, 11). In mouse BALB/c 3T3 fibroblasts, p27 plays an essential role for restriction point control by growth factors in serum, but the role of p21 remains unclear (12, 13). We previously reported that cell adhesion is required for the G1/S phase transition in BALB/c 3T3 fibroblasts (5). In this paper, we report that cell adhesion critically down-regulates p21 expression to promote mouse BALB/c 3T3 fibroblast cell cycle.

MATERIALS AND METHODS

Materials. Poly(HEMA) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Autoradiographic emulsion type NR-M2 was from Konica Co. (Tokyo, Japan). Antibodies specific for CDK2, p27, and cyclin D1 were from Transduction Laboratories Co. (Lexington, KT). Antibodies specific for p21, cyclin E, and cyclin A, and Protein A/G PLUS-Agarose™ were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ECL™ Western blotting detection reagents were from Amersham Japan (Tokyo, Japan).

Cell culture. Mouse BALB/c 3T3 fibroblast cells were cultured with Dulbecco's MEM medium. Cells were synchronized to the quiescent state by serum starvation and quiescent cells were stimulated with 10% FCS by the procedures previously described (5). Cells were detached from the culture dishes by treatment with trypsin/EDTA solution and cultured on poly(HEMA)-coated dishes to prevent cell adhesion.

Measurement of DNA synthesis. Newly synthesized DNA in the cells was detected by labeling with [6-^{3}H]thymidine. Cells were cultured in medium containing [6-^{3}H]thymidine (1 μCi/ml, 0.66 Ci/mmol), fixed with pure methanol, and covered with autoradiographic emulsion NR-M2. After 2 days exposure, the emulsion was developed and labeled nuclei were counted with a microscope.

Immunoblot analysis. Total proteins were extracted from cultured cells with lysis buffer (25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 1% NP-40, 1 mM EDTA, 100 mM NaF, 200 μM $NaVO_3$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluo-

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Abbreviations: cyclin-dependent kinase, CDK; fetal calf serum, FCS; poly(2-hydroxyethyl methacrylate), poly(HEMA).

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ride). Equal amounts of extracted proteins were separated by SDS-polyacrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane. The membrane was incubated with TBST (25 mM Tris-HCl (pH 7.6), 125 mM NaCl, 0.1% Tween-20) containing 10% dried milk at 37°C for 1 h for blocking non-specific reaction. The membrane was incubated with the specific antibodies in TBST containing 5% dried milk (for detection of p21, TBST containing 1% dried milk was used) at room temperature for 2 h, followed by incubation with peroxidase-conjugated anti-mouse or anti-rabbit IgG in TBST containing 5% dried milk for 1 h. After washing the membrane, the bands reactive to the specific antibodies were detected on X-ray film with ECL™ Western blotting detection reagents.

Immunoprecipitation. Equal amounts of proteins extracted with lysis buffer were immunoprecipitated with anti-cyclin E antibodies followed by addition of Protein A/G PLUS-Agarose™. After washing with lysis buffer, the immunoprecipitates were used for measurement of cyclin E-associated histone H1 kinase activity.

Cyclin E-associated histone H1 kinase activity. Immunoprecipitates with anti-cyclin E antibodies were incubated in 20 ml of kinase assay solution (25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 10 mM $MgCl_2$, 5 μg histone H1, 20 μM ATP, 5 μCi [γ-^{32}P-ATP]) at 37°C for 1 h. Labeled histone H1 was separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

Northern blot analysis. Total RNAs were extracted by the acid guanidinium thiocyanate-phenol-chloroform method (14). Northern blot analysis was performed as previously described (5). A p21 probe (345 bps) was generated by an amplification of mouse p21 exon 2 by polymerase-chain-reaction method (15). A p53 probe (0.8 kbps) was prepared from cloned mouse p53 cDNA (16).

RESULTS

We previously reported that loss of cell adhesion blocked the G1/S phase transition (5). Here we confirmed this observation counting the number of nuclei labeled with [^{3}H]thymidine. When quiescent cells were stimulated with 10% FCS, S phase started at around 12 h after serum stimulation (Fig. 1A), as previously described (5). Over 90% of nuclei were labeled with [^{3}H]thymidine when quiescent cells were stimulated with 10% FCS (Fig. 1B, N). When cells were cultured without adhesion during late G1 and S phases (6-22 h after serum stimulation), only 10-15% nuclei were labeled (Fig. 1B, A(−)).

Since activation of cyclin E / CDK2 is a crucial event for the transition of G1 to S phase, we examined cyclin E-associated histone H1 kinase activity in the cells without adhesion. Cyclin E-associated histone H1 kinase was activated at around the G1/S boundary (10-14 h after serum stimulation) and the activation was abrogated by loss of cell adhesion (Fig. 1C). We confirmed that CDK2 proteins were co-immunoprecipitated with anti-cyclin E antibodies (data not shown).

