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Morphological Adjustment of Senescent Cells by Modulating Caveolin-1 Status*

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Morphological change is one of the cardinal features of the senescent phenotype; for example, senescent human diploid cells have a flat large shape. However, the mechanisms underlying such senescence-related morphological alterations have not been well studied. To investigate this situation, we characterized the senescence-dependent changes of cellular structural determinants in terms of their levels and activities. These determinants included integrins, focal adhesion complexes, and small Rho GTPases, and special emphasis was placed on their relationships with caveolin-1 status. We observed that the expression integrin β_1 and focal adhesion kinase (FAK) were increased and that the phosphorylations of FAK and paxillin, hallmarks of focal adhesion formation, were also increased in senescent human diploid fibroblast cells. Moreover, the Rho GTPases Rac1 and Cdc42 were found to be highly activated in senescent cells. In addition, focal adhesion complexes and Rho GTPases were up-regulated in the caveolin-rich membrane domain in the senescent cells. Activated Rac1 and Cdc42 directly interacted with caveolin-1 in senescent cells. Interestingly, caveolin-1 knock-out senescent cells, achieved by using small interfering RNA and antisense oligonucleotide, showed disrupted focal adhesion formation and actin stress fibers via the inactivation of FAK, which resulted in morphological adjustment to the young cell-like small spindle shape. Based on the results obtained, we propose that caveolin-1 plays an important role in senescence-associated morphological changes by regulating focal adhesion kinase activity and actin stress fiber formation in the senescent cells.

Morphologic changes are characteristic features of the senescent phenotype that occur at both the cellular and organism level. Senescent cells show morphologically flattened and enlarged cell shapes. However, the molecular mechanisms underlying such morphological alterations in senescent cells have not been elucidated; instead, this phenotype has become an accepted phenomenon. Without doubt, such structural changes can limit functional efficiency. Therefore, it may be assumed that the functional deterioration of senescent cells may be intimately associated with such morphological changes. In our previous study, we observed that the senescent phenotypes can be reversed by simply reducing caveolin-1 status, at least functionally, in terms of mitogenic signaling and DNA synthesis reentry (1). During this study, we observed that the modulation of caveolin status in senescent cells may induce morphological changes. Therefore, in this work, we focused on the possibility of morphological adjustment in association with caveolin status modulation.

To study the structural changes of senescent cells, we examined the cytoskeletal regulatory proteins, *i.e.* Rac1, RhoA, and Cdc42, and the integrins and integrin-mediated focal adhesion complex molecules. Associations between these molecules were traced with respect to their roles in the determination of aging-specific structural phenotypes.

The activation of RhoA, Rac1, and Cdc42 leads to the formation of actin stress fibers, which results in the formation of membrane ruffles/lamellipodia and filopodia. They are also involved in the network of integrin-mediated focal adhesion complexes (2–4). Various cytoskeletal proteins and signaling molecules, such as Shc, focal adhesion kinase (FAK),[1] paxillin, vinculin, talin, and tensin, are associated with the cytoplasmic portion of integrins in focal adhesion complexes (5), and of these, FAK, a 125-kDa cytoplasmic tyrosine kinase localized in focal adhesions, is a key component (6–8). FAK obtains its kinase activity through autophosphorylation and phosphorylates other focal adhesion molecules, such as paxillin (5). In addition, the morphological changes of senescent HDF cells involve the activation of Rb family proteins, the enhancement of actin stress fibers, and the redistribution of focal adhesion proteins (9). Recently, the α-chain of integrins was suggested to be associated laterally with the oligomeric membrane protein caveolin-1 (10).

Caveolin, the principal component of caveolae, is a 21–24-kDa integral membrane protein. Caveolin-1 has a scaffolding domain within its NH_2-terminal region. Through this domain, caveolin-1 interacts with G-protein α-subunits, H-Ras, Src family tyrosine kinases, protein kinase C isoforms, EGF-R, Neu, and endothelial nitric oxide synthase (11–13). Moreover, the targeted down-regulation of caveolin-1 would be sufficient to drive the transformation of cells and to hyperactivate the Erk kinase cascade (14, 15). In contrast, senescent HDFs show high levels of caveolin expression and of its colocalization with

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[1] The abbreviations used are: FAK, focal adhesion kinase; HDF, human diploid fibroblast; EGF, epidermal growth factor; PBS, phosphate-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; siRNA, small interfering RNA; GST, glutathione *S*-transferase.

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EGF-R. The overexpression of caveolin-1 in young HDFs suppresses the activation of Erk-1/2 upon EGF stimulation, indicating that caveolin has a direct role in EGF signaling, which results in premature cellular senescence in primary cultures of murine fibroblasts transgenically overexpressing caveolin-1 (16–19). Moreover, the down-regulation of caveolin-1 can restore Erk and Elk activation and can lead to the re-initiation of DNA synthesis upon EGF stimulation (1). These results demonstrate that caveolin-1 has a central role in the process of cellular senescence. Therefore, it might be of significance to identify the role of caveolin in aging-related morphological change, to establish its relationship with small GTPases and cytoskeletal modulating molecules, and to determine its role in aging-related functional efficiency.

MATERIALS AND METHODS

Reagents—Human diploid fibroblasts were isolated from foreskin. Monoclonal antibodies against caveolin-1 (C43420), phosphocaveolin-1 (Y14), FAK (F15020), paxillin (P13520), flotillin-1 (F65020), Rac1 (R56220), and RhoA (R73920) were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibodies against phospho-FAK (sc11765-R), Cdc42 (8401), and actin (sc-1616) were purchased from Santa Cruz Biotechnology, Inc. Monoclonal antiphosphopaxillin (Tyr-118) was purchased from Cell Signaling Technologies, Inc. Secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Zymed Laboratories Inc.; chemiluminescent detection systems were from Pierce, and other biochemical reagents were from Sigma.

Cell Culture and Transfection—Human diploid fibroblasts were kept in a 100-mm dish in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Cells were passed serially *in vitro*, and young cells, proliferating cells, and senescent or near-senescent cells were collected at population doublings 15 and 60. Cellular senescence was confirmed by delayed population doubling times and by using a senescence-associated β-galactosidase activity assay (20).

For studies on the Rho GTPases, young cells were seeded in 100-mm dishes and transfected with active mutant RhoA-V12, Rac1-V12, or Cdc42-V12 by using LipofectAMINE PLUS reagent (Invitrogen) in serum-free medium. Four h after transfection, the cells were placed in complete medium in a CO_2 incubator for 2 days before harvest.

