Phosphorylation of Ezrin by Cyclin-Dependent Kinase 5 Induces the Release of Rho GDP Dissociation Inhibitor to Inhibit Rac1 Activity in Senescent Cells

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Abstract

Normal somatic cells enter a state of irreversible proliferation arrest designated cellular senescence, which is characterized by biochemical changes and a distinctive morphology. Cellular stresses, including oncogene activation, can lead to senescence. Consistent with an antioncogenic role in this process, the tumor suppressor pRb plays a critical role in senescence. Reexpression of pRb in human tumor cells results in senescence-like changes, including cell cycle exit and cell shape alteration. Here, we show that pRb-induced senescent SAOS-2 cells and senescent human diploid fibroblasts are accompanied by increased phosphorylation of ezrin at T235 by cyclin-dependent kinase 5 and consequent dissociation of Rho GDP dissociation inhibitor (Rho-GDI) from an ezrin/Rho-GDI complex. The release of Rho-GDI results in increased interaction with Rac1 GTPase and inhibition of Rac1 GTPase activity. In addition, reduction of Rho-GDI by small interfering RNA in pRb-transfected cells prevented senescence-associated flat cell formation, suggesting that Rho-GDI plays an important role in contributing to cellular morphology in the process of senescence. (Cancer Res 2006; 66(5): 2708-15)

Introduction

A fundamental characteristic of normal cells is the limited ability to proliferate in culture. Normal somatic cells withdraw from the cell cycle after a finite number of divisions and enter an irreversible arrest designated cellular senescence (1). A number of phenotypes have been associated with senescence. Cells attain an enlarged, flattened shape and display increased senescent-associated β-galactosidase activity (2). Senescent cells undergo an irreversible G1 growth arrest involving the repression of genes that drive cell cycle progression and the up-regulation of cell cycle inhibitors, like p16INK4a, p27Kip1, p53, and its transcriptional target p21CIP1 (3, 4). The irreversible arrest of cell division may be tumor suppressive, and escape of cells from senescence results in immortalization and oncogenesis (1, 5). Recently, several reports confirmed that senescent cells accumulate in tumors in vivo and act to suppress tumor growth (6-8). Thus, the senescence process is believed to be a mechanism that prevents the accumulation of deleterious mutations that lead to cellular immortality and malignant transformation (9). Furthermore, senescence may play a significant role in response to cancer therapy (10).

Methods

Many studies have shown that senescent cells are characterized by the accumulation of the hypophosphorylated, active form of the retinoblastoma tumor suppressor protein, pRb, which blocks passage from G1 to S phase of the cell cycle (11-13). Indeed, reintroduction of pRb into Rb−/− tumor cell lines induces senescence, even in cells that do not contain wild-type p53 (4, 14, 15). Similarly, overexpression of p16INK4a can induce senescence in pRb-positive tumor cells (16). Loss of p16INK4a or pRb function seems to be required for immortalization of at least some human cell types, apparently as an obligate step in preventing senescence (17-19).

Studies done in our laboratory and by many others have indicated that reintroduction of pRb into pRb-deficient SAOS-2 cells results in a typical senescent phenotype, including flat cell formation and increased β-galactosidase activity (4, 14, 15, 20-22). This unique cellular morphologic change is accompanied by alterations in the cytoskeletal proteins of the ERM family (ezrin, radixin, and moesin), which have been implicated in reorganization of actin filaments (20). ERM proteins in senescent cells induced by pRb are phosphorylated by cyclin-dependent kinase 5 (cdk5), which is also involved in a reduction of Rac1 activity and increased actin polymerization associated with the senescent cell shape change (20, 23). It is not at present clear how cdk5-dependent modulation of ERM proteins might influence Rac1 activity. However, others have found that ERM proteins can regulate the Rho subfamily of GTPases, which function in actin organization (24), suggesting that regulation of ERM activity by cdk5 might affect Rac1 activity directly.

The small G proteins of the Rho family, consisting of the Rho, Rac, and Cdc42 subfamilies, are involved in various cell functions, such as cell morphologic change, cell motility, and cytokinesis (25). The small G proteins have two interconvertible forms: the GDP-bound inactive and GTP-bound active forms (26). The conversion from the inactive form to the active form is mediated by two types of regulators: GDP/GTP exchange protein (GEP), which stimulates this reaction, and GDP dissociation inhibitor (GDI), which inhibits this conversion by directly binding to the small G proteins (25). Studies have shown that GDI can bind to the NH2-terminal domain of ERM. The interaction leads to the activation of members of the Rho subfamily by relieving Rho-GDI inhibition (27). Thus, ERM proteins play an important role in the activation of the Rho family members by recruiting their negative regulators.

