RhoA Regulates G1-S Progression of Gastric Cancer Cells by Modulation of Multiple INK4 Family Tumor Suppressors

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Abstract

RhoA, a member of the Rho GTPase family, has been extensively studied in the regulation of cytoskeletal dynamics, gene transcription, cell cycle progression, and cell transformation. Overexpression of RhoA is found in many malignancies and elevated RhoA activity is associated with proliferation phenotypes of cancer cells. We reported previously that RhoA was hyperactivated in gastric cancer tissues and suppression of RhoA activity could partially reverse the proliferation phenotype of gastric cancer cells, but the underlying mechanism has yet to be elucidated. It has been reported that RhoA activation is crucial for the cell cycle G1-S procession through the regulation of Cip/Kip family tumor suppressors in benign cell lines. In this study, we found that selective suppression of RhoA or its effectors mammalian Diaphanous 1 and Rho kinase (ROCK) by small interfering RNA and a pharmacologic inhibitor effectively inhibited proliferation and cell cycle G1-S transition in gastric cancer lines. Down-regulation of RhoA-mammalian Diaphanous 1 pathway, but not RhoA-ROCK pathway, caused an increase in the expression of p21Waf1/Cip1 and p27Kip1, which are coupled with reduced expression and activity of CDK2 and a cytoplasmic mislocalization of p27Kip1. Suppression of RhoA-ROCK pathway, on the other hand, resulted in an accumulation of p16INK4a, p16INK4a, p18INK4c, and p19INK4d, leading to reduced expression and activities of CDK4 and CDK6. Thus, RhoA may use two distinct effector pathways in regulating the G1-S progression of gastric cancer cells. (Mol Cancer Res 2009;7(4):570–80)

Introduction

Gastric cancer is a disease of poor prognosis and high mortality. Although a recent decline in both incidence and mortality has been noted, it is the fourth most commonly occurring cancer and the second leading cause of cancer-related death worldwide (1, 2). There is a marked geographic variation in incidence of gastric cancer: China, Japan, and Korea have high rates of gastric cancer and account for more than half of all cases worldwide, whereas countries in North America, western Europe, Australasia, and Africa have found lower gastric cancer rates (2).

Rho family GTPases contribute to the regulation of multiple biological processes including cell size, cell cycle progression, apoptosis/survival, morphology, cell polarity, cell adhesion, and membrane trafficking (3). There are 23 mammalian members of Rho GTPases, with RhoA, Rac1, and Cdc42 as the best characterized ones. Like Ras, Rho GTPases act as molecular switches to control signal transduction pathways by cycling between a GDP-bound, inactive form and a GTP-bound, active form. Once activated, Rho GTPases bind to and activate several downstream effectors, leading to their biochemical responses (4). For RhoA GTPase, several proteins including protein kinase N, rhophilin, rhotekin, mammalian Diaphanous 1 (mDia1), Rho kinase (ROCK), and citron kinase have been identified as direct effectors. Both mDia1 and ROCK have been recognized as key mediators of the RhoA effect on cell actin reorganization, cell proliferation, and cytokinesis (5). Up-regulation of RhoA activity has often been associated with tumorigenesis (6). Overexpression of either RhoA itself or its upstream or downstream signaling elements has been detected in several human tumors, including pancreatic cancer, non-inflammmatory breast cancer, melanomas, lung cancer, colorectal cancer, and gastric cancer (7, 8). Inhibitory mutants of RhoA can prevent Ras-induced transformation of fibroblasts, whereas overexpression of a constitutively active mutant of RhoA can transform NIH 3T3 cells and promote the invasive potential of cultured rat hepatoma cells (9, 10). When RhoA was inhibited by the Clostridium botulinum C3 toxin or by a dominant-negative mutant in Swiss 3T3 cells, the G1-S cell cycle progression was significantly impaired. Furthermore, active RhoA mutant could induce G1-S-phase progression in serum-starved quiescent cells (11, 12). RhoA has thus been suggested to contribute to cancer cell transformation.

Abundant observations indicate that RhoA may regulate cell cycle progression by affecting several critical components of...
the cell cycle machinery, including the activities of cyclin-dependent kinases (CDK) and CDK inhibitors (CKI). Activated Ras was unable to promote the G1-S progression in Swiss 3T3 fibroblasts when RhoA activity was suppressed, which in part was attributed to an accumulation of high level of p21Waf1/Cip1 (13, 14). Similarly, there is evidence suggesting that RhoA activation is crucial for p27Kip1 down-regulation and RhoA-mediated activation of ROCK and mDia1 can promote G1 progression through Skp2-mediated degradation of p27Kip1 (15-18). However, dominant-negative CDK2 can prevent the down-regulation of p27Kip1 in response to the expression of an active RhoA mutant, which suggests that RhoA may also promote p27Kip1 degradation via the activation of cyclin E/CDK2 complex (19). It is possible that RhoA regulates cell proliferation and cell cycle progression through p21Waf1/Cip1 and p27Kip1.