Senescence-associated β-Galactosidase Staining—Senescent cells were washed with phosphate-buffered saline (PBS) and fixed for 3~5 min (at room temperature) in 3% formaldehyde in PBS. After washing with PBS, cells were incubated at 37 °C with fresh senescence-associated β-galactosidase staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$). Sixteen h later, senescent cells were identified under a light microscope.

Immunofluorescence Analysis—Cells cultured on glass coverslips were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Nonspecific protein binding sites were then saturated with 2% bovine serum albumin in PBS for 30 min. The cells were then washed with PBS and incubated with polyclonal antibodies against caveolin-1 or monoclonal antibodies against paxillin. Rhodamine-conjugated phalloidin (Sigma) was used to stain the actin cytoskeleton. Unbound proteins were removed by washing, and cells was incubated with Texas Red- or fluorescein isothiocyanate-labeled secondary antibodies (Molecular Probes and Santa Cruz Biotechnology, Inc.) for 1 h. Nuclei were then fluorescently labeled with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). The coverslips were then washed and mounted on glass slides. Fluorescent images were obtained using a confocal microscope.

Immunoprecipitation Analysis—Cells were lysed with lysis buffer (25 mM Tris, pH 7.4, 10 mM NaCl, 1% Triton X-100, 1% cholic acid, 1 mM protease inhibitor (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate) and immunoprecipitated. Cell lysates were clarified by centrifugation at 7,300 × *g* for 10 min at 4 °C, and protein concentrations were determined using the Bradford assay. Cell lysates were incubated with 2.5 μg/ml monoclonal anticaveolin-1 antibody for 15 h at 4 °C and then with 10 μl of protein G-agarose for 3 h at 4 °C. Immunoprecipitates were extensively washed in washing buffer (25 mM Tris, pH 7.4, 10 mM NaCl, 1% Triton X-100, 1 mM protease inhibitor (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate), resuspended in 250 mM Tris-Cl (pH 6.8), 357.7 mM β-mercaptoethanol, 10% SDS, 0.5% bromphenol blue, 50% glycerol (5× sample buffer), and analyzed by immunoblotting.

Immunoblot Analysis—Immunoprecipitated proteins, gradient fractions, and cell lysates were analyzed by immunoblotting using the indicated antibodies, *i.e.* anticaveolin-1 monoclonal antibody (0.5 μg/ml), anti-FAK (1.25 μg/ml), antiphospho-FAK (1.25 μg/ml), antipaxillin (1.25 μg/ml), antiphosphopaxillin (1.25 μg/ml), antiflotillin-1 (1.25 μg/ml), anti-Cdc42 (1 μg/ml), anti-Rac1 (1.25 μg/ml), anti-Rho (1.25 μg/ml), and antiactin antibody (0.4 μg/ml). The density of the visualized band was quantified using an image analyzer (Bio-Rad, model GS-700). At least three independent experiments were conducted.

Purification of Caveolin-rich Membrane Fractions—Cells were washed in ice-cold PBS and scraped into 2 ml of 500 mM sodium carbonate, pH 11.0. Homogenization was performed using a loosely fitting Dounce homogenizer (20 strokes), a Polytron (three 10-s bursts; output, 50), and a sonicator (three 10-s bursts).

The homogenate was then adjusted to a 40% sucrose concentration by adding 2 ml of 80% sucrose solution prepared in MBS buffer (25 mM MES, pH 6.5, 0.15 M NaCl, 1 mM EDTA) and placed in an Ultracentrifuge tube. A 5–30% (w/v) discontinuous sucrose gradient was formed above, with both gradients in MBS containing 250 mM sodium carbonate, and was centrifuged at 39,000 rpm (200,000 × *g*) for 16 h in an SW41 rotor (Beckman Instruments). A light-scattering band confined to the 5–30% sucrose interface confirmed the presence of caveolin. From the top of each gradient, 12 1-ml fractions were collected. The protein concentrations of the gradient fractions were determined by Bradford assay.

RNA Interference and Transfection—Synthetic siRNA duplexes were used as described by Elbashir *et al.* (21). A synthetic siRNA duplex corresponding to the caveolin-1 mRNA sequence 5′-AACCAGAAGGGACACACAGUU-3′ was used to inhibit caveolin-1 protein expression (1), and a synthetic siRNA duplex corresponding to the firefly luciferase (GL2) mRNA sequence 5′-AACGUACGCGGAAUACUUCGA was used as a negative control. The siRNA duplexes were purchased from Dharmacon Research (Lafayette, CO) as duplexed 2′ unprotected, desalted, and purified siRNA. Transfection of 21-nucleotide siRNA duplexes was carried out using Oligofectamine™ (Invitrogen). Two days before transfection, the cells were trypsinized and counted (5×10^4 cells/100-mm dish) so that they were 50% confluent on the day of transfection. siRNA-transfection reagent complexes were prepared as prescribed by the manufacturer. The cells were then transfected with 0.5 nmol (7 μg) of siRNA and Oligofectamine™ reagent in serum-free medium and incubated for 4 h at 37 °C in a CO_2 incubator. Following incubation, the cells were supplied with growth medium containing 10% fetal bovine serum and harvested 48 or 72 h later for further assay.

Activity Analysis of the Rho Family GTPases—RhoA, Rac1, and Cdc42 activity assays were performed as described previously (22). The relative amount of active GTPases was determined by measuring the amount of GTPases sedimented by the glutathione *S*-transferase-Rac1/Cdc42-binding domain of p21-activated kinase or by the glutathione *S*-transferase-RhoA-binding domain of rhotekin relative to the amount in whole-cell lysates. Bound proteins were resolved by 15 or 17.5% SDS-PAGE and immunoblotted using anti-RhoA, anti-Rac1, or anti-Cdc42 antibodies.

RESULTS

Morphological Changes of Senescent Cells Caused by Caveolin-1 Down-regulation—Senescent cells were confirmed by using senescence-associated β-galactosidase staining as a biomarker (Fig. 1*A*) and showed irregular large flat shapes *versus* young cells and increased levels of focal adhesion, lamellipodia, and filopodia (Fig. 1*B*). Previously, we reported that the caveolin level is significantly increased in senescent cells (16). In this study, we observed that a simple reduction in the caveolin-1 status, as determined by the siRNA method, induced in senescent cells morphological changes corresponding to the young cell-like shape, *i.e.* a small and spindle shape (Fig. 1, *C* and *D*). These data suggest that caveolin-1 might be actively involved in the morphogenesis of HDF cells during the cellular aging process.