In this study, we found that the association of ezrin with Rho-GDI was dramatically reduced in senescent SAOS-2 cells induced by pRb and in senescent human diploid fibroblasts (HDF). The phosphorylation of ezrin at Thr235 by cdk5 kinase induced the dissociation of Rho-GDI from the ezrin/Rho-GDI complex. The released Rho-GDI inhibited Rac1 activity, contributing to the cellular morphology change characteristic of senescence. Thus, activation of cdk5 downstream of pRb can lead to alteration of...
ezrin function as a small G protein regulator. These studies underscore a role for cdk5 and Rho-GDI in senescence and thus possibly in tumor suppression by pRb.

Materials and Methods

Cell culture. The human osteosarcoma cell line SAOS-2, subclone 2.4, was used for these studies (21). Cells were maintained at 37 °C, 5% CO2, and grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 15% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin. IMR90 HDFs were maintained in DMEM supplemented with 10% FBS. The human breast carcinoma cell line MDA-MB-468 (American Type Culture Collection, Rockville, MD) was maintained in Leibovitz L-15 medium supplemented with 10% FBS.

Antibodies. Antibodies to ezrin and Rho-GDI were purchased from Upstate Biotechnology (Lake Placid, NY). Anti–phospho-ezrin (Thr(567))/radixin (Thr(564)) was obtained from Cell Signaling Technology (Beverly, MA). Anti–phospho-Thr(235) of ezrin was generated as described (20). Anti-cdk5 DC17, anti-RhoA, and anti-Cdc42 antibodies were provided from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag M2 antibody was obtained from Sigma (St. Louis, MO). Anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody was purchased from Chemicon International (Temecula, CA). Anti-Rac1 antibody was provided from BD Science (San Diego, CA).

Immunoblot and immunoprecipitation. Cells were rinsed twice with PBS and lysed for 25 minutes on ice in ELB lysis buffer [50 mmol/L HEPES (pH 7.2), 250 mmol/L NaCl, 2 mmol/L EDTA, 0.1% NP40] and freshly added protease and phosphatase inhibitors (50 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 1 mmol/L DTT, 25 μg/mL aprotinin, 25 μg/mL trypsin inhibitor, 25 μg/mL leupeptin, 2 mmol/L β-glycerophosphate). The lysates were clarified by centrifugation for 10 minutes at 14,000 × g and transferred to an Eppendorf tube. Cell lysates were heated for 5 minutes at 95 °C in 2× sample buffer [100 mmol/L Tris (pH 6.8), 2% SDS, 20% glycerol, 0.04% bromophenol blue, 2% β-mercaptoethanol]. Proteins were separated by SDS-PAGE and electroblotted onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham, Piscataway, NJ). The blots were incubated with peroxidase-conjugated AffiniPure goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 to 60 minutes and visualized by ECL detection (Amersham). For immunoprecipitation, the lysates were incubated with appropriate antibody overnight at 4 °C. Immune complexes were collected with protein A/G-Sepharose and washed twice with lysis buffer. Bound proteins were released by heating for 5 minutes at 95 °C in sample buffer. The immunoprecipitates were resolved by SDS-PAGE, then transferred to nitrocellulose, and subjected to immunoblot analysis as above.

Assessment of Rac1 activity. Rac1 activity was determined using an assay developed for RhoA activity (28) and modified for Rac1 activity in the laboratory of Jeffrey Settleman (Department of Medicine, Massachusetts General Hospital, Charlestown, MA) using GST-Pak1 as bait. The pEBG-Pak1 plasmid, a gift of Jeffrey Settleman, was bacterially expressed, bound to glutathione S-transferase (GST) beads, and the assay was done as described (28).

Short hairpin RNA (hairpin small interfering RNA) synthesis. DNA-based small interfering RNA (siRNA) vectors were constructed in pBS/U6 (gift of Yang Shi, Harvard Medical School, Boston, MA) as described (29). Briefly, 22-oligonucleotide-long inverted repeats, separated by a 6-nucleotide linker, were inserted downstream of the U6 promoter. The transcribed RNA thus comprised a 22-bp hairpin RNA. Five thymidines were inserted downstream of the antisense strand to provide a stop signal for the polymerase III RNA polymerase. The sense strand of the hairpin was identical to a 22-nucleotide region in the target Rho-GDIα gene (558-576). These sequences were determined to be specific to the target gene by BLAST search. Scramble 22-nucleotide was generated as control shRho-GDI. In addition, control siRNA constructs, including pBS/U6 control shGFP (scramble 22-nucleotide), pBS/U6 green fluorescent protein (GFP) hairpin siRNA (shRNA) were kindly provided by Yang Shi (29). cdk5 shRNA was generated as described (23).