Within the CKI family, whether INK4 proteins have a role in the RhoA-mediated cell cycle progression has yet to be examined. In the present study, by using specific small interfering RNA (siRNA) and small-molecule inhibitors, we have assessed the effect of the suppression of RhoA or its effectors on cell growth, cell cycle progression, and the associated cell cycle protein alterations in the human gastric AGS cell line, which has elevated RhoA expression and activity. Our results show that suppression of RhoA or its effectors, mDia1/ROCK, inhibits the gastric cancer cell growth and causes G1-S cell cycle arrest. This effect is due to increased INK4 family proteins as well as an accumulation of the Cip/Kip family proteins. We conclude that RhoA may use ROCK and mDia effector pathways in regulating G1-S progression of gastric cancer cells by modulation of the INK4 family cell cycle inhibitors.

Results
RhoA Expression in Gastric Cancer Cell Lines and the Normal Gastric Mucosal Epithelial Cell Line GES-1
RhoA expression in three gastric cancer cell lines and normal gastric mucosal epithelial cell line GES-1 were investigated by Western blotting (Fig. 1A). Significantly increased expression of RhoA in all three gastric cancer cell lines, SGC7901, AGS, and MKN45, by comparison with that of the GES-1 cell line, was observed. This is consistent with our previous results showing that RhoA-GTP level in these cancer cells is also elevated (7). We chose to use the AGS cell line as our experimental model in the following studies.

Effect of RhoA and Effector Knockdown on Gastric Cancer Cell Proliferation
Previous studies have shown that RhoA contributes to cell proliferation in many cell types. We used specific siRNA against RhoA or mDia1, and the ROCK inhibitor Y27632, to specifically block RhoA and its signaling pathways. The efficiency of inhibition by the respective siRNA oligonucleotides was >90% as determined by immunoblotting (Fig. 1B). After treatment with the siRNAs or Y27632, cell growth rates were quantified as described in Materials and Methods. All treated cells showed significantly reduced growth (P < 0.01) compared with the untreated groups. Although both the siRNAs and Y27632 can inhibit AGS cell growth, a significantly stronger inhibitory effect was observed in the cells with double suppression of mDia1 and ROCK compared with inhibition of mDia1 alone. In addition, the growth inhibition of Y27632-treated cells was more evident than the mDia1 knockdown group (Fig. 2). These results indicate that RhoA, through its effectors mDia1 and ROCK, play a key role in AGS cell proliferation.

Regulation of Cell Cycle Progression
To further understand the mechanisms of the growth inhibition, the effect of the suppression of RhoA or its effectors on the cell cycle regulation of AGS cells was analyzed by propidium iodide staining and flow cytometric analysis (Fig. 3). To varying extents, the cell cycle was found accumulated at the G1 phase after the application of the RhoA, mDia1, or ROCK inhibitors, which is associated with a corresponding decrease in S and G2-M phases when compared with the untreated control cells. Consistent with the results of cell growth modulation, double suppression of mDia1 and ROCK showed an accumulation of 53.0% in the G1 phase, whereas 49.2%, 46.3%, and 50.3% cells in the G1 phase were found in the RhoA, mDia1, and ROCK suppressed cells, respectively. There is a statistical significance between each of treated groups and the untreated groups. Furthermore, double suppression of mDia1 and ROCK

FIGURE 1. Expression of RhoA in gastric cancer cell lines and effects of RhoA and mDia1 siRNAs. A. Expression of RhoA in three gastric cancer cell lines and normal gastric mucosal epithelial cell line GES-1 were examined by Western blotting. There were higher expressions of RhoA in the three gastric cancer cell lines, SGC7901, AGS, and MKN45, compared with that of the GES-1 cell line. B. Gastric cancer AGS cells were transiently transfected with three Stealth duplexes of siRNA of RhoA or mDia1, respectively, by Lipofectamine and nonspecific duplex was taken as the control. Forty-eight hours later, total proteins were extracted and subjected to analysis on 15% or 8% SDS-PAGE followed by Western blotting. Membranes were probed with anti-RhoA, mDia1, and β-actin antibodies followed by peroxidase-conjugated appropriate secondary antibodies and visualized by enhanced chemiluminescence detection system. Efficiency of inhibition of siRNA oligonucleotides of RhoA and mDia1 was >90%. Representative experiment of three determinations with similar results.
group of cells showed significant difference from that of the cells treated with siRNA of mDia1 alone. These results suggest that the growth-inhibitory effect of RhoA inhibition is the result of a block in the cell cycle at G1 phase and that both ROCK and mDia1 contribute to the RhoA regulated G1-S-phase progression.