Colocalization of Caveolin-1 and Focal Adhesion Complex—Caveolin-1 is associated with focal adhesion complex by bind-

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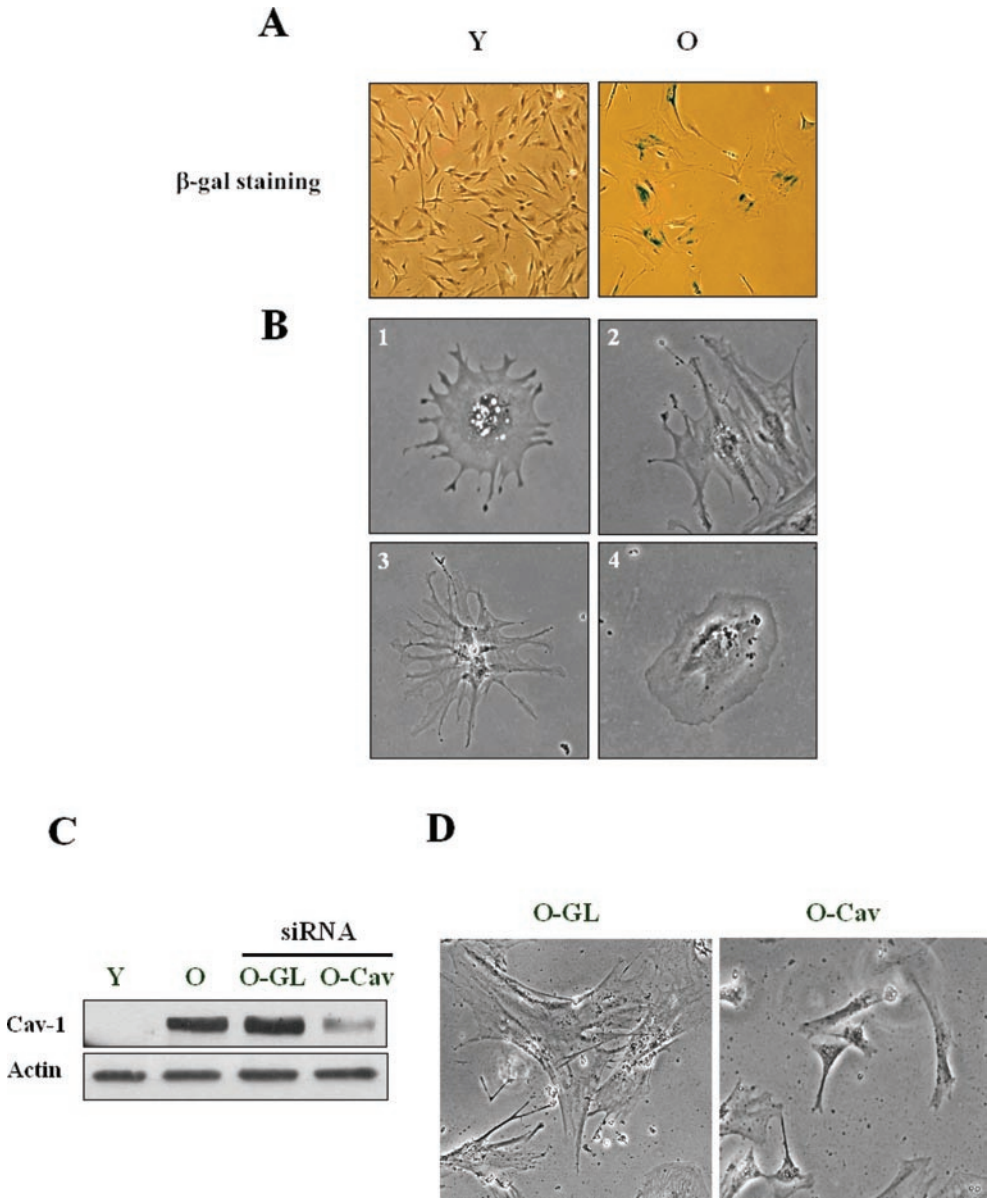

FIG. 1. **Morphologies of young, senescent, and caveolin-1 siRNA-transfected senescent cells.** *A*, young (*Y*) and senescent (*O*) HDF cells were fixed with 4% paraformaldehyde and then incubated with senescence-associated β-galactosidase (*β-gal*) staining solution. After 16 h, the cells were analyzed under a light microscope. *B*, various senescent cell morphologies were observed. *C*, senescent cells were transfected with the caveolin-1 siRNA (*O-Cav*) and firefly luciferase siRNA (*O-GL*) as a control. After 48 h, the transfected senescent cells were harvested with young and untransfected senescent cells. The expression levels of caveolin-1 (*Cav-1*) were analyzed by Western blotting with mouse monoclonal anticaveolin-1 antibody. *D*, the morphologies of young and senescent cells after caveolin-1 down-regulation were determined by light microscopy.

ing to integrin in the membrane (23, 24). The activity of the focal adhesion kinase is significantly reduced by caveolin-1 down-regulation (25). To elucidate the role of caveolin in senescence-associated morphological changes, young and senescent HDF cells in culture were fractionated by sucrose gradient density centrifugation in the absence of detergent, a procedure widely used to isolate caveolae-enriched membrane domains (26). Immunoblot analysis of the fractions showed that the majority of the caveolin was present at the low density, 5%/30% sucrose interface of the gradient (Fig. 2*B*, Fractions 5–8). Protein amounts in each fraction showed that the senescent cells contained higher levels of protein in the caveolae domain (Fig. 2*A*). A significant proportion of focal adhesion molecules, *e.g.* FAK and paxillin, was recruited to the caveolae fractions in senescent cells, whereas flotillin, a membrane lipid raft marker, was similar in young and senescent HDF cells (Fig. 2*B*). These data showed that two kinds of microdomains differentially existed in young and senescent cells. When young and senescent cells were co-immunostained with antipaxillin and anticaveolin-1 antibodies (Fig. 2*C*), paxillin was found to be colocalized with caveolin-1 in the membrane of senescent cells but not in young cells. Although the expression level of paxillin was not increased in senescent HDF cells, it was recruited to a focal adhesion region in the senescent cell membrane. These data suggest that the observed increase in focal adhesion proteins might be related to the up-regulation of caveolin-1.

