Rac1-dependent transcriptional up-regulation of p27Kip1 by homophilic cell–cell contact in vascular endothelial cells

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Abstract

The mechanism for the transcriptional up-regulation of p27Kip1 due to the formation of the cell–cell contact was investigated in vascular endothelial cells. The induction of the cell–cell contact by adding an extra number of endothelial cells activated Rac1, up-regulated p27Kip1 mRNA and protein, and also facilitated the cell cycle arrest. Transduction of the Rac1 inhibitor protein using the cell-penetrating peptide or treatment with a Rac1 inhibitor NSC23766 inhibited the p27Kip1 up-regulation and delayed the cell cycle arrest. Rac1 was therefore suggested to mediate the contact-induced transcriptional up-regulation of p27Kip1. The role of Rac1 in the regulation of the p27Kip1 promoter activity was next examined with a luciferase reporter assay. The promoter activity was increased by inducing the cell–cell contact, which was significantly inhibited by the Rac1 inhibitory protein and NSC23766. The evaluation of various truncated promoter regions determined region −620 to −573 nucleotides from the initiation codon to be responsible for the contact-induced, Rac1-dependent activation of the p27Kip1 promoter. The present study thus demonstrated for the first time that the activation of Rac1 due to the cell–cell contact plays a critical role in the transcriptional up-regulation of p27Kip1 in vascular endothelial cells.

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1. Introduction

The growth of vascular endothelial cell is known to be regulated by homophilic cell–cell contact both in situ and in culture [1], thus contributing to the formation of a monolayer lining of endothelial cells, angiogenesis and the functional differentiation in vascular tissues [2]. A disruption of the cell–cell contact re-initiates the endothelial growth, which may be related to the endothelial dysfunction seen in such vascular diseases as atherosclerosis. However, the mechanism of the contact-induced growth arrest still remains to be elucidated.

A cyclin-dependent kinase inhibitor p27Kip1 has been shown to play a crucial role in the contact-induced growth arrest [3–6]. The expression of p27Kip1 is regulated by both transcriptional and post-transcriptional mechanisms. The ubiquitin-proteasomal or other proteolytic mechanisms plays a major role in the rapid degradation and inactivation of p27Kip1 seen before the quiescent cells return to the cell cycle and progress to the S phase [7–10]. The transcriptional repression also contributes to the down-regulation of p27Kip1 prior to the platelet-derived growth factor-induced progression to the S phase in smooth muscle [11]. On the other hand, the mechanism of the up-regulation of p27Kip1 during the contact-induced growth arrest still remains to be elucidated. We have previously demonstrated the transcriptional up-regulation and the stabilization of mRNA to be associated with an increase in the expression of p27Kip1 during the contact-induced growth arrest in vascular endothelial cells [4]. However, the precise mechanisms of the transcriptional up-regulation of p27Kip1 by cell–cell contact still remain to be elucidated.

The genomic structure of the p27Kip1 gene has been partially determined in humans and mice [12–15]. As a result, at least three exons have been suggested to exist. The coding region of p27Kip1 resides on exons 1 and 2, while exon 3 contains only an untranslated region [12]. The p27Kip1 gene is TATA-less, and the region required for basal promoter activity has been determined to
be highly conserved between humans and mice [13,14]. Vitamin D, interferon-β and triiodothyronine have been shown to activate the transcription of p27^Kip1 [16–20]. Synergistic action of Sp1 and NF-Y has been reported to play an important role in the vitamin D-induced transcriptional activation [20]. However, the intracellular signal transduction and the cis- and trans-acting elements involved in the contact-induced transcriptional up-regulation of p27^Kip1 still remain to be elucidated.

The formation of the cell–cell contact has been shown to activate Rho family proteins [21]. The type of Rho protein that is activated due to the cell–cell contact varies with the type of cell and the type of adhesion molecule involved [21]. The engagement of VE-cadherin or nectin has been shown to be associated with the activation of Rac1 but not RhoA [22,23]. The activation of Rho family proteins is considered to regulate cell adhesion complex, cytoskeletal organization, cell polarity and gene expression [21]. A role of Rac1 in the contact-induced up-regulation of the p27^Kip1 transcription thus remains to be investigated.

In the present study, we investigated the role of Rac1 in the transcriptional up-regulation of p27^Kip1 due to the homophilic cell–cell contact in vascular endothelial cells. The involvement of Rac1 was examined by introducing a Rac1 inhibitory protein into the intact endothelial cells. We herein demonstrated, for the first time, that the Rac1 mediates the cell–cell contact-induced activation of the p27^Kip1 promoter.