**Alteration of Cip/Kip Family Proteins and INK4 Family Proteins**

CKIs are well known to interfere with cell cycle progression and their deregulation can cause cell cycle phase-specific arrest. These cell cycle kinase inhibitors perturb the phosphorylation regulation of their target proteins, CDKs. The protein levels of Cip/Kip family members are crucial for the regulation of G1-S progression. We next investigated the expression patterns of the Cip/Kip family members under the RhoA pathway-specific inhibitors by Western analysis (Fig. 4A and B). Both siRNAs against RhoA and mDia1 caused a significantly accumulation of p21Waf1/Cip1 and p27Kip1 proteins compared with AGS control cells. On the contrary, Y27632 did not significantly alter the expression of p21Waf1/Cip1 or p27Kip1. These findings suggest that RhoA-mediated p21Waf1/Cip1 and p27Kip1 expression is likely through mDia1-dependent, but not the ROCK-dependent, pathway in the AGS cells.

The INK4 family members are differentially expressed during mammalian development. The diversity in the patterns of expression of INK4 genes suggests that this family of cell cycle inhibitors may have cell lineage-specific or tissue-specific functions. Apart from their physiologic roles, the INK4 proteins are often inactivated in cancer, and they are established tumor suppressors (20, 21). Although prior studies have shown that INK4 proteins have an important role in the regulation of the cell cycle, little is known with respect to whether they are involved in the regulation of cell cycle progression by RhoA. Next, we investigated their possible alteration at both mRNA and protein levels in our model system. By real-time PCR (RT-PCR), we found that the mRNA levels of all INK4 family members were significantly increased in the RhoA or effector inhibited cells comparing with control cells (Fig. 4E-H). This phenomenon is more evident in the mDia1 and ROCK double-blocking cells with an increase of \( \sim 10.24 \)-fold in p15INK4b, 17.9-fold in p16INK4a, and 18.05-fold in p18INK4c, and higher mRNA expression of p19INK4d was also found in the Y27632-treated cells. The changes of the INK4 family member expression appear to be more modest in the mDia1 cells than that of other experimental groups. In support of these observations, the protein expression patterns of the four INK4 family members were found to be altered in a similar trend as the mRNAs (Fig. 4C and D). These results suggest that RhoA may modulate the INK4 family members through the ROCK pathway, which is distinct from the mechanism of regulation of the Cip/Kip family proteins.

**Subcellular Localizations of p27Kip1 and p21Waf1/Cip1**

The subcellular localizations of p27Kip1 and p21Waf1/Cip1 are closely tied to their functions. p27Kip1 is mostly localized in the nuclei under normal physical conditions but is largely excluded from the nuclei in many tumor cells. There is considerable evidence showing that the cytoplasmic localization of p27Kip1 correlates with high tumor grade and poor prognosis (22-24). With this in mind, we performed immunofluorescence assays to investigate the subcellular localization of p21Waf1/Cip1 and p27Kip1 proteins in AGS cells after blocking RhoA pathways (Fig. 5). The number of p21Waf1/Cip1- and p27Kip1-positive...
cells, as well as increased staining intensity of p21<sub>Waf1/Cip1</sub> and p27<sub>Kip1</sub>, was found higher in the RhoA or mDia1 siRNA-treated cells than in control cells. Abundant p27<sub>Kip1</sub> appeared in the cytoplasm after RhoA or mDia1 suppression, whereas p21<sub>Waf1/Cip1</sub> was mostly localized in the nucleus. As controls, both p21<sub>Waf1/Cip1</sub> and p27<sub>Kip1</sub> were found only in the nucleus with significantly lower levels in Y27632-treated cells or untreated control cells. These observations raise the possibility that the RhoA-mDia1 pathway is involved in regulating p27<sub>Kip1</sub>, but not p21<sub>Waf1/Cip1</sub>, nucleus localization.