Senescence-associated β-galactosidase staining assay. For the senescence-associated β-galactosidase assay, SAOS-2 cells were cotransfected with the indicated plasmids and pBabe puro plasmid. The cells were maintained in selective media containing puromycin at 0.5 μg/mL for 10 days, and the senescence-associated β-gal assay was done as described previously (2). Briefly, cells were washed in PBS and fixed in 2% formaldehyde/0.2% glutaraldehyde. Then cells were washed and incubated at 37 °C overnight with fresh senescence-associated β-gal stain solution [1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per mL, 40 mmol/L citric acid/sodium phosphate (pH 6), 150 mmol/L NaCl, 2 mmol/L MgCl2, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide].

Results

Expression and association of Rho-GDI with ezrin in senescent cells. Previous studies have found that ectopic expression of pRb in the SAOS-2 osteosarcoma cells induced senescent-associated flat cell formation (4, 14, 15, 20, 21, 30, 31). The cellular morphologic alteration was accompanied by up-regulation of ERM proteins and actin reorganization (20). ERM proteins have been found to regulate the function of the Rho subfamily of small GTPases involved in cellular morphology in part by binding Rho-GDI, a negative regulator of the small G proteins (25). Because pRb-induced senescence leads to inhibition of Rac1 activity (23), we surmised that ERM proteins and Rac1 activity might be linked in senescent cells. To further understand the mechanism of pRb-mediated cell shape change, we first examined the expression levels of Rho-GDI in pRb-transfected cells. The endogenous expression of Rho-GDI increased in a time-dependent manner as SAOS-2 cells became senescent in the presence of pRb (Fig. 1A). Similarly, ezrin expression was also up-regulated, consistent with previous data (20).

Ezrin possesses two conserved domains that have been termed N-ERM and C-ERM association domains, or ERMADs (32, 33). The intramolecular N/C-ERMAD interaction maintains ezrin in a closed, inactive form and results in masking of binding sites for adhesion molecules and filamentous-actin (34, 35). Regulation of ezrin is thought to occur through conformational changes consequent to posttranslational modifications that inhibit association of the N-ERMAD with the C-ERMAD (34, 35). Phosphorylation of T567 in ezrin has been found to be critical for conversion of ezrin to the active, open form competent for membrane localization and actin binding (36). In addition, our previous work showed that the up-regulated ezrin in senescent cells was phosphorylated by cdk5 at Thr(235) in the NH2-terminal region. This phosphorylation of T235 also prevents the intermolecular N/C-ERMAD association and contributes to senescence-related morphologic alterations (20). Here, we compared the phosphorylation of ezrin at T235 and T367 in senescent cells. T567 phosphorylation was observed 2 days after pRb expression and persisted as pRb-expressing cells underwent senescence, whereas phosphorylation of T235 was dramatically increased in senescent SAOS-2 cells 5 and 10 days following pRb expression (Fig. 1A).

Studies have found that the NH2-terminal halves of ERM proteins could directly bind to Rho-GDI (24, 27). Therefore, we assessed the physical interaction of Rho-GDI with ezrin in senescent cells. Cell lysates from pRb-transfected SAOS-2 cells were prepared 2, 5, and 10 days following transfection. These lysates were subjected to immunoprecipitation with anti-ezrin

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Rho-GDI Inhibits Rac1 in Senescent Cells

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Figure 1. Dissociation of ezrin from Rho-GDI in SAOS-2 cells transfected with pRb. A, SAOS-2 cells were transfected with empty vector (v) or pRb and selected with puromycin. 2, 5, 10, indicates days after transfection before cell lysis. Protein lysates of control or pRb-expressing SAOS-2 cells were prepared and subjected to immunoblot analysis for Rho-GDI, ezrin, phospho-ezrin (Thr567)/moesin (Thr558)/radixin (Thr564), phospho-Thr235, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The immunoprecipitates (IP) with anti-ezrin antibody were then detected with anti-phospho-Thr235 ezrin antibody. B, cell lysates were immunoprecipitated with anti-ezrin antibody followed with anti-Rho-GDI antibody. C, cell lysates were incubated with immobilized GST/Rho-GDI. Bound proteins were analyzed with anti-ezrin antibody. WB, Western blot.