2. Materials and methods

2.1. Cell cultures

The primary cultures of bovine aortic endothelial cells (BAECs) and porcine aortic endothelial cells (PAECs) were obtained as previously reported [4,24]. BAECs, PAECs and HeLa cells were cultured and maintained in Dulbecco’s modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) containing 10% (v/v) fetal bovine serum and antibiotics.

2.2. Protocols to study the effect of the formation of the cell–cell contact on the Rac1 activity, the S phase progression, the level of p27Kip1 mRNA and protein, and p27^Kip1 promoter activity

The experimental protocols are summarized in Fig. 1. The cells at confluence were harvested by trypsinization and then were replated at a confluent density of 25% (4 × 10^5 cells/60-mm dish). When the promoter activity was studied, the cells were transfected 24 h later with 1 μg pGL3-reporter plasmid and 0.5 μg phRL-TK in 2.5 ml DMEM containing 10 μg lipofectamine (Invitrogen, Carlsbad, CA, USA) and 2 μg lipofectamine PLUS™ reagent (Invitrogen). After the 4-h transfection, the cells were washed in PBS twice, and then the cell culture was resumed in DMEM containing 10% (v/v) serum and antibiotics, either with or without the addition of the extra number (8 × 10^5 cells/60-mm dish) of endothelial cells or HeLa cells. When the effects of a Rac1 inhibitor protein TATHA-PBD or a Rac1 inhibitor NSC23766 were examined, it was added 2 h after adding the extra cells. When studying the Rac1 activity, the S phase progression, and the p27Kip1 expression, the transfection step was omitted.

Fig. 1. The experimental protocols to induce the formation of the cell–cell contact. BAECs were plated at 4 × 10^5 cells/60-mm dish (25% of the confluent density) at time 0. The cells were transfected with reporter plasmids 24 h later. After 4-h transfection step, indicated by shaded boxes, the cell culture was resumed either with or without adding the extra number of BAECs or HeLa cells (8 × 10^5 cells/60-mm dish), and with or without TATHA-PBD. TATHA-PBD was added to the culture 2 h after adding the extra number of cells. The transfection step was omitted in the experiments without the promoter assay. The formation of cell–cell contact was evaluated by immunofluorescence staining of β-catenin at the time point indicated by arrows in each protocol (a–f). The images obtained 24 h after plating BEACs (g) and HeLa cells (h) at 8 × 10^5 cells/60-mm dish are shown for comparison purposes. Scale bar=25 μm.
2.3. Analysis of cell cycle progression with propidium iodide staining

The cells were harvested by trypsinization and fixed in 50% (v/v) methanol at –20 °C for more than 20 min. The cells were then stained with 50 μg/ml propidium iodide (Sigma) in PBS containing 50 μg/ml RNase A (Sigma) at room temperature for 20 min, as previously described [4]. A total of 20,000 cells were analyzed with a flow cytometer FACSCalibur (Becton Dickinson, San Jose, CA, USA). ModFit LT ver 2.0 software program (Verity Software House, Topsham, Maine, USA) was used to determine the fraction of the S phase of the cell cycle.

2.4. Analysis of S phase entry by 5-bromo-2′-deoxy-uridine incorporation

The S phase entry of the cells was examined by the nuclear incorporation of 5-bromo-2′-deoxy-uridine (BrdU) as previously described [4]. In brief, the cells were fixed with 10 mM BrdU for 1 h, and then fixed in 70% (v/v) ethanol, 50 mM glycine (pH 2.0) at –20 °C. The incorporated BrdU was detected using a BrdU labeling and detection kit (Roche Diagnostics, Tokyo, Japan). The fraction of BrdU-positive cells was determined by counting >500 cells in one experiment, as previously described [4].

2.5. Immunofluorescence staining of β-catenin

Immunofluorescence staining of β-catenin in BAECs was performed as previously described [4]. In brief, the cells were fixed with ethanol at –20 °C for 20 min, and then blocked with 5% (w/v) bovine serum albumin in PBS at room temperature for 60 min. β-Catenin was detected by the mouse monoclonal antibody 15B8 (Sigma), followed by a Texas red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The fluorescence images were obtained under a fluorescence microscope (Leica DMRXA; Leica, Wetzlar, Germany) with a 63× objective lens and a CCD camera ProgRes C14 (Jenoptik, Jena, Germany), and saved as TIFF files.