To investigate how the cyclin E / CDK2 activity is abrogated by loss of cell adhesion, we examined the effect of loss of cell adhesion on CDK2-related protein abundance by immunoblot analysis. The abundance of CDK2 did not significantly change during G1 phase, and loss of cell adhesion did not significantly affect the abundance of CDK2 (Fig. 2A). Cyclin E increased at the G1/S boundary, but the abundance of cyclin E was

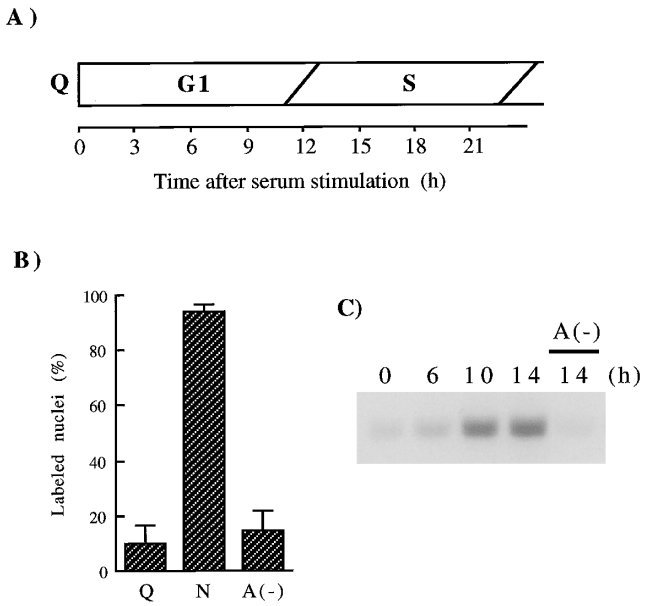

FIG. 1. DNA synthesis and cyclin E-associated histone H1 kinase activity are suppressed by loss of cell adhesion. A) A scheme for progression of BALB/c 3T3 fibroblast cell cycle when quiescent cells were stimulated with 10% FCS. B) DNA synthesis. Quiescent BALB/c 3T3 fibroblasts in monolayer culture were stimulated with 10% FCS. [^{3}H]Thymidine was added to the culture medium at 6 h after serum stimulation. Cells were fixed with methanol at 22 h after serum stimulation and labeled nuclei were counted with a microscope. Q, quiescent cells (not stimulated with serum); N, serum-stimulated cells with no further treatment; A(−), cells detached at 6 h after serum stimulation and cultured on poly(HEMA)-coated dishes. C) Cyclin E-associated histone H1 kinase activity. Quiescent cells were stimulated with 10% FCS. At each time point after stimulation, total cellular proteins were extracted with lysis buffer. Immunoprecipitates formed with anti-cyclin E antibodies were analyzed for cyclin E-associated histone H1 kinase activity as described in MATERIALS AND METHODS. A(−), cells detached at 6 h after serum stimulation and cultured on poly(HEMA)-coated dishes for more 8 h.

not significantly affected by loss of cell adhesion (Fig. 2A). A CDK2 inhibitor, p27, was abundant in quiescent cells and was down-regulated by serum. The down-regulation of p27 by serum was partially prevented by loss of cell adhesion (Fig. 2A and 2B). In contrast, another CDK2 inhibitor, p21, was not detected in quiescent cells, and loss of cell adhesion markedly increased p21 abundance (Fig. 2B). By loss of cell adhesion, the abundance of cyclin A was suppressed, but that of cyclin D1 was not affected (Fig. 2B). These results suggest that abrogation of cyclin E / CDK2 activity by loss of cell adhesion was mediated by increase of the CDK inhibitors, p27 and p21.

We further examined whether cell adhesion affects p21 and p27 expression not only in serum-stimulated cells but also in exponentially growing cells. Loss of cell adhesion remarkably increased p21 proteins and p21 mRNAs within 2 h after detachment in exponentially growing cells (Fig. 3, A(−)). The induction of p21

was reversed within 2 h after suspension cells were re-attached to ordinary culture dishes (Fig. 3, ReA). p21 was not induced by treatment with trypsin/EDTA solution if cells were not detached (Fig. 3, T). In contrast to p21, the abundance of p27 was not significantly affected by loss of cell adhesion, which indicates that p27 is not critically regulated by cell adhesion (Fig. 3A). Transcription of p21 gene is reported to be activated by tumor suppressor gene product, p53 (15). Loss of cell adhesion did not affect the mRNA level of p53 in this experiment (Fig. 3B). All results shown above suggest that loss of cell adhesion up-regulates p21 expression, which plays an essential role for arresting the BALB/c 3T3 fibroblast cell cycle.