antibody followed by immunoblot with anti–Rho-GDI antibody. The association of Rho-GDI with ezrin was low in parental SAOS cells transfected with empty vectors. Intriguingly, endogenous Rho-GDI was highly coupled with ezrin in 2-day pRb-transfected SAOS cells. This interaction decreased in a time-dependent pattern in pRb-expressing senescent cells (Fig. 1B), despite the increase in Rho-GDI steady-state levels, suggesting that the fraction of Rho-GDI not associated with ezrin is much higher in senescent cells than in proliferating SAOS-2 cells. To further study this interaction, lysates from SAOS-2 cells transfected with pRb were incubated with Rho-GDI GST fusion protein and then detected with anti-ezrin antibody. Similar to the coimmunoprecipitation result, only a very small amount of ezrin interacted with GST-Rho-GDI in lysates of SAOS-2 cells transfected with empty vectors, possibly due to the low expression level of ezrin and the presence of endogenous Rho-GDI. However, interaction between GST-Rho-GDI and ezrin strongly increased in cells transfected with pRb for 2 days. Interestingly, GST/Rho-GDI bound poorly to ezrin in extracts derived from cells undergoing pRb-induced senescence (Fig. 1C), despite high ezrin levels and low levels of endogenous Rho-GDI/ezrin complex. These results suggest that phosphorylation of ezrin might be critical for controlling the association of ezrin with Rho-GDI. Phosphorylation of T567 site in the COOH-terminal domain of ezrin at the early time point (2 days) may change the closed configuration of ezrin to the open form, which then exposes the NH2-terminal fragment of ezrin to allow coupling with Rho-GDI. However, as cells become senescent in the presence of pRb, NH2-terminal phosphorylation of ezrin at T235 occurs as a result of cdk5 activation (20). This modification might prevent the binding of ezrin to endogenous and to GST-Rho-GDI as observed above.

In attempt to show that the senescence-associated dissociation of the ezrin/Rho-GDI complex occurs in other, unperturbed settings, we assayed extracts of serially passaged IMR90 HDFs. Immunoblot of extracts of these cells shows that the expression levels of Rho-GDI and ezrin do not change appreciably with passage of these normal fibroblasts. Interestingly, T235 phosphorylation of ezrin increased in senescent cells, and T567 phosphorylation persisted (Fig. 2A), just as is observed in the SAOS-2 model system. In addition, lysates from early passage, middle passage, and senescent IMR90 cells were subjected to immunoprecipitation with anti-ezrin antibody followed by immunoblot with anti–Rho-GDI antibody. Rho-GDI could be clearly detected in ezrin immunoprecipitates from early passage cells but was absent from ezrin immunoprecipitates from senescent cell lysates (Fig. 2B). Similarly, the coupling of ezrin with Rho-GDI was also decreased in immunocomplexes using anti–Rho-GDI primary antibody followed by immunoblot with anti-ezrin antibody (Fig. 2C). These data show that phosphorylation of ezrin on T235 by cdk5 is a general property of cells

Figure 2. Dissociation of ezrin/Rho-GDI complex in IMR90 cells. A, IMR90 cells were passaged in culture and harvested for lysates at early passage (EP), middle passage (MP), or after undergoing senescence (S). Cell lysates were detected by immunoblot using anti-Rho-GDI, ezrin, phospho-ezrin (Thr567)/moesin (Thr558)/radixin (Thr564), phospho-Thr235, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. B, immunoprecipitates (IP) with anti-ezrin antibody were subjected to anti–Rho-GDI and anti-ezrin antibodies. C, cell lysates were precipitated with anti-Rho-GDI in lysates of serially passaged IMR90 HDFs. Immunoblot of extracts of these cells shows that the expression levels of Rho-GDI and ezrin do not change appreciably with passage of these normal fibroblasts. Interestingly, T235 phosphorylation of ezrin increased in senescent cells, and T567 phosphorylation persisted (Fig. 2A), just as is observed in the SAOS-2 model system. In addition, lysates from early passage, middle passage, and senescent IMR90 cells were subjected to immunoprecipitation with anti-ezrin antibody followed by immunoblot with anti–Rho-GDI antibody. Rho-GDI could be clearly detected in ezrin immunoprecipitates from early passage cells but was absent from ezrin immunoprecipitates from senescent cell lysates (Fig. 2B). Similarly, the coupling of ezrin with Rho-GDI was also decreased in immunocomplexes using anti–Rho-GDI primary antibody followed by immunoblot with anti-ezrin antibody (Fig. 2C). These data show that phosphorylation of ezrin on T235 by cdk5 is a general property of cells
undergoing senescence, and this modification of ezrin correlates with a reduction in ezrin/Rho-GDI complexes in senescent cells.