2.6. Preparation of recombinant proteins

A fragment containing the Rac1/Cdc42-binding domain (PBD; residues 67–150) of human p21-activated protein kinase-1 and a fragment containing the RhoA-binding domain (RB; residues 941–1075) of Rho kinase were expressed as recombinant proteins containing a hexahistidine tag, either with (TATHA-RB) or without ((His)6-PBD, (His)6-RB) a cell-penetrating peptide of the human immunodeficiency viral Tat protein and a hemagglutinin tag at their N-terminus, as previously described [24–27]. The recombinant proteins were expressed in Escherichia coli JM109, and then affinity-purified through Ni²⁺-loaded Hi-Trap chelating column, as previously described [25]. The recombinant proteins were eluted in 20 mM Tris–HCl, 500 mM imidazole, 500 mM NaCl, 1 mM β-mercaptoethanol, pH 8.0 and directly used in further analyses. The nucleotide sequence data of the full-length porcine p27Kip1 gene with a various length was obtained by PCR amplification with a genomic clone in pEGFP-N1 as a template. PCR products were then subcloned into a pGL3-basic vector (Promega, Madison, WI, USA). The reporter plasmids thus obtained were pGL3-Kip1 (–686 to –19), pGL3-Kip1 (–648 to –19), pGL3-Kip1 (–620 to –19), pGL3-Kip1 (–573 to –19), pGL3-Kip1 (–501 to –19), pGL3-Kip1 (–335 to –19), pGL3-Kip1 (–164 to –19), pGL3-Kip1 (–130 to –19) and pGL3-Kip1 (–106 to –19). The number in parentheses indicates the residue number at the 5′- and 3′-end of the promoter region, while the first base of the translation initiation codon was assigned to be +1 (Fig. 8A). The overlapping extension PCR [29] was used to construct pGL3-Kip1

2.7. Isolation of genomic clones for porcine p27Kip1 genes

A plaque hybridization technique and the probes labeled with [α-32P]-dCTP were utilized to screen for the p27Kip1 genes in a porcine liver genomic library (Stratagene, La Jolla, CA, USA). A 340-bp PCR product corresponding to region 147–486 nt of the p27Kip1 cDNA clone (accession number: AB031955, [28]) or a 682-bp product corresponding to region 473–648 nt of the p27Kip1 cDNA clone (accession number: AB031958, [28]) was used as screening probes. The phage DNA (~40 μg) was isolated from the plate lysate (~100 ml) of the positive clones obtained after repeated hybridization. The library inserts were excised out by digestion with NotI and then were ligated to the NotI-digested pEGFP-N1 (Clontech, Palo Alto, CA, USA), for sequencing and further analyses. The nucleotide sequence data of the full-length porcine p27Kip1 gene have been deposited into the GeneBank/EMBL/DDBJ databases under the accession number of AB083336.

2.8. Construction of the luciferase reporter plasmids for the porcine p27Kip1 promoter assay

The promoter region of the porcine p27Kip1 gene with a various length was obtained by PCR amplification with a genomic clone in pEGFP-N1 as a template. PCR products were then subcloned into a pGL3-basic vector (Promega, Madison, WI, USA). The reporter plasmids thus obtained were pGL3-Kip1 (–686 to –19), pGL3-Kip1 (–648 to –19), pGL3-Kip1 (–620 to –19), pGL3-Kip1 (–573 to –19), pGL3-Kip1 (–501 to –19), pGL3-Kip1 (–335 to –19), pGL3-Kip1 (–164 to –19), pGL3-Kip1 (–130 to –19) and pGL3-Kip1 (–106 to –19). The number in parentheses indicates the residue number at the 5′- and 3′-end of the promoter region, while the first base of the translation initiation codon was assigned to be +1 (Fig. 8A). The overlapping extension PCR [29] was used to construct pGL3-Kip1

Fig. 2. Effect of the induction of cell–cell contact on the cell cycle progression in PAECs. (A) Representative time courses of the changes in the S phase fraction after plating PAECs at 4 × 10⁶ cells/60-mm dish (open circles). Closed circles, the S phase progression following the addition of the extra number of cells (8 × 10⁶ cells/60-mm dish) at 24 h after the initial plating at 4 × 10⁶ cells/60-mm dish. Closed triangles, the cell cycle progression when the same number of cells (8 × 10⁶ cells/60-mm dish) was plated in the absence of the pre-seeded cells. Data obtained with the triplicate measurement in one series of experiment are shown. (B) Comparison of the fraction of the S phase at the time points indicated after plating PAECs at 4 × 10⁶ cells/60-mm dish in the presence of cell–cell contact (solid bars) and in its absence (open bars). The number in parentheses indicates the residue number at the 5′- and 3′-end of the promoter region, while the first base of the translation initiation codon was assigned to be +1 (Fig. 8A). The overlapping extension PCR [29] was used to construct pGL3-Kip1