Focal Adhesion and Cytoskeletal Proteins in Senescent HDF Cells—To confirm the roles of the focal adhesion complex in the senescent phenotype, young and senescent cells were examined for focal adhesion and actin stress fiber formation. Confocal microscopic analysis indicated that focal adhesion and actin stress fiber formation, monitored by antipaxillin antibody (*green*) and phalloidin (*red*), respectively, were significantly increased in senescent cells (Fig. 3*A*).

As shown in Fig. 3*B*, senescent HDF cells showed higher expressions of integrin β_1, FAK, and caveolin-1 than young

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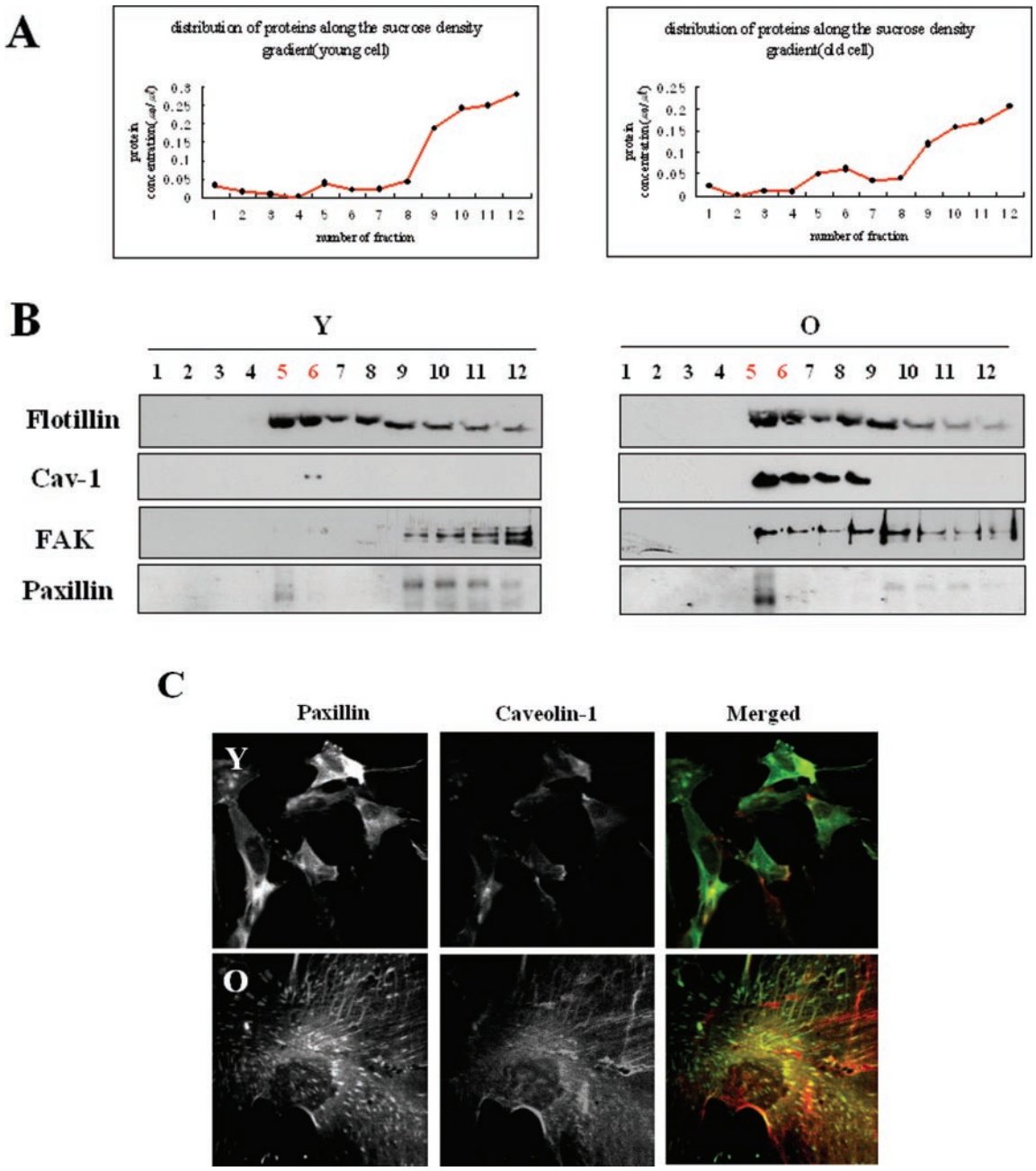

FIG. 2. **Localization of caveolin-1 and focal adhesion complexes.** Young and senescent cells were suspended in sodium carbonate buffer and lysed by homogenization and sonication. A 5–35% discontinuous sucrose gradient was formed and centrifuged at 175,000 × *g* for 16–20 h in an SW41 rotor. A light-scattering band confined to the 5–35% sucrose interface was observed and collected. *A*, protein levels in each fraction from young and senescent cells. *B*, localization of caveolin and focal adhesion complexes was determined by Western blotting. *Y*, young; *O*, old; *Cav-1*, caveolin-1. *C*, the colocalization of caveolin-1 and focal adhesion was confirmed by confocal microscopy. Young and senescent cells were stained with polyclonal anticaveolin-1 antibody and monoclonal antipaxillin antibody, a marker of focal adhesion. *Green*, focal adhesion; *red*, caveolin-1.

cells but did not show higher expression of paxillin. However, in senescent cells, the phosphorylation statuses of FAK and paxillin were increased (Fig. 3, *C* and *D*), suggesting increased focal adhesion formation. These data indicate that an increase of integrin and FAK in senescent cells recruits paxillin into the focal adhesion and that FAK, after being activated by integrin, phosphorylates paxillin.

Inactivation of FAK and the Disruption of Focal Adhesion and Actin Stress Fiber Formation by the Down-regulation of Caveolin-1 in Senescent Cells—To explore the importance of caveolin-1 in senescent morphological changes via focal adhesion formation and Rho GTPases, senescent HDF cells were transfected with siRNA and antisense oligonucleotides of caveolin-1. Although the expression of FAK was not significantly changed, the phosphorylation of FAK was dramatically reduced by the down-regulation of caveolin-1 in caveolin siRNA-transfected senescent cells *versus* the control firefly luciferase siRNA-transfected senescent cells (Fig. 4, *A* and *B*). The tyrosine phosphorylation of FAK and of the other signaling molecules responsible for cytoskeletal reorganization and cell spreading after ligand engagement of β_1 integrins may depend on interactions between FAK and Src family kinases. Because caveolin-1 has been shown previously to interact with c-Src (27), it may be presumed that the integrin-mediated tyrosine phosphorylation of FAK is affected by caveolin-1 status.