**Phosphorylation of ezrin at T235 releases Rho-GDI.** To assess the effect of phosphorylated ezrin on the association of ezrin with Rho-GDI, we used Flag-tagged wild-type ezrin and a series of mutants, including Flag-tagged T235A, T235D, T567A, T567D, T235A/T567A (TATA), or T235D/T567D (TDTD) ezrin. A, lysates were subjected to immunoprecipitation with anti–Rho-GDI antibody followed by immunoblot with either anti-Flag or anti-Rho-GDI antibody. B, cell lysates were incubated with immobilized GST/Rho-GDI and analyzed with anti-Flag antibody. As a control, an equal amount of lysates used for the pulldown was immunoblotted with anti-Flag antibody for total flag-tagged wild-type ezrin and its mutants. C, lysates were subjected to a GST-Pak pull down assay. The level of active Rac1 was detected with anti-Rac1 antibody. As a control, an equal amount of lysates used for the pulldown was immunoblotted with Rac antibody for total Rac1. WB, Western blot.

The decreased ability of ezrin T235D to couple with Rho-GDI might be due to an ability of 235D to mimic phosphorylation of T235. To test if phosphorylation of ezrin by cdk5 in transfected cells alters Rho-GDI binding in a manner similar to the T235D mutation, cell lysates from SAOS-2 cells transfected with or without p35/cdk5 were incubated with the NH2 terminus of ezrin, or NH2-terminal mutants T235A or T235D fused to GST. The complexes were then detected with Rho-GDI antibody. When equivalent amounts of GST/N-ezrin, GST/N-T235A, and GST/N-T235D were used in these assays (data not shown), only wild-type T235 ezrin strongly interacted with endogenous Rho-GDI in the absence of cdk5; T235A or T235D showed decreased binding to Rho-GDI (Fig. 4A). Interestingly, GST/N-ezrin failed to bind to Rho-GDI in the presence of cdk5, suggesting that cdk5 could phosphorylate the NH2-terminal fragment of ezrin at T235, preventing the interaction between ezrin and Rho-GDI (Fig. 4A). The binding of T235A and T235D, although weak, was not further decreased by cdk5 treatment. To further confirm that the dissociation of ezrin from Rho-GDI is due to the phosphorylation of ezrin by cdk5, cdk5 shRNA was generated as described (23). SAOS-2 cells transfected with shRNA vector, control shRNA, and cdk5 shRNA alone or pRB with control shRNA and cdk5 shRNA were subjected to selection with puromycin for 5 days. Immunoblot experiments revealed that cdk5 shRNA was able to efficiently inhibit the expression of endogenous cdk5, whereas various control shRNAs, including shRNA vector and control shRNA, had no effect on the expression of cdk5 (Fig. 4B). In addition, the presence of pRB stimulated cdk5 expression in cells transfected with pRB and control shRNA constructs (Fig. 4B), consistent with our previous observation (23). SAOS-2 cells were then transfected with pRB alone or with pRB plus cdk5 shRNA, dominant-negative cdk5, or cdk5 for 7 days. Cell lysates were incubated with GST/Rho-GDI and precipitated proteins detected with anti-ezrin antibody. Reduction of active cdk5 resulted in increased association of ezrin and Rho-GDI (Fig. 4C). Together, these results support the notion that phosphorylation of ezrin on T235 by cdk5 disrupts the coupling of ezrin with Rho-GDI, and this effect can be mimicked by the T235D mutation.

**Rho-GDI binds Rac1 in senescent cells and inhibits its activity.** In the course of cell activation, proteins of the Rho family (Rac, Rho, and Cdc42) are released from Rho-GDI. The release of expression levels of Flag-tagged ezrin and its mutants were constant as determined by direct immunoblot with Flag antibody. Rho-GDI binding was nearly undetectable when T235D and TDTD mutant forms of ezrin were used; in comparison, the coupling of wild-type ezrin with Rho-GDI was clear (Fig. 3B). Furthermore, the interaction between Rho-GDI and T235A or TATA mutants was slightly reduced, suggesting that T235A may not confer the fully wild-type function of ezrin (Fig. 3B). In addition, the pseudophosphorylated T567D variant slightly stimulated the association with Rho-GDI compared with the association of wild-type ezrin with Rho-GDI (Fig. 3B).