Alteration of Cyclins and CDKs

It is well known that CDK2, CDK4/6, cyclin D, and cyclin E cooperate to promote G1-phase progression. Generally, these CDKs are negatively regulated by CKIs, including the INK4 family members. We found that by blocking RhoA, mDia1, or ROCK, the protein levels of CDK2 and CDK4/6 were downregulated (Fig. 6A and B), whereas the cyclin D1 and cyclin E levels appeared unaffected (data not shown). Additionally, the activities of CDK2 and CDK4/6 were analyzed by an in vitro kinase assay using a Rb-C fusion protein as a substrate. This
substrate fusion protein contains maltose-binding protein, the COOH-terminal region (701-928) of pRb, which can be phosphorylated by various cyclin-CDK complexes. Because pRb residue Ser795 is preferentially phosphorylated by CDK2 or CDK4/6 (25), we measured CDK activities by detecting Ser795-specific phosphorylation of Rb-C by Western blot analysis. As shown in Fig. 6C and D, the activity of CDK2 was strongly reduced in the siRNA mDia1 cell group as well as in the RhoA knockdown cells but not in the ROCK-targeted cells, suggesting that RhoA regulates CDK2 activity through the mDia1 pathway. A significant inhibition of CDK4/CDK6 expression and activities in the Y27632-treated cells was observed, similar to the RhoA-targeted cells, suggesting that CDK4/CDK6 is modulated by the RhoA-ROCK pathway. Overall, our data suggest that RhoA and its effectors mDia1 and ROCK regulate the CDK2 and CDK4/6 at the expression and/or activity level, which may involve multiple inputs through cyclins and CKIs as well as transcriptional machineries.

Phosphorylation and Induction of Rb Family Proteins

CKIs regulate the activity of CDKs, which in turn regulate phosphorylation of several substrates, including pRb. Rb is a key regulator of cell cycle G1-S progression. Phosphorylation of specific pRb serine or threonine residues has been shown to regulate the binding of Rb with different targets, including E2F family members, and inhibit their activities. Next, the phosphorylation status of Rb was examined by immunoblotting using antibodies directed against phosphorylated Rb (Ser780, Ser795, and Ser807/Ser811) and Rb (Fig. 7A and B). As expected, phosphorylation at Ser795 and Ser807/Ser811 was preferentially inhibited by RhoA or mDia1 knockdown, whereas a relatively milder change was found in the ROCK inhibitor-treated cells. In contrary, the changes of phosphorylation at Ser780 did not appear to be significantly affected. These observations suggest that phosphorylation of Rb at Ser795 and Ser807/Ser811 can be regulated by the RhoA-mDia1 pathway in AGS cells.

Regulation of the E2F Family Transcriptional Factors

The E2F/Rb pathway is a major regulatory component for genes that are required for S-phase entry. Hypophosphorylated Rb binds to and occludes the transactivation potential of E2F transcription factors. To determine the functional consequences of persistently hypophosphorylated Rb proteins at late G1-S transition, during which a complex is formed by Rb with E2F, the expressions of E2F1, E2F2, and E2F3 in the whole-cell lysates were investigated, and the Rb-bound E2Fs were analyzed by immunoprecipitation. There was a marked increase in the complex formation of Rb/E2F1, Rb/E2F2, and Rb/E2F3 in all treated cells compared with control cells (Fig. 8C and D), whereas the protein expression levels of E2F1, E2F2, and E2F3 were unaffected (Fig. 8A and B). These results indicate that RhoA and its effectors regulate the binding of Rb to the E2F family members but do not affect the expression of these proteins. The complex formation of Rb with E2F regulated by RhoA requires both the mDia1 and the ROCK pathways.

Discussion

Several studies have shown that RhoA is overexpressed in cancer tissues compared with that of normal tissues (7, 8). Overexpression of RhoA in NIH 3T3 cells causes the cells to become tumorigenic in nude mice. Transformation by RasV12 can be suppressed by dominant-negative RhoA. These evidence strongly suggest that RhoA plays an important role in tumorigenesis (6, 26-28). Our previous work has shown that RhoA was overexpressed and highly activated in seven gastric cancer cell lines (7). In the present study, we further show that there is elevated expression of RhoA in three gastric cancer cell lines and found that RhoA-specific siRNA could significantly inhibit the proliferation and tumorigenesis of AGS cells (29). Previous studies have indicated that RhoA mainly regulates the G1-S cell cycle progression through Cip/Kip family (18, 30), and RhoA has also been linked to transcription of p21Waf1/Cip1 (13) and cyclin D1 (31, 32) and the degradation
of p27Kip1 through regulation of cyclin E/CDK2 activity (19, 32, 33). However, these studies were conducted in fibroblast cells, and the mechanistic role of RhoA in the proliferation of cancer cells remains unclear. By using the gastric cancer cell line AGS as a cell model, we show here that not only p21Waf1/Cip1 and p27Kip1, but also the INK4 family members, may be intimately involved in RhoA regulation of G1-S cell cycle progression, and RhoA depends on mDia1 and ROCK differentially in this process.