In addition, focal adhesion and actin stress fiber formation were reduced in caveolin siRNA-transfected senescent cells (Fig. 4*C*). Reductions of focal adhesion and actin stress fiber

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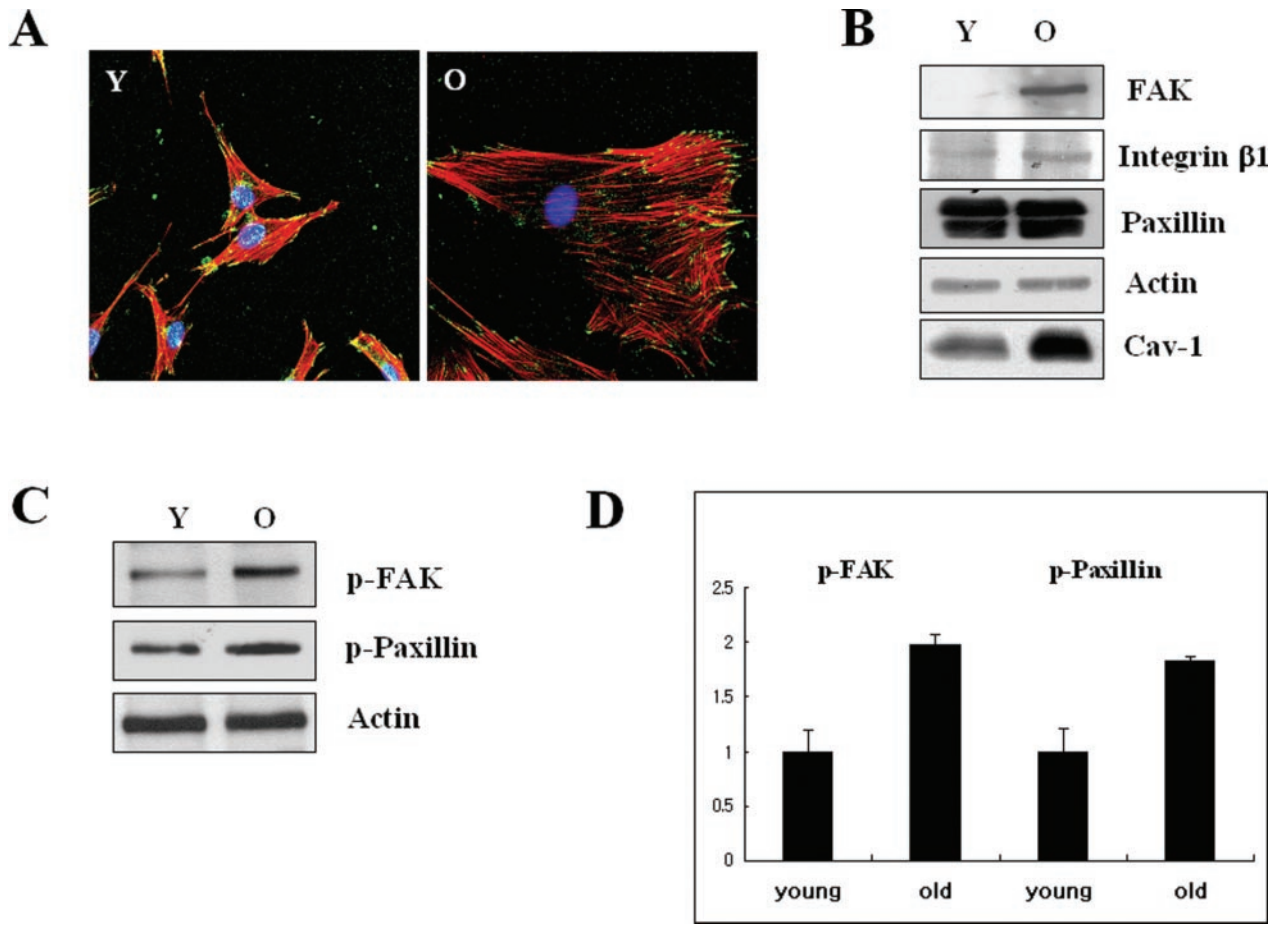

FIG. 3. **Focal adhesion formation in young and senescent cells.** *A*, focal adhesion and actin stress fiber formation were analyzed using antipaxillin, caveolin-1 antibody, and phalloidin. *Green*, focal adhesion; *red*, actin stress fiber and caveolin-1; *blue*, nuclei. *B*, the expressions of focal adhesion complexes, such as integrin β_1, FAK, or paxillin, were determined using their respective antibodies in young and senescent cells. *C*, phosphorylation of FAK and paxillin was determined using phospho-specific anti-FAK and antipaxillin antibodies. *D*, quantitative graph of phosphorylation of FAK and paxillin. The blots shown are representative of at least 3–5 independent experiments, and the histograms represent the average and standard deviations.

formation via the inactivation of FAK by caveolin-1 status reduction resulted in morphological alterations toward the young cell phenotype (Fig. 4*D*). These results suggest that morphological changes shown by senescent cells via focal adhesion complexes and actin stress fibers could be modulated by caveolin-1 status.

The Role of Rho Family GTPases in Senescent HDF Cells—Rho family GTPases, including RhoA, Rac1, and Cdc42, regulate the morphogenesis and cytoskeletal reorganization of cells. As shown in Fig. 1*B*, senescent cells showed higher levels of focal adhesion and of lamellipodia and filopodia formation. These results suggest that Rho GTPases might play important roles as morphological determinants in senescent cells. Fig. 5, *A* and *B*, showed increased Rac1 and Cdc42 expressions and activities in senescent HDF cells but did not show such increases for RhoA. Rho family proteins cycle between inactive GDP-bound forms and active GTP-bound forms and serve as molecular switches for a variety of intracellular signaling pathways. When young and senescent cell lysates were subjected to activity analysis using GST-p21-activated kinase and GST-rhotekin, respectively, senescent HDF cells showed elevated Rac1 and Cdc42 GTPase activity (Fig. 5*C*).