Studies have found that ezrin was involved in the regulation of small GTPase Rac1 activity (38). Here, we analyzed the effect of wild-type ezrin and its mutants on the activity of Rac1. Cell lysates pulled down with GST-conjugated PAK beads were detected with anti-Rac1 antibody. Total Rac1 expression showed no change in the direct immunoblot with Rac1 antibody (Fig. 3C). Intriguingly, T235D and TDTD variants of ezrin reduced the activity of Rac1, whereas T567D mutant slightly stimulated its activation. (Fig. 3C) These results indicated that phosphorylation of ezrin might play an important role in the mediation of Rac1 biological function.

**Rho-GDI Inhibits Rac1 in Senescent Cells**
GDI from these complexes is an important step, allowing the activation by guanine nucleotide exchange factors and membrane association of the GTP-bound members of Rho family through their isoprenyl group (39). Indeed, GDI binds in vitro to GDP-bound forms of these proteins with a 10-fold higher affinity than for the GTP-bound forms (40). We thus wished to determine if elevated levels of “free” Rho-GDI in senescent cells could contribute to the reduction of Rac1 activity we had previously observed (23). First, the expression levels of Rho family proteins were determined in SAOS-2 cells transfected with pRb for the indicated days. MDA-MB-468 cell lysates were used as positive control. Rac1 protein was expressed at high levels and had little change with or without pRb. However, the amount of RhoA and Cdc42 could not be visualized in SAOS cells while they are highly expressed in MDA-MB-468 cells (Fig. 5A). We postulated that Rho-GDI released from ezrin interaction following ezrin phosphorylation by cdk5 might interact with Rac1 in SAOS cells. Thus, cell lysates from SAOS-2 cells transfected with pRb or pRb with dominant-negative cdk5, cdk5, or cdk5 shRNA were immunoprecipitated with Rac1 antibody. The immunocomplexes were then subjected to immunoblot with Rho-GDI antibody. Expression of pRb or coexpression of cdk5 with pRb in senescent cells stimulated the interaction between Rho-GDI and Rac1. In contrast, shcdk5 reduced this association as did dominant-negative cdk5 albeit less effectively (Fig. 5B). Total Rac1 expression showed no change in the immunoprecipitates. These results strongly suggest that cdk5 is involved in the regulation of coupling between Rho-GDI and Rac1 at the expense of ezrin/Rho-GDI complexes. As we have previously shown that reduction in cdk5 activity in senescent cells increases Rac1 activity (23), these results together suggest that a cdk5-dependent decrease in ezrin-bound Rho-GDI can lead to increased Rho-GDI/Rac1 complexes and consequent Rac1 inhibition.

Maintenance of Rho-GDI expression is required for inhibition of Rac1 activity and senescent cell morphology. To test the function of Rho-GDI in senescence, a DNA-based hairpin siRNA construct (shRNA) specifically inhibiting the production of Rho-GDI was used. SAOS-2 cells transfected with Rho-GDI shRNA and control shRNA plasmids alone or pRb with Rho-GDI shRNA and control Rho-GDI shRNA were subjected to selection for 5 days. Immunoblot experiments showed that siRNA for Rho-GDI markedly reduced the expression of endogenous Rho-GDI, whereas various control siRNAs, including pBS/U6 control shGFP, GFP shRNA (shGFP), shRNA vector, and control shRho-GDI, had no effect on the expression of Rho-GDI (Fig. 6A). The presence of pRb stimulated Rho-GDI in cells transfected with pRb and control shRho-GDI constructs (Fig. 6A). Importantly, the decrease of Rho-GDI by shRNA reversed the loss of Rac1 activity in senescent cells mediated by pRb as indicated in cell lysates pulled down with GST-Pak followed by immunoblot with anti-Rac1 antibody (Fig. 6B). In contrast, pRb-induced senescent cells remained low in Rac1 activity in the presence of control Rho-GDI shRNA (Fig. 6B). In addition, SAOS-2 cells transfected with control shRho-GDI and Rho-GDI shRNA with or without pRb were selected with puromycin for 10 days, stained for senescence-associated β-gal expression, and photographed. The data show that even the partial reduction in protein expression of Rho-GDI by shRNA significantly reduced flat cell formation in the presence of pRb, but expression of nonspecific, control shRNA did not. The senescent cells with
pRb plus Rho-GDI shRNA continued to express senescence-associated \( \beta \)-gal but were markedly altered in morphology displaying a more retractile body with long projections, although Rho-GDI shRNA alone had little effect on the phenotype of parental cells in the absence of pRb (Fig. 7). These data support a role for Rho-GDI in the acquisition of the senescent morphology in this system.