Accumulating evidence suggest that the INK4 family could have important roles in tumor suppression. The INK4 class of cell cycle inhibitors, p15INK4b, p16INK4a, p18INK4c, and p19INK4d, are homologous inhibitors of the CDKs, CDK4 and CDK6, which promote cell proliferation (21, 34, 35). Knockout studies of mice specifically deficient for p15INK4b and p16INK4a have revealed that they are more prone to spontaneous cancers than wild-type littermates (36). Overexpression of p15INK4b and p16INK4a in mice also maintains its role in tumor suppression (37). Latres et al. reported that p18INK4c-null mice also exhibit hyperplasia and pituitary tumors (38). In the current study, we found the expression of four INK4 family members all increased to a different extent, in both the protein and the mRNA levels, after the RhoA pathways were blocked. In fact, loss of p15INK4b, p16INK4a, and p18INK4c by gene mutation, deletion, and/or methylation has been reported in a variety of human cancers (39, 40). Clinical findings have revealed that loss of p16INK4a function is correlated with a poor prognosis in some human cancers (41). These studies have suggested that INK4 family members might play a negative role in the progression and prognosis of human cancers. Accordingly, our study indirectly supports these findings. We found that there were mild elevations of the INK4 family members in RhoA or mDia1 knockdown cells, but remarkable changes were found in the double suppression of mDia1 and ROCK or ROCK alone, suggesting that RhoA regulates the expression of INK4 family members through the ROCK pathway.

In addition to the INK4 proteins, up-regulation of p21Waf1/Cip1 and p27Kip1 was found in RhoA or mDia1 knockdown cells but

FIGURE 6. Effects of inhibition of RhoA and its effectors on the expression and activities of CDKs in AGS cells. Cells were treated with the different inhibitors separately. At the end of the treatments, whole-cell lysates were prepared and subjected to immunoblotting and immunoprecipitation. Loading volume was normalized with respective protein contents. A. Expression of CDK2, CDK4, and CDK6 was analyzed by Western blotting. β-Actin was used as a loading control. Representative blot of each protein from three independent experiments that yielded similar results. B. Protein expression levels of CDK2, CDK4, and CDK6 were measured by quantitative Western blotting. Densitometry data presented in graphical form are “fold change” compared with AGS cells control after normalization with respective loading control (β-actin). Mean ± SD (n = 3), * P < 0.05 versus AGS cells control. C. CDK2, CDK4, and CDK6 were immunoprecipitated from whole-cell lysates by using specific antibodies. Their activities were tested by the kinase assay employing Rb fusion protein as a substrate. Samples were subjected to be analyzed by 12% SDS-PAGE and immunoblotting with anti-pRb-Ser780 antibody. β-Actin was used as a loading control. Representative blot from three independent experiments that yielded similar results. D. Activities of CDK2, CDK4, and CDK6 were quantified by densitometry. All data of relative activity presented in graphical form are “fold change” compared with AGS cells control after normalization with respective loading control (β-actin). Mean ± SD (n = 3), * P < 0.05 versus AGS cells control.
not in the Y27632-treated cells. There is abundant evidence suggesting that RhoA regulates the expression of p21Waf1/Cip1 and p27Kip1, which influence the progression of the G1-S cell cycle. For example, RhoA represses p21Waf1/Cip1 expression in oncogenic Ras-induced G1-S-phase progression in Swiss 3T3 fibroblasts (13). Inhibition of RhoA and ROCK resulted in increased p21Waf1/Cip1 expression through an extracellular signal-regulated kinase-dependent pathway and decreased smooth muscle cell proliferation (42, 43). However, similar to our results, in Ras-transformed fibroblasts or colon cancer cell lines, suppression of p21Waf1/Cip1 mediated by RhoA does not require ROCK, suggesting that the modulation of p21Waf1/Cip1 expression by RhoA is mediated by cell type-dependent signaling pathways (44-46). Suppression of RhoA has been reported to elevate p27Kip1 protein levels (19, 33), but when shape-restricted capillary cells on low fibronectin were treated with Y27632, the p27Kip1 expression decreased (18). Zuckerbraun et al. reported that RhoA did not appear to be involved in the regulation of p27Kip1 expression in smooth muscle cells (42), whereas Mamamoto et al. reported that RhoA-mediated activation of ROCK and mDia1 can promote G1 progression by inhibiting p27Kip1 (18). Our results suggest that RhoA modulates the expression of p21Waf1/Cip1 and p27Kip1 through the mDia1 pathway in AGS cells. The inconsistencies in the literature may be due to the fact that different cell lines were examined and suggest that the modulation mechanism of p21Waf1/Cip1 and p27Kip1 expression by RhoA warrants further study in specific cell lineages.