* P < 0.05.
Representative photos and the summary of the immunoblot detection of the total and GTP-bound forms of RhoA and Rac1 48 h after the initial plating of PAECs, with (EC+EC) and without (EC) the addition of the extra number of cells. After the immunodetection, actin and the pull-down probes were visualized by naphtol blue black staining. To normalize the possible loading variation, the level of total RhoA or Rac1 was normalized by the level of actin (total RhoA or Rac1/actin), and the level of assigned a value of 1. The data are the mean±S.E.M. (normalized by the level of total RhoA or Rac1 (GTP-RhoA or Rac1/total RhoA or Rac1). The value obtained without adding the extra number of cells (EC) was GTP-bound forms of RhoA and Rac1 was normalized by the level of the pull-down probe (GTP-RhoA or Rac1/Probe). The level of GTP-RhoA or Rac1 was further normalized by the level of total RhoA or Rac1 (GTP-RhoA or Rac1/total RhoA or Rac1). The value obtained without adding the extra number of cells (EC) was assigned a value of 1. The data are the mean±S.E.M. (n=5). *P<0.05 vs. EC.

2.9. Luciferase assay

The promoter activity was evaluated 48 h after the initial plating, i.e., 20 h after terminating the transfection of the reporter plasmid (Fig. 1). The cells were washed in PBS twice, and then they were lysed in the passive lysis buffer (Promega). The lysates were kept frozen at −80 °C until use. After thawing and clarification of the lysates by a brief centrifugation on a tabletop microcentrifuge (13,000×g, 30 s, room temperature), the luciferase activity was determined using dual-luciferase reporter assay system (Promega) and a luminometer Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany). The activity of firefly luciferase was normalized by that of Renilla luciferase, and the value obtained with pGL3-Kip1 (−686 to −19) without the addition of the extra cells was then assigned a value of 1 (Fig. 5A).

2.10. Pull-down assay for the GTP-bound forms of Rac1 and RhoA

The cell extract (500 μg protein) was prepared in the lysis buffer (50 mM Tris–HCl, pH 7.2, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 500 mM NaCl, 10 mM MgCl2, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μM 4-aminidophenylmethane sulfonyl fluoride, pH 7.2), and the bound protein was directly eluted in 50 μl SDS-sample buffer (50 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.01% (w/v) NaN3, 0.01% (w/v) bromophenol blue) by heating at 100 °C for 5 min. The equal volume (20 μl) of the resin eluates was subjected to SDS-PAGE on 7.5–20% (w/v) gradient polyacrylamide gel and immunoblot analysis using anti-Rac1 (No.610650; BD Bioscience, San Jose, CA, USA) and anti-RhoA (sc-418; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. The total lysates were also subjected to immunoblot analysis for the expression of RhoA, Rac1 and Cdc42 (anti-Cdc42 antibody; sc-87, Santa Cruz Biotechnology). The immune complex was detected with an enhanced chemiluminescence technique (ECL plus kit, Amersham, Buckinghamshire, UK). The light emission was detected and analyzed with ChemiDoc XRS-J and Quantity One software (BioRad, Tokyo, Japan). After immunoblot detection, the polyvinylidene difluoride membranes were stained with naphthol blue black to detect the bands corresponding to actin in the total lysate and the pull-down probes in the bound fraction. The density of RhoA and Rac1 in the total lysate (total RhoA and Rac1) was normalized by the density of actin, while that seen in the bound fraction (GTP-bound forms) was normalized by the density of the pull-down probes and the density of total RhoA or Rac1 (Fig. 3).

2.11. RT-PCR analysis of p27kip1 mRNA expression

The total RNA was isolated and then subjected to the RT reaction for p27kip1 and β-actin as previously described [4]. Thereafter, the RT products were subjected to either real-time PCR or regular PCR followed by agarose gel electrophoresis. The purified DNA of the pEGFP-Kip1 plasmid [29] and the RT-PCR product of β-actin obtained with the total RNA of PAECs were used as a standard template for p27kip1 and actin, respectively, to evaluate the linearity of the analysis. The real-time PCR was performed in a 20-μl reaction mixture with a LightCycler (Roche Diagnostics, Mannheim, Germany). The PCR reaction for both p27kip1 and β-actin was composed of the initial 1-min denaturation at 95 °C, followed by the subsequent amplification step consisting of 15-s denaturation at 95 °C, 10-s annealing at 55 °C and 20-s extension at 72 °C. The linear relationship between the