Discussion

The present study shows that senescent morphogenesis is regulated by specific molecular changes. Senescent cell shape alteration induced by pRb involves the up-regulation of cytoskeleton ERM proteins that mediate actin organization. The phosphorylation of ezrin at T235 by cdk5 resulted in the dissociation of Rho-GDI from an ezrin/Rho-GDI complex. This released Rho-GDI then bound to and inhibited Rac1 activity to contribute to the senescence-associated cellular morphologic change.

ERM proteins exist in a closed configuration so that the NH\(_2\)-terminal and COOH-terminal domains interact with each other and mutually mask binding sites, preventing the interaction with actin filaments in the COOH-terminal region (41). When the NH\(_2\)-terminal domain of ERM is exposed, Rho-GDI binds to it, resulting in the release of GDP-bound members of Rho family. Indeed, GDI-1 directly interacts with the NH\(_2\) terminus of ERM proteins \textit{in vitro}, and the exogenous NH\(_2\) terminus of ezrin, introduced into intact COS-7 cells, replaced endogenous RhoA in the GDI-1 complex isolated from the cells (27). The displaced GDP-bound forms are attacked by GEP and efficiently converted to GTP-bound forms, which interact with downstream targets and ultimately regulate reorganization of the actin cytoskeleton (25, 42). In this way, ezrin can contribute to proliferative signaling by helping to activate Rho family GTPases. However, our work reported here suggests that regulation of GDI by ERM in senescent cells is much more complicated. Phosphorylation of T235 in the NH\(_2\)-terminal domain of ezrin by cdk5 and T567 in the COOH terminus by other kinases was increased in pRb-induced senescent SAOS-2 cells and senescent IMR90 HDFs, although T567 phosphorylation seems to be induced earlier (Figs. 1 and 2). These phosphorylation activities result in the dissociation of intermolecular N/C-ERMAD in senescent cells. The open form of ezrin then contributes to senescence-related flat cell formation (20). Interestingly, we have found that the interaction between ezrin and Rho-GDI decreased significantly in senescent SAOS-2 cells transfected with pRb and in senescent IMR90 HDFs, although Rho-GDI strongly coupled with

Figure 6. Effect of “knock-down” of endogenous expression of Rho-GDI on Rac1 activity. A, immunoblot for Rho-GDI was done with lysates from SAOS-2 cells or cells transfected with pBS/U6 control shGFP, pBS/U6 GFP shRNA, pBabe-puro/pBS/U6 vector, pBS/U6 Rho-GDI shRNA, pBS/U6 control shRho-GDI, pRb with pBS/U6 shRho-GDI, or pRb with pBS/U6 control shRho-GDI at 5 days after transfection and selection. B, cell lysates were pulled down with GST-Pak. The activation of Rac was detected with anti-Rac1 antibody.

Figure 7. Inhibition of the expression of Rho-GDI disrupted senescent cell shape. SAOS-2 cells were transfected with pBabe-puro plus pBS/U6, control shRho-GDI, shRho-GDI, pRb expression vector, pRb with control shRho-GDI, or pRb plus shRho-GDI. Cells selected with puromycin for 10 days were stained to detect expression of senescence-associated \( \beta \)-galactosidase (blue). Photomicrographs of representative fields are shown to illustrate effects of pRb or Rho-GDI RNAi constructs on cell morphology. Magnification, \( \times 60 \).
ezrin in SAOS-2 cells after a 2-day pRb introduction and in early-passaged IMR90 HDFs. This association might result from the early exposure of the NH2 terminus of ezrin because T567 phosphorylation precedes phosphorylation of T235, which seems strongly concomitant with the onset of the senescent phenotype.

An important role for phosphorylation of T235 in the prevention of the interaction of ezrin with Rho-GDI is supported by our observations using mutant forms of ezrin. The T235D mutant that mimics phosphorylation of Thr235 of ezrin bound poorly to Rho-GDI (Fig. 3). Furthermore, in vitro phosphorylation of ezrin by cdk5 resulted in the dissociation of Rho-GDI from Rho-GDI/ezrin complex, and blockade of the activation of cdk5 by cdk5 shRNA or dominant-negative cdk5 prevented dissociation of Rho-GDI and ezrin in senescent cells (Fig. 4). These findings suggest that ezrin (or other ERM proteins) can interact with Rho-GDI under normal growth conditions. As cells become senescent, elevated kinase activity of cdk5 (23) phosphorylates ezrin to disrupt the coupling of ezrin with Rho-GDI. The released Rho-GDI is then free to inhibit Rac1 activity in senescent cells. Interestingly, our data further suggest that in the absence of cdk5 or Rho-GDI, Rac1 activity increases dramatically following Rb reintroduction and senescence induction, perhaps as a consequence of continued mitogen signaling in pRb-arrested cells (23).