Recent studies provide in vivo evidence that, in addition to their roles as tumor suppressors, p21Waf1/Cip1 and p27Kip1 may also function as oncogenes in certain tumor cells when they mislocalize to the cytoplasm (47, 48). Cytoplasmic localization of p27Kip1 was shown to correlate with high tumor grade and poor prognosis in human cancers, which suggests an active tumor-promoting function of p27Kip1 in the cytoplasm (22-24). Likewise, when p21Waf1/Cip1 translocated to the cytoplasm, it can inhibit caspase-3, thus inhibiting apoptosis (49). In our study, significant cytoplasmic localization of p27Kip1 was observed in AGS cells on RhoA down-regulation. However, p21Waf1/Cip1 was found mostly in the nucleus in these cells. We caution that the anti-p27 antibody we employed for immunofluorescence studies needs more careful examination for specificity. Besson et al. found that the p27Kip1CK-/CK- knock-in mice, a mutant p27Kip1 that can no longer bind with cyclin/CDKs, developed a whole range of hyperplastic and neoplastic lesions, including lung adenocarcinomas and pituitary tumor. Intriguingly, the p27Kip1CK-/CK- mutant also localizes in the cytoplasm, further suggesting that a cytoplasmic function might mediate the oncogenic effect (48). How our observed cytoplasmic localization of p27Kip1 in the RhoA or mDia1 knockdown AGS cells affects the RhoA-mediated function will require further examination by p27Kip1 suppression.

Consistent with our previous observations, siRNA suppression of RhoA slowed the growth of gastric cancer AGS cells. The suppression of either ROCK or mDia1 can also inhibit AGS cell growth, but it seems that Y27632 is more efficient than the mDia1 siRNA in the inhibition of the AGS cell proliferation. Fluorescence-activated cell sorting analysis revealed that the cell cycle was arrested by suppression of RhoA and its downstream effectors at the G1-S-phase transition. Other studies have shown that RhoA and its effectors promoted cell cycle progression of G1-S (18, 32). Our data indicate both mDia1 and ROCK are important mediators in this pathway and both the Cip/Kip family and the INK4 family are involved in the regulation of G1-S transition in AGS cells. It is possible that the RhoA-ROCK-INK4 pathway plays an important role in the G1-S transition in the gastric cancer AGS cells. Another study reported that p27Kip1 showed a greater antitumor effect than that of the other CKIs (p16INK4a, p18INK4c, p19INK4d, and Ser780/Ser811 antibodies and anti-Rb antibody. Loading volume was normalized with respective protein contents. A. Representative Western blots. B. Quantification of three independent experiments. Densitometry data presented in graphical form are “fold change” compared with AGS cells control after normalization with respective “loading control” (total protein of Rb). Mean ± SD (n = 3), *, P < 0.05 versus AGS cells control.

FIGURE 7. Suppressions of RhoA, mDia1, and/or ROCK inhibit Rb phosphorylation in AGS cells. Cells were treated with the different inhibitors separately. Forty-eight hours later, total cell lysates were prepared and subjected to immunoblotting with Phosphoplus anti-Rb: Ser780, Ser795, and Ser807/Ser811 antibodies and anti-Rb antibody. Loading volume was normalized with respective protein contents. A. Representative Western blots. B. Quantification of three independent experiments. Densitometry data presented in graphical form are “fold change” compared with AGS cells control after normalization with respective “loading control” (total protein of Rb). Mean ± SD (n = 3), *, P < 0.05 versus AGS cells control.
Our data revealed that the expression and activities of CDK2, CDK4, and CDK6 were reduced to a different extent by blocking of RhoA, mDia1, or ROCK, and this effect was associated with the hypophosphorylation of Rb protein. Previous studies have reported that RhoA activation is associated with activation of the cyclin D1 promoter in mammary epithelial cells and functions as the master regulator of cyclin D1 expression and G1 progression in NIH 3T3 cells (30, 32). We did not find detectable changes of cyclin D1 or cyclin E but observed an inactivation of related CDKs in our system. Furthermore, we found that the phosphorylation of Rb at Ser780, Ser795, and Ser807/Ser811 sites were decreased, and the binding of Rb to E2F was elevated. Because Rb sequesters the E2F family transcription factors, the E2F family regulating proteins required for S-phase DNA synthesis are reduced by RhoA inhibition, affecting G1-phase progression.

In summary, suppression of RhoA and its effectors, mDia1 and/or ROCK, results in G1-S cell cycle arrest in gastric cancer AGS cells. The INK4 family members, p15INK4b, p16INK4a, p18INK4c, and p19INK4d, as well as the p21Waf1/Cip1 and p27Kip1, are all up-regulated after the suppression. In this process, RhoA regulates the INK4 family members through the ROCK pathway, but the Cip/Kip family members are regulated by the RhoA-mDia1 pathway. The alterations cause changes in the expression and activation of related CDKs, allowing Rb proteins to sequester E2F family transcription factors. Our study provides insight into the regulatory function of RhoA in gastric cancer cell growth and suggests new molecular targeting therapies in the fight against gastric cancer.