### Fig. 3. Activation of Rac1 due to the formation of the cell–cell contact in PAECs. (A) Representative photos of three separate immunoblot detection of RhoA, Rac1 and Cdc42 in the cell lysates (10 μg proteins) of B/ACEs and PAECs at confluence. The chemiluminescence images were obtained with the same exposure time (5 min). (B) Representative photos and the summary of the immunoblot detection of the total and GTP-bound forms of RhoA and Rac1 48 h after the initial plating of PAECs, with (EC+EC) and without (EC) the addition of the extra number of cells. After the immunodetection, actin and the pull-down probes were visualized by naphthol blue black staining. To normalize the possible loading variation, the level of total RhoA or Rac1 was normalized by the level of actin (total RhoA or Rac1/actin), and the level of GTP-bound forms of RhoA and Rac1 was normalized by the level of total lysate (GTP-RhoA or Rac1/total RhoA or Rac1). The value obtained without adding the extra number of cells (EC) was assigned a value of 1. The data are the mean±S.E.M. (n=5). *P<0.05 vs. EC.
logarithms of the amount of the input standard template and the crossing cycle number was obtained with a range of 1 pg to 10 ng for p27Kip1, and 1 fg to 1 pg for β-actin (data not shown). However, the RT products of the cell samples did not give the crossing cycle number within the linear range for p27Kip1 (data not shown).

We thus conducted a PCR analysis as previously described with minor modifications [4]. The PCR reaction for p27Kip1 was composed of the initial denaturation at 94 °C for 2 min and the subsequent 30-cycle amplification step consisting of 1-min denaturation at 94 °C, 1-min annealing at 52 °C and 1-min extension at 72 °C. The PCR reaction for β-actin was composed of the initial denaturation at 94 °C for 2 min and the subsequent 25-cycle amplification step consisting of 1-min denaturation at 94 °C, 1-min annealing at 55 °C and 1-min extension at 72 °C. The PCR products were separated on 3% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide. The densities of the bands of the fluorescent images obtained with a gel documentation system equipped with a CCD camera (Printograph; Atto, Tokyo, Japan) were analyzed using the Quantity One software program (Bio-Rad). The linear relationship between the amount of the input standard template and the density of the band was obtained with a range of 0 to 1.0 pg for p27Kip1 and 0 to 100 fg for actin (data not shown). The density of the PCR products obtained with the cell samples was within this linear range for both p27Kip1 and β-actin. The levels of p27Kip1 were then normalized by the level of β-actin obtained from the corresponding RT product, and the value obtained without addition of the extra number of endothelial cells and TATHA-PBD was assigned a value of 1.

2.12. Immunoblot analysis of p27Kip1 protein expression

The level of p27Kip1 protein was analyzed by an immunoblot analysis as previously described [4]. The cell homogenate was prepared in the same lysis buffer as that used for the pull-down assay, and then the equal amount of protein was subjected to SDS-PAGE on 15% polyacrylamide gel and the following immunoblot detection using anti-p27Kip1 antibody (No. 610242; BD Bioscience) and the horseradish peroxidase-conjugated secondary antibody (Sigma). The immune complex was detected and analyzed as described above. After the immunodetection, the membrane was stained with naphthol blue black to visualize the band of actin. The density of p27Kip1 as detected by immunoblot was normalized by the density of actin, and then the value obtained under control condition was assigned a value of 1.

2.13. Other materials

NSC23766, a Rac inhibitor [31], was purchased from Calbiochem (San Diego, CA, USA), and dissolved at 10 mM in distilled H2O.
2.14. Statistical analysis

The data are the means±S.E.M. of the indicated number of independent experiments. The analysis of variance (ANOVA) evaluated any statistical significance. A value of \( P<0.05 \) was considered to be significantly different.

3. Results

3.1. Effect of the formation of the cell–cell contact on the cell cycle progression in PAECs

The formation of the cell–cell contact was induced according to a protocol shown in Fig. 1, while the transfection step was omitted when evaluating the cell cycle progression. After plating PAECs at \( 4 \times 10^5 \) cells/60-mm dish (\(~25\%\) of the confluent density), the cell cycle reached the first peak of the S phase at 24 h, and the lower second peak at 36 h. Thereafter, the fraction of the S phase gradually decreased (Fig. 2A, lower panel). When plated at \( 8 \times 10^5 \) cells/60-mm dish (\(~50\%\) of the confluent density), the cell cycle phase reached the peak of the S phase 24 h after the plating, and then the fraction of the S phase rapidly decreased (Fig. 2A, upper panel). These observations were consistent with the findings of our previous report [4]. When \( 8 \times 10^5 \) cells/60-mm dish were added 24 h after the initial plating at \( 4 \times 10^5 \) cells/60-mm dish, the subsequent cell cycle progression to the S phase was substantially suppressed (Fig. 2A, lower panel). The fraction of the S phase seen 24 h after the addition of the cells was much lower than that seen 24 h after plating at \( 8 \times 10^5 \) cells/60-mm dish, while it was also significantly lower than that seen in the mixture of the time-matched samples obtained 48 h after the plating at \( 4 \times 10^5 \) cells/60-mm dish and those obtained 24 h after the plating at \( 8 \times 10^5 \) cells/60-mm dish (Fig. 2B).