DISCUSSION

It has been established that loss of cell adhesion suppresses cyclin A gene expression at the transcriptional level (9, 17, 18). Anchorage-dependent cyclin A gene transcription in NIH 3T3 cells is reported to be mediated by cyclin E / CDK2 and p27 (9). Here, we show

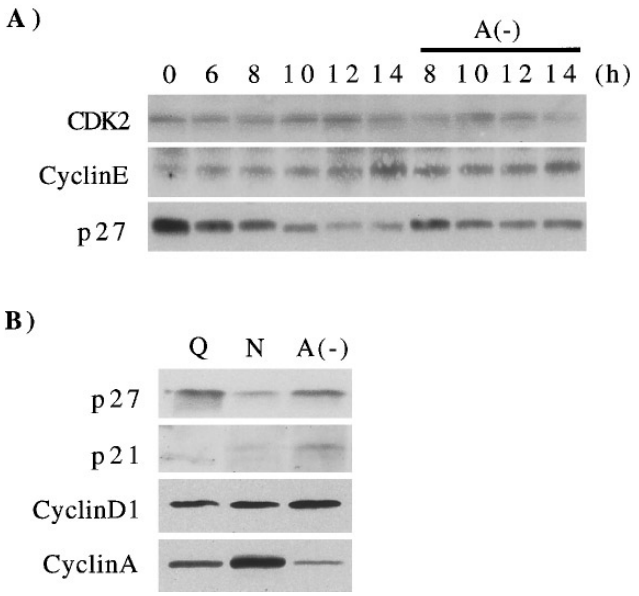

FIG. 2. Loss of cell adhesion partially prevents the down-regulation of p27 by growth factors in serum and induces p21 expression. A) Quiescent cells were stimulated with 10% FCS. At each time point, total cellular proteins were extracted with lysis buffer and analyzed by immunoblot analysis as described in MATERIALS AND METHODS. A(−), cells detached at 6 h after serum stimulation and cultured on poly(HEMA)-coated dishes for the rest of time period. B) Quiescent cells were stimulated with 10% FCS. Total proteins were extracted at 14 h (for p27 and p21) and at 18 h (for cyclin D1 and cyclin A) after serum stimulation and were analyzed by immunoblot analysis as described in MATERIALS AND METHODS. Q, quiescent cells (not stimulated with serum); N, serum-stimulated cells with no further treatment; A(−), cells detached at 6 h after serum stimulation and cultured on poly(HEMA)-coated dishes for the rest of time period.

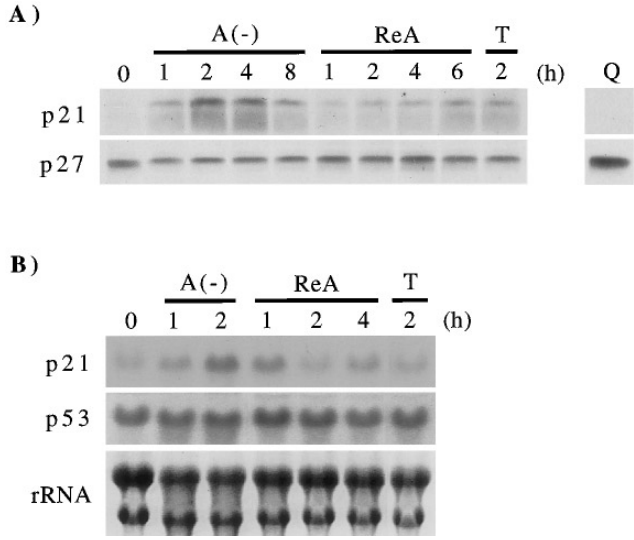

FIG. 3. Loss of cell adhesion induces p21 expression but not p27 in exponentially growing BALB/c 3T3 fibroblasts. A) Immunoblot analysis. A(−), exponentially growing cells detached from culture dishes by treatment with trypsin/EDTA solution and then cultured on poly(HEMA)-coated dishes; ReA, cells cultured without adhesion for 2 h and re-attached to ordinary culture dishes (cells were cultured in monolayer). T, cells treated with trypsin/EDTA solution for short time (cells were not detached); Q, quiescent cells. At each time point after treatments, total cellular proteins were extracted and analyzed by immunoblot analysis as described in MATERIALS AND METHODS. B) Northern blot analysis. Exponentially growing cells were treated by the same procedures described in (A). Total RNAs were extracted at each time point after treatments and analyzed by Northern blot analysis as described in MATERIALS AND METHODS.

that expression of cyclin A and cyclin E / CDK2 activity in BALB/c 3T3 cells are suppressed by loss of cell adhesion and that loss of cell adhesion up-regulates p21 expression in serum-stimulated cells and in exponentially growing cells. However, in exponentially growing cells, loss of cell adhesion does not affect the abundance of p27. p27 is reported to be an essential component of the pathway that connects mitogenic signals by growth factors to the cell cycle in BALB/c 3T3 cells (12, 13). These findings suggest that growth factors critically down-regulate p27 and cell adhesion critically down-regulates p21 to proceed BALB/c 3T3 fibroblast cell cycle. In contrast to these results, several reports show that cell adhesion does not affect cyclin E / CDK2 activity nor p27 and p21 abundance in some rat fibroblast cell lines (7, 10, 11). It is possible that cell adhesion regulates cell cycle through the other pathways that do not include activation of CDK2 or CDK2 inhibitors, p21 and p27. Further study is needed to understand the precise regulatory mechanisms of cell adhesion for growth regulation.

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