Materials and Methods

Antibodies

Antibodies against p15INK4b (sc-612), p16INK4a (sc-9968), p18INK4c (sc-9965), p19INK4d (sc-1063), mDia1 (sc-10886), and RhoA (sc-418) were from Santa Cruz Biotechnology. Antibodies to p21Waf1/Cip1 (2946), p27Kip1 (2552), CDK2 (2546), Rb (Ser780, Ser795, and Ser807/Ser811), Kit (9300), and β-actin

![FIGURE 8. Effect of suppression of RhoA, mDia1, and ROCK on the expression of E2F and the binding of Rb to E2F in AGS cells. Cells were treated with the different inhibitors separately. At the end of the treatments, whole-cell lysates were prepared and subjected to immunoblotting and immunoprecipitation. Loading volume was normalized with respective protein contents. A. Expression of E2F1, E2F2, and E2F3 were analyzed by Western blotting. β-Actin was used as a loading control. Representative blot of each protein from three independent experiments that yielded similar results, respectively. B. Protein expression levels of E2F family members were quantified by densitometric analysis of Western blotting. Densitometry data presented in graphical form are “fold change” compared with AGS cells control after normalization with respective loading control (β-actin). Mean ± SD (n = 3). *, P < 0.05 versus AGS cells control. C. Total protein extracts were subjected to immunoprecipitation with anti-Rb antibody followed by SDS-PAGE on 12% gels and Western blotting. Membranes were probed with anti-E2F1, anti-E2F2, anti-E2F3, and anti-Rb antibody separately followed by peroxidase-conjugated appropriate secondary antibody and visualization by the enhanced chemiluminescence detection system. Representative blot of each protein from three independent experiments that yielded similar results, respectively. D. Binding levels of Rb to E2F family members were quantified by densitometric analysis. Densitometry data presented in graphical form are “fold change” compared with AGS cells control after normalization with total Rb protein. Mean ± SD (n = 3). *, P < 0.05 versus AGS cells control. IP, immunoprecipitation; WB, Western blotting.](mcr.aacrjournals.org/article-fig8)
(4970) were from Cell Signaling Technology. Antibodies against CDK4 (06-139) and E2F3 (05-551) were from Upstate Biotechnology. Antibodies against E2F1 (554312) and E2F2 (556525) were from BD Pharmingen Technical. Antibody to CDK6 (AHZ0242) was from Biosource. Antibodies against cyclin D1 and cyclin E are from Calbiochem. Goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated antibodies were from Pierce.

Cell Lines and Cell Culture

Gastric cancer cell lines SGC7901, AGS, and MKN45 and immortal gastric mucosal epithelial cell line GES-1 were all preserved in our institute (7). All cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Life Technologies), 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Sigma). Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

siRNA Transfection

Three duplexes of siRNA sequences against RhoA and mDia1 molecules were chemically synthesized by Invitrogen. siRNA oligonucleotides were prepared following the manufacturer's instructions. In preliminary experiments, we investigated the expression of RhoA in the gastric cancer cell lines and normal GES-1 cell line with Western blotting. Subsequently, we optimized conditions for efficient transfection and determined the efficacy of siRNA oligonucleotides using Western blotting. We selected one duplex from the three, which had the strongest interfering effect. The efficiencies of inhibition of both RhoA (sense 5′-UGAGCAAGCAUCCACAGGC-3′ and antisense 5′-GCCUGUGGAAAGACUGCUUGCUCA-3′) and mDia1 (sense 5′-AAACUCUCACUCUACGUAAC-3′ and antisense 5′-GGAGUUAAGAGAGUAGGAGCUCU-3′) were >90%.

In transfection experiments, cells were plated on dishes containing antibiotic-free medium and incubated overnight. When the cells were at 40% to 50% confluence, siRNA oligonucleotides transfection was done with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, and a nonspecific duplex was used as the control. After 24 h, the medium was replaced with the fresh medium. Y27632 (Sigma), an inhibitor of ROCK, was dissolved in water and used at a concentration of 2 μM for 24 h.