To confirm the roles of Rac1 and Cdc42 in the production of the senescent phenotype, we transfected active mutants of Rac1 and Cdc42 into young HDF cells. The active mutants of Rac1 and Cdc42 (Fig. 5*D*) were found to be able to induce morphological changes similar to those of senescent HDF cells. These results indicate that the activated Rho GTPases, especially Rac1 and Cdc42, might regulate the morphological alterations associated with the cellular aging process.

Linkage of Caveolin-1 with Rac1 and Cdc42 in Senescent Cells—Because Rho GTPases regulate morphological changes via cytoskeletal reorganization, we examined the localization of Rac1 and Cdc42 and the interaction between caveolin-1 and Rac1 and Cdc42 in young and senescent HDF cells. As shown in Fig. 6*A*, Rho GTPases were recruited into caveolin fractions and were directly linked with caveolin-1 in senescent HDF cells by immunoprecipitation analysis (Fig. 6*B*). This result suggests that the activations of Rac1 and Cdc42 require their colocalization and interaction with caveolin-1 in the caveolin-rich membrane to facilitate senescence-associated morphological alteration.

DISCUSSION

Senescent cells show morphological changes, such as a flat large cell shape in addition to functional decay and growth arrest (28, 29). In various cellular processes, morphological changes play an important role in the cellular function. Recently, it was reported that the structural alterations of senescent cells are intimately related with an increase in focal adhesion and Rb family protein expression (9). However, the basic mechanism for this aging-associated morphological change has not been well studied.

In this study, we found that the morphologies of senescent

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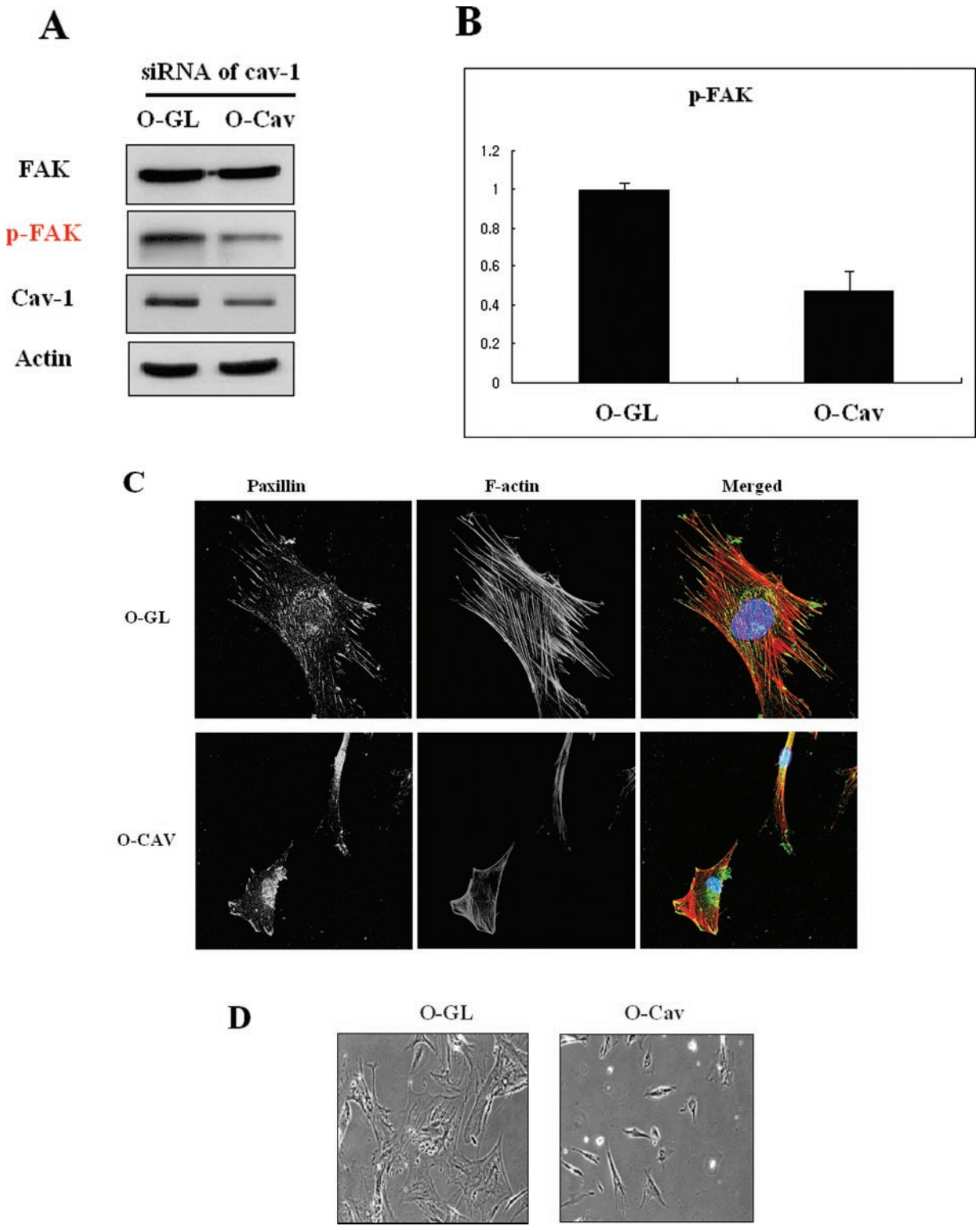

FIG. 4. **The effect of caveolin-1 down-regulation in senescent cells.** *A*, after transfecting senescent cells with the siRNAs of caveolin-1 (*O-Cav*) and firefly luciferase (*O-GL*), the cells were harvested and analyzed with phospho-specific anti-FAK antibody. *B*, quantitative graph of phosphorylation of FAK after siRNA transfection. The blots shown are representative of at least 3–5 independent experiments, and the histograms represent the average and standard deviations. *C*, after siRNA transfection, senescent and young cells were placed on coverslips in a 24-well plate. Focal adhesion and actin stress fiber were examined by confocal microscopy by using antipaxillin. *D*, morphological changes induced by caveolin-1 down-regulation in senescent cells were determined by light microscopy.

cells became young cell-like simply by down-regulation of caveolin-1 status (Fig. 1*D*). This result suggests that caveolin-1 is involved in the morphological implications of the cellular aging process.