3.2. Activation of Rac1 and up-regulation of p27Kip1 by the formation of the cell–cell contact in PAECs

RhoA and Rac1 are readily detected in PAECs and BAECs, while Cdc42 was scarcely detected (Fig. 3A). We thus examined the effect of the formation of the cell–cell contact on the activity of Rac1 and RhoA proteins in PAECs. Because of the difference in the cell number due to the experimental protocol, the same

![Graph A](image1)

![Graph B](image2)

Fig. 5. Activation of the p27\(^{Kip1}\) promoter activity by the homophilic cell–cell contact in BAECs. (A) The p27\(^{Kip1}\) promoter activities shown as a function of the initial plating density (\(16 \times 10^5\) cells/60-mm dish corresponded to the confluent density). The promoter activity was expressed as the relative values, while assigning the value obtained with a plating density of \(4 \times 10^5\) cells/60 mm to be 1. The data are the mean±S.E.M. (n=3). *\( P<0.05 \) vs. the value obtained with a plating density of \(4 \times 10^5\) cells/60-mm dish. (B) The activity of the various promoter regions without (EC) and with the addition of the extra number of BAECs (EC+EC) or HeLa cells (EC+HeLa). The promoter activity was expressed as the relative values, while assigning the value obtained with region \(-686\) to \(-19\) and without addition of the extra number of cells to be 1. The data are the mean±S.E.M. of the indicated number of experiments. †\( P<0.05 \); *\( P<0.05 \) vs. the values obtained without adding the extra number of cells for each construct. The reporter constructs are schematically shown; gray boxes, the p27\(^{Kip1}\) promoter region with the residue numbers at \(5'\)- and \(3'\)-end; boxes with LUC, a coding sequence of the firefly luciferase.
The amount of the cell extract (500 μg protein) was subjected to the assay. The addition of the extra number of cells had no effect on the total amount of RhoA or Rac1. However, it increased the amount of the GTP-bound form of Rac1 approximately 2-fold of that seen without addition of the cells (Fig. 3B), while it had no significant effect on the amount of the GTP-bound form of RhoA (Fig. 3B). The addition of the extra number of cells also up-regulated the level of p27Kip1 mRNA, and this up-regulation of p27Kip1 was abolished by the transduction of 100 nM TATHA-PBD (Fig. 4A). The level of p27Kip1 protein was also up-regulated by the addition of the extra number of cells, and this up-regulation was inhibited by either TATHA-PBD or NSC23766 (Fig. 4B). The functional significance of the Rac1-dependent p27Kip1 up-regulation was supported by the observation that TATHA-PBD significantly increased the S phase fraction seen 24 h after adding the extra number of PAECs, as evaluated by the BrdU incorporation (Fig. 4C).

The results shown above suggested that the formation of the cell–cell contact activated Rac1, thereby up-regulating the p27Kip1 mRNA and protein, and also facilitating the cell cycle arrest. In the following studies, the role of Rac1 in the regulation of the p27Kip1 promoter activity was investigated.

3.3. Density-dependent activation of p27Kip1 promoter in BAECs

The effect of the plating density on the p27Kip1 promoter activity was first investigated. Since the pilot experiments showed the transfection efficiency of PAECs to be too low to quantitatively investigate the promoter activity, BAECs were thus used to evaluate the activity of the p27Kip1 promoter. As shown in Fig. 5A, the promoter activity of region −686 to −19 increased depending on the plating density, thus reaching a maximum at the density of 12 × 10⁵ cells/60-mm dish, which corresponded to approximately 75% of the confluence density. Lowering plating density below 4 × 10⁵ cells/60-mm dish caused no significant decrease in the promoter activity. The activity obtained with the plating density of 4 × 10⁵ cells/60-mm dish was thus used as the basal activity and it was assigned a value of 1.

3.4. Activation of the p27Kip1 promoter by induction of the homophilic cell–cell contact

We further investigate the cell–cell contact-induced activation of the p27Kip1 promoter, according to the protocol shown in Fig. 1. The addition of BAECs increased the promoter activity of region −686 to −19 approximately 2-fold compared to that seen...
without addition of the cells (Fig. 5B, EC vs. EC+EC). The addition of HeLa cells significantly increased the promoter activity of the region \(-686\) to \(-19\), but to the much lesser extent than that seen with BAECs (Fig. 5B, EC+HeLa). The addition of BAECs did induce the formation of the cell–cell contact to the higher level than that seen without addition of the extra BAECs (Fig. 1a, c). HeLa cells do not express \(\beta\)-catenin to the appreciable level (Fig. 1h). The addition of HeLa cells to BAECs did not induce formation of the dense cell–cell contact (Fig. 1e).