Although most cells produce multiple Rho family members, we have found that only Rac1 is inhibited in senescent cells. Recent studies have linked ezrin with the regulation of Rac1. Production of ezrin T567D in nonconfluent Madin-Darby canine kidney cells increases the amount of GTP-bound Rac1, whereas the level of GTP-bound RhoA or Cdc42 does not change (38). Interestingly, our observations using mutant forms of ezrin have revealed that the T235D mutant that mimics phosphorylation of Thr235 of ezrin reduced Rac1 activity (Fig. 3). This decreased GTP-bound form of Rac1 was accompanied by an overall reduction in the amount of Rho-GDI bound to exogenous ezrin. Together, with our previous studies showing reduction of Rac1 activity but not RhoA and Cdc42 activity in senescent cells (23), these results suggest that ezrin might specifically function upstream of Rac1 activation. Ezrin might act as a scaffold by recruiting the Rac1-negative regulator GDI to the membrane at sites where actin remodeling takes place under normal conditions. However, as cells undergo senescence, phosphorylation of ezrin by cdk5 may release GDI from a local ezrin/GDI complex at the membrane, and this GDI then interacts with Rac1 to inhibit its activation. Furthermore, phosphorylation of ezrin by cdk5 in senescent cells also favors the "open" configuration of ezrin, which exposes the actin binding site likely contributing to the senescence-associated, flattened phenotype (20).

The inhibition of Rac1 activation in senescent cells through the regulation of Rho-GDI and ezrin by cdk5 might contribute to the tumor suppressive function of cellular senescence. Rac activity has been reported to be involved in integrin-mediated cell motility and invasion (43). Indeed, such a role of Rac1 may partly explain recent findings that suggest a role for ezrin in metastasis (42, 44, 45). Furthermore, biochemical experiments have connected Rac1 with merlin, an ERM-related protein. Merlin is regulated by phosphorylation in a Rac/cdc42-dependent fashion. The phosphorylation of merlin at Ser510 induced by the p21-activated kinase PAK results in the inactivation of merlin’s activity as a tumor suppressor (46, 47). In addition, loss of merlin exhibits characteristics of cells expressing activated Rac (46–48). Thus, the reduced activity of Rac1 GTPase might regulate merlin/ezrin association or other functions of merlin in senescent cells and favor the antiproliferative role of merlin. Indeed, our preliminary results indicate that an interaction between ezrin and merlin occurs concomitant with the onset of senescence.

Our data from Rho-GDI knockdown experiments further indicates that the presence of Rho-GDI is necessary for the senescent morphology. Blockade of Rho-GDI production by shRNA efficiently prevented the flat cell formation of senescent cells induced by pRb. Cells expressing Rho-GDI shRNA displayed long projected protrusions (Fig. 7), indicating that Rho-GDI–mediated inhibition of Rac1 plays an important role in the senescence-related morphology, but this down-regulation of Rac1 is likely to be only one aspect of multiple biochemical changes required to generate the complete senescent phenotype. Indeed, our previous studies showed that cotransfection of pRb with activated Rac1V12 reduced flat cell formation and senescence-associated β-gal activity (23). However, cells expressing pRb and Rac1V12 or pRb and shRho-GDI are not of identical shape, suggesting that high levels of Rac1 may have additional effects on the cells or that Rho-GDI may have roles that extend beyond regulation of Rac1 in this system. Recent studies show that blockade of the ability of Rho-GDI to bind to constitutively active Cdc42 results in a transformation defect in NIH 3T3 fibroblasts (49). Moreover, the introduction of Rho-GDI siRNA into active Cdc42-transfected cells also inhibits their transformation, indicating that Rho-GDI has both negative and positive functions in the regulation of Rho proteins (49). Thus, further investigation of the details of Rho-GDI’s role in the process of cellular senescence is important and will lead to a better understanding of senescence as a tumor-suppressive process.

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References

Phosphorylation of Ezrin by Cyclin-Dependent Kinase 5 Induces the Release of Rho GDP Dissociation Inhibitor to Inhibit Rac1 Activity in Senescent Cells

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