Caveolin-1 is associated with focal adhesion complex through integrin in the membrane and controls the activity of focal adhesion kinase (10, 23, 24). These results indicate that caveolin can play an important role in focal adhesion formation and thereby participate in the adhesion signal cascade as well. We observed that the formation of focal adhesion and the formation

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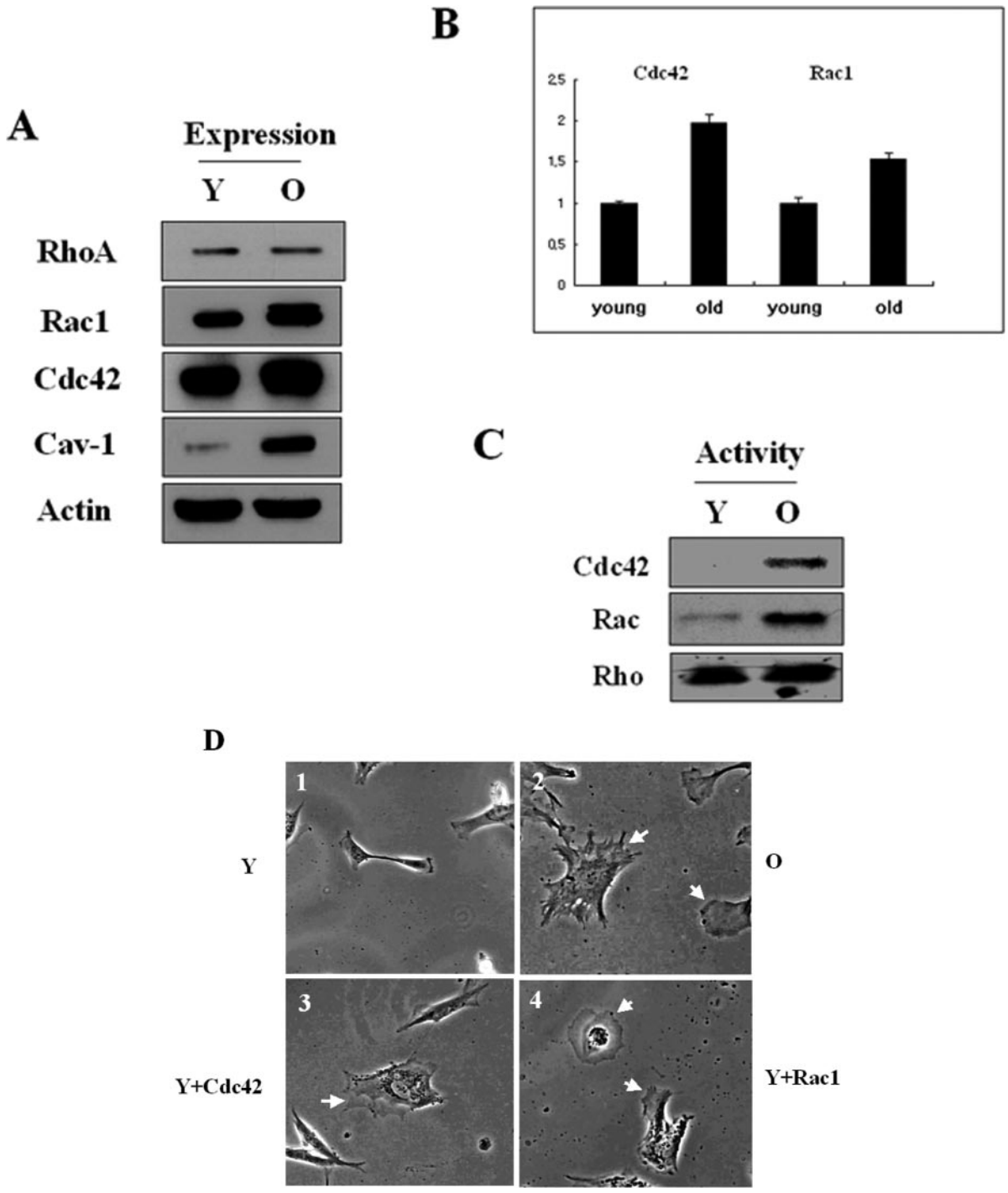

FIG. 5. **The expression and activation of Rho family GTPases in young and senescent cells.** *A*, the expression levels of Rho GTPases in young (*Y*) and senescent (*O*) cells were determined by Western blotting using anti-RhoA, anti-Rac1, or anti-Cdc42 antibodies. *B*, the quantitative graph of expression levels of Rac1 and Cdc42. The blots shown are representative of at least 3–5 independent experiments, and the histograms represent the average and standard deviations. *C*, Rac1, Cdc42, or RhoA activity assays were performed as described under "Materials and Methods" using a GST fusion protein derived from p21-activated kinase or rhotekin, which selectively binds GTP-bound RhoA, Rac1, or Cdc42. GST-Rhotekin precipitates were immunoblotted with monoclonal antibodies recognizing RhoA, Rac1, or Cdc42. *D*, constitutively active mutants Rac1 and Cdc42 were transfected in young cells. After 48 h, the cell morphologies were examined by light microscopy. *Arrows* indicate the morphological changes caused by transfecting these active mutants of Rac1 and Cdc42. *1*, young cells; *2*, senescent cells; *3*, active mutant of Cdc42-transfected young cells; *4*, active mutant of Rac1-transfected young cells.

of actin stress fiber are both increased and tend to be anchored in the membrane after interaction with caveolin-1 in senescent cells (Figs. 2 and 3). In addition, caveolin-1 down-regulation caused the inactivation of FAK, which led to the disruption of focal complexes and actin stress fibers (Fig. 4). These findings show that the simple reduction of caveolin-1 in senescent cells can reverse their morphological form and that they revert to small, spindle-shaped cells via the modulation of focal adhesion complexes. As shown in Fig. 2*B*, the levels of focal adhesion molecules, such as integrin β_1 and FAK, were elevated in the caveolin-rich membrane, suggesting the possibility of their interaction. Interaction between integrin and caveolin-1 has been suggested previously (10, 24), and it is well known that integrins activate various protein tyrosine kinases, including FAK