The basal activity seen without addition of the extra number of cells gradually decreased as the promoter regions were shortened (Fig. 5B). The promoter activity of the regions \(-501\) to \(-19\) and \(-335\) to \(-19\) significantly increased in response to the addition of BAECs by approximately 1.7- and 1.6-fold, respectively, while
the addition of HeLa cells had no significant effect (Fig. 5B). A further deletion of −335 to −165 abolished the responsiveness to the addition of BAECs (Fig. 5B).

3.5. Inhibition of the Rac1 signaling inhibited the contact-induced activation of the p27Kip1 promoter

TATHA-PBD concentration dependently inhibited the activation of the promoter activity of region −686 to −19 induced by the addition of the extra number of BAECs (Fig. 6). The basal activity that was seen without the addition of the cells was also slightly, but significantly, inhibited by TATHA-PBD (Fig. 6). The maximal inhibitory effect on both basal and activated promoter activity was observed with 30–100 nM TATHA-PBD. Such effective concentrations are thus consistent with those seen for the inhibitory effect on the cell cycle progression in our previous report [24]. The addition of the cells in the presence of 100 nM TATHA-PBD increased the promoter activity to the level similar to that seen with the addition of HeLa cells (Fig. 5 vs. Fig. 6). In contrast, the control recombinant protein, (His)6-PBD, which lacks a cell-penetrating peptide, had no significant effect on both basal and activated promoter activity (Fig. 6). The addition of TATHA-PBD had no apparent effect on the immunofluorescence staining of β-catenin (Fig. 1a vs. b; c vs. d; e vs. f). The contact-induced activation of the p27Kip1 promoter activity was also inhibited by a Rac1 inhibitor, NSC23766 in a concentration-dependent manner (Fig. 7). An inhibition similar to that seen with 100 nM TATHA-PBD was obtained with 50 μM NSC23766.

3.6. The promoter region responsible for the contact-induced, Rac1-dependent transcriptional activation of p27Kip1 gene

All promoter regions −686 to −19, −501 to −19 and −335 to −19 increased their activity in response to the formation of the cell–cell contact (Figs. 5 and 6). However, the inhibitory effect of TATHA-PBD on the promoter activity was observed only with region −686 to −19 (Fig. 5B). We thus further investigated region −686 to −501 for the sequences responsive to Rac1 (Fig. 5B). All regions investigated increased their promoter activity in response to the formation of the cell–cell contact (Fig. 8B). However, TATHA-PBD significantly inhibited the contact-induced activation of the promoter activity seen with regions −686 to −19, −648 to −19 and −620 to −19 (Fig. 8B). A deletion of region −620 to −574 (regions −573 to −19) abolished such an inhibitory effect of TATHA-PBD. TATHA-PBD also significantly inhibited the promoter activity of pGL3-Kip1 (−686 to −19:TT), which contained mutations in the binding sites for Sp1 [15] and MZF1 [30] (Fig. 8B).

4. Discussion

We previously reported that the formation of the cell–cell contact induced a transcriptional up-regulation of p27Kip1 mRNA and protein, and also facilitated the cell cycle arrest, all of which were inhibited by inhibiting the Rac1 activity. A pull-down assay did reveal an increase in the activity of Rac1 but not RhoA upon formation of the homophilic cell–cell contact. Collectively, our observations suggest that the formation of the homophilic cell–cell contact activated Rac1, which in turn induced the transcriptional up-regulation of p27Kip1, thereby increasing the p27Kip1 expression and facilitating the contact-induced growth arrest. Our previous report demonstrated a temporal correlation between the p27Kip1 up-regulation and the cell cycle arrest [4]. The observations of the present study therefore suggest the existence of a functional link between the p27Kip1 up-regulation and the cell cycle arrest. Rac1 has been reported to be involved in the angiotensin II-induced up-regulation of p27Kip1 [32]. The present study thus provides the first evidence for the involvement of Rac1 activation in the contact-induced transcriptional up-regulation of the p27Kip1 gene.

It is noteworthy that the addition of HeLa cells slightly, but significantly, increased the promoter activity. However, such an activation of the HeLa cells was much lower than that seen with the endothelial cells. These observations suggest that some mechanisms other than the homophilic cell–cell contact may also contribute to the activation of the p27Kip1 promoter. Such additional mechanisms may include non-homophilic but still specific cell adhesion mechanisms or some non-specific mechanism caused by cell crowding [33]. It should also be noted that the inhibition of Rac1 did not completely inhibit the activation of the p27Kip1 promoter by the formation of the homophilic cell–cell contact. This observation suggests the involvement of some pathways other than Rac1 in the cell–cell contact-induced activation of the p27Kip1 promoter. However, the promoter activity obtained with TATHA-PBD was similar to that obtained with HeLa cells. These observations thus suggest that Rac1 may be specifically involved in the activation of the p27Kip1 promoter due to the homophilic cell–cell contact.