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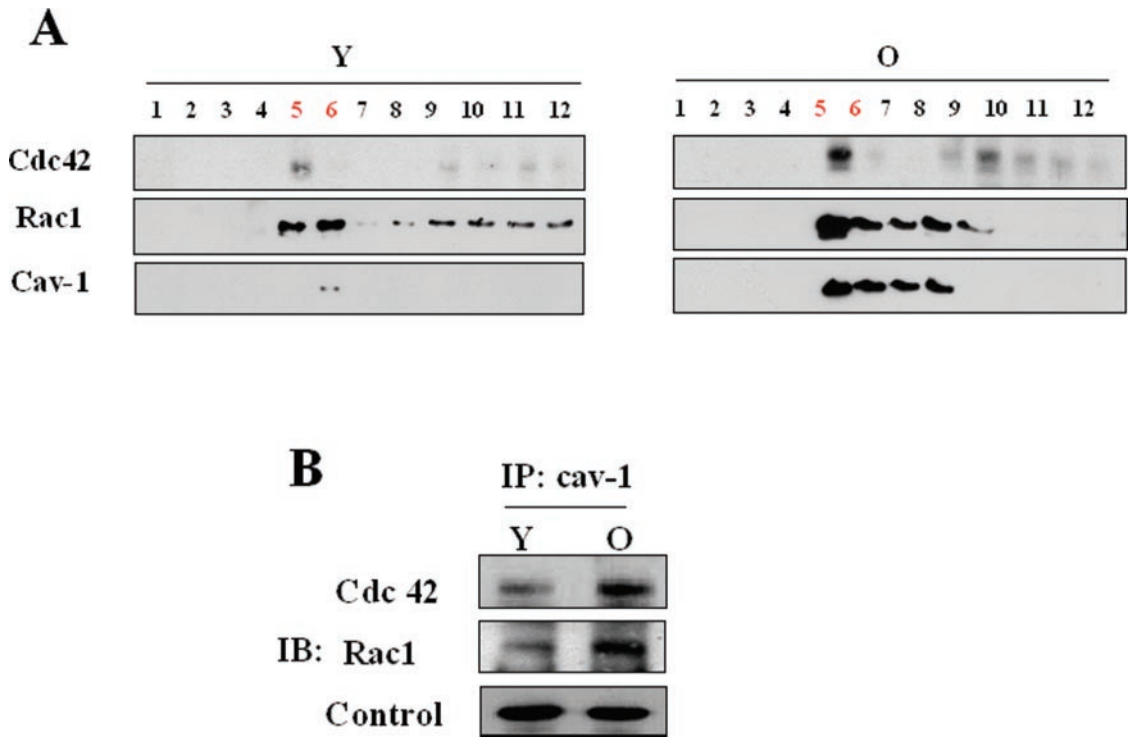

FIG. 6. **Colocalization and the interactions of Rac1 and Cdc42 with caveolin-1 in senescent cells.** *A*, the localizations of Rac1 and Cdc42 within caveolin-rich membrane factions were analyzed by immunoblotting with anti-Rac1 and anti-Cdc42 antibodies. *Y*, young; *O*, old; *Cav-1*, caveolin-1. *B*, direct interaction between Rac1 and Cdc42 with caveolin-1 was determined by immunoprecipitation (*IP*). The lysates from young and senescent cells were incubated with anticaveolin-1 antibody, and complexes were examined by immunoblotting (*IB*) with anti-Rac1 and anti-Cdc42 antibodies.

and Src family kinases (8, 10). Therefore, it seems reasonable to propose that the strong interaction between caveolin-1 and integrins and focal adhesion complex molecules determines the morphology of senescent cells.

Members of the Rho family of small GTPases act as intracellular molecular switches that transduce signals from extracellular stimuli to the actin cytoskeleton and the nucleus (30, 31). The most characteristic function of Rho GTPases is that they act as regulators of various aspects of actin cytoskeleton dynamics in fibroblasts (2, 3). The activations of RhoA, Rac1, and Cdc42 result in the formation of focal adhesion, actin stress fibers, membrane ruffles/lamellipodia, and filopodia, respectively. As shown in Fig. 5, in senescent HDF cells Rac1 and Cdc42 were up-regulated in terms of their expressions and activities. To confirm the role of Rac1 and Cdc42 in the determination of senescent morphologic changes, young HDF cells were transfected with their respective constitutive active mutants, and this was found to result in senescence-like changes (Fig. 5, *C* and *D*). Therefore, we suggest that the morphological changes of senescent cells that occur via increased focal adhesion, actin stress fiber, and membrane ruffles can be explained by increased Rac1 and Cdc42 activity in the cellular aging process.

We found that the activities of Rac1 and Cdc42 are related to caveolin-1 status in senescent cells by analyzing the colocalization and interaction between caveolin-1 and Rho GTPases. As shown in Fig. 6, activated Rac1 and Cdc42 were enriched in caveolae-related membrane and directly interacted with caveolin-1 in senescent cells. These results probe a novel relationship between caveolin-1 and Rho GTPases with respect to the morphogenesis of cellular senescence. The role of Rac1 and Cdc42 in the regulation of neuronal morphogenesis, including migration, polarity, axon growth and guidance, dendrite elaboration and plasticity, and synapse formation, has been elaborated previously (32). However, the regulatory mechanisms of Rac1 and Cdc42 have not been elucidated clearly. Based on the results of the present study, we suggest that caveolin might be involved in the activations of Rac1 and Cdc42 during cellular processes such as senescence and differentiation. The novel role of caveolin in aging-associated morphologic differentiation might be explained in terms of its regulation of Rac1 and Cdc42 activities and the subsequent cytoskeletal reorganization.

Our data show the increased expression and phosphorylation of FAK in senescent cells (Fig. 3). Although the expression level of paxillin did not change, paxillin was recruited to focal complexes, and this resulted in the assembly of actin stress fibers in senescent cells. Because active Rho GTPases, especially Rac1 and Cdc42, induce morphological changes via the regulation of focal adhesion and actin cytoskeletal reorganization (33, 34), the recruitment of Rac1 and Cdc42 in the caveolin-rich membrane fractions and their interaction with caveolins in senescent cells (Fig. 6) *may* imply that the aging-associated morphological alterations are influenced by caveolin-1 status. Therefore, it seems likely that a reduction of caveolin-1 status in senescent cells could disrupt the aging-associated morphological alterations. In other words, caveolin-1 status might play a prime role as a determinant of the phenotypic morphological changes associated with senescence. In addition, we have suggested previously that caveolin-1 status may modulate the functional efficiency of senescent cells exposed to mitogenic signals (1) and that the modulation of caveolin-1 status in senescent cells adjusts functional efficiency and restores morphological appearance.

Therefore, it appears that caveolin-1 status may be a principle determinant of the senescent phenotype not only in terms of functional deterioration but also in terms of morphological alteration, as if it plays the role of a gatekeeper at the boundary between the cell and the external environment. Taken together, our results indicate the possibility that the senescent phenotype is capable of flexible adjustment and that caveolin-1 status is a determinant of this process.

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