An analysis of variously truncated promoter regions of the p27Kip1 gene revealed two functional regions: regions −335 to −165 and −620 to −574. The deletion of region −335 to −165 caused a loss of responsiveness to the formation of the cell–cell contact. However, this region is considered to be included in the transcript of p27Kip1 as a 5′-untranslated region, according to the transcription initiation sites determined in humans and mice [13,15,34]. Therefore, this region could be involved in the post-transcriptional regulation of p27Kip1 in response to the formation of the cell–cell contact. However, it is also possible that the effect of the deletion of this region on the luciferase activity may not be specific. Both the basal activity and the responsiveness to the formation of the cell–cell contact decreased as the promoter region was shortened (Fig. 5B). Some degree of responsiveness may be linked to the basal activity. The deletion of region −335 to −165 might have decreased the basal activity to the critically lower level to support the responsiveness to the formation of the cell–cell contact.

On the other hand, region −620 to −574 is suggested to contain sequences which are responsible for the Rac1-dependent activation of the p27Kip1 promoter. Many investigations independently
identified region −573 to −501 to contain binding sites for such transcription factors as Sp1, NF-Y, CTF, or E2F1 and also to contribute to the up-regulation of p27Kip1 in response to vitamin D in U937 cell, tamoxifen in human lung cancer cells, or galectin-1 in HT-29 cells [13–15,20,35–38]. The region −620 to −574 identified in the present study is thus located just upstream of this hot region. The database search [39] identified a binding site for MZF1 [30,40] and δEF1 [41] in region −620 to −573 (Fig. 8A). However, the precise mechanism regarding how Rac1 regulates the transcription of p27Kip1 gene, and the transcription factors that are involved thus remain to be determined in a future study.

Rho proteins have been reported to play an important role in the cell cycle progression [42]. We have reported that Rho proteins, RhoA and Rac1, play a critical role in the S phase progression in BAECs [24]. Adhesion to the extracellular matrix has been shown to be associated with the activation of RhoA and Rac1, the down-regulation of p27Kip1, and the stimulation of the cell growth [43,44]. RhoA and Rac1, on one hand, were reported to facilitate the proteosomal degradation of p27Kip1 and p21Cip1 [43,45]. It is thus conceivable that the adhesion to the extracellular matrix activates Rho proteins, thereby down-regulating p27Kip1. Our finding of the involvement of Rac1 in the contact-induced up-regulation of p27Kip1 is thus apparently inconsistent with these reports. The effect of Rac1 on the level of p27Kip1 may differ depending on the situation of either cell adhesion or cell–cell contact. The formation of cell–cell contact, as well as the cell adhesion, have been reported to activate Rac1 [46,47]. The spatio-temporal regulation of the Rac1 activity should thus play a critical role in the differential control of the adhesion-induced cell growth and the contact-induced growth arrest.

The cell-penetrating peptide-mediated protein transduction technique [48] was used to introduce the Rac1 inhibitory protein into the endothelial cells. A rapid introduction of the inhibitory protein is essential when investigating the time-dependent events such as cell cycle progression. The conventional transfection with the plasmid or viral vectors or the knock-down of the protein expression with an RNA interference technique causes the delayed effects. It is also difficult to control the level of the protein by these methods. We therefore took advantage of the cell-penetrating peptide-mediated protein transduction. We and others have demonstrated that proteins can be introduced into the cells in as short a time as 10–15 min and also in a quantitative manner [24–27,48]. These advantages made it possible to specifically investigate the role of Rac1 in the contact-induced activation of the p27Kip1 promoter.

In conclusion, the present study demonstrated for the first time that the activation of Rac1 due to the formation of the homophilic cell–cell contact induced an activation of the p27Kip1 promoter activity, thereby up-regulating the p27Kip1 expression and facilitating the cell cycle arrest. Region −620 to −573 of the p27Kip1 promoter has been identified to play a primary role in the Rac1-dependent activation of the p27Kip1 transcription. Our findings thus suggest the existence of a new mechanism for the transcriptional regulation of p27Kip1 due to the cell–cell contact, which plays an important role in the contact-induced growth arrest in vascular endothelial cells. The transcription factor involved in this Rac1-dependent up-regulation of the p27Kip1 transcription, however, remains to be elucidated. The cell–cell contact-induced growth arrest is the most important physiological mechanism regulating the growth arrest not only in endothelial cells but also in epithelial cells. Our discovery would shed some light on the mechanism of the contact-induced growth arrest.

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References