Western Blotting

For analysis of cell cycle proteins, cultured cells were prepared as described above, placed on ice, and rinsed three times with 10 mL ice-cold PBS. The cells were lysed on the plate by the addition of radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP-40, 50 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 mM leu-2-μM NaCl, 100 mM Na3VO4, 1 μg/mL leupeptin] containing a cocktail of protease inhibitors and phosphatase inhibitors as described previously. Samples of total protein (1 mg) were incubated with anti-Rb primary antibody and rotated end-up overnight at 4°C followed by incubation with Gammabind plus Sepharose (GE Healthcare) for 2 h. The protein complexes were washed three times with immunoprecipitation buffer and released from the Sepharose beads by boiling in loading buffer for 5 min and then centrifuged. The immune complexes were subjected to 12% SDS-PAGE and transferred to polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat milk and incubated with primary anti-E2F1, E2F2, E2F3, and Rb antibodies.

CDK Activity Assay

The cells were incubated in a nondenaturing cell lysis buffer [20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaN3, 1 μg/mL leupeptin] containing a cocktail of protease inhibitors and phosphatase inhibitors. Total lysates were prepared and immunoprecipitated with anti-CDK2, anti-CDK4, and anti-CDK6 antibodies as described above followed by incubation with Gammabind plus Sepharose. The bead complexes were washed three times in immunoprecipitation buffer and then three times in kinase buffer [25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, and 0.1 mM Na3VO4, 10 mM MgCl2]. Kinase reactions were done at 30°C for 30 min in a kinase buffer by using Rb-C fusion protein (Cell Signaling Technology) as a substrate at a concentration of 2 μg/20 μL reaction containing 200 μM ATP followed as per the manufacturer's protocol. The reaction was halted by adding 5× SDS loading buffer. After boiling for 5 min, the reaction products were electrophoretically separated on a 12% SDS-PAGE gel, and phosphorylated proteins were detected by anti-Ser(95) antibody.

Densitometry Analysis

 Autoradiograms of the immunoblots were scanned using a Bio-Rad Image Analyzer System. The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed using the Bio-Rad Quantity One Analyzer software. β-Actin was used as a loading control. Samples after siRNA oligonucleotide or Y27632 treatment were normalized to the densitometry value of the AGS cells without treatment, and the comparative data are presented as “fold change.” Both autoradiograms and respective

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1. **Immunoprecipitation**

Harvested cells were incubated in immunoprecipitation buffer [50 μM Tris-HCL (pH 7.4), 200 μM NaCl, 0.1% Triton X-100] containing a cocktail of protease inhibitors and phosphatase inhibitors as described previously. Samples of total protein (1 mg) were incubated with anti-Rb primary antibody and rotated end-up overnight at 4°C followed by incubation with Gammabind plus Sepharose (GE Healthcare) for 2 h. The protein complexes were washed three times with immunoprecipitation buffer and released from the Sepharose beads by boiling in loading buffer for 5 min and then centrifuged. The immune complexes were subjected to 12% SDS-PAGE and transferred to polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat milk and incubated with primary anti-E2F1, E2F2, E2F3, and Rb antibodies.

2. **CDK Activity Assay**

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Table 1. Sequences of PCR Primers, Length of PCR Products, and Optimal Annealing Temperature

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers (5′-3′)</th>
<th>Template (bp)</th>
<th>Annealing (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p15</td>
<td>F: CAGCCGAGCGTGCAAGAGT</td>
<td>100</td>
<td>60</td>
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<tr>
<td></td>
<td>R: CTTGCTCCTCCGCGCATTC</td>
<td>89</td>
<td>59.3</td>
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<tr>
<td>p16</td>
<td>F: CAGACATCCCCGATTGAAAGAAC</td>
<td>183</td>
<td>57.6</td>
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<tr>
<td></td>
<td>R: CTTGTTCTCCTCGCGCATTC</td>
<td>195</td>
<td>62.5</td>
</tr>
<tr>
<td>p18</td>
<td>F: TGGAACTTGGAAATCCCGAG</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: GGTAGGGGGGGAGGCATATCT</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>p19</td>
<td>F: CATCAGGCACGTTGACATCAG</td>
<td>183</td>
<td>57.6</td>
</tr>
<tr>
<td></td>
<td>R: GCAGGTTCCCTTCATTATCC</td>
<td>195</td>
<td>62.5</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: GAAGGTGAAAGGAGGAGGATC</td>
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<td>60.1</td>
</tr>
<tr>
<td></td>
<td>R: GAAGATGTTAGGGAGGATTC</td>
<td>225</td>
<td>61.2</td>
</tr>
</tbody>
</table>

densitometry data are representative of three independent experiments with reproducible findings.

Quantitative RT-PCR

Total cellular RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s protocol and quantified by spectrophotometer (A260/A280; Bio-Rad). The primers used for SYBR Green PCR were designed with the assistant of Primer Software version 5.0, listed in Table 1. All primers were checked by running a virtual PCR, and the amplifications were analyzed for the expected product. RT-PCR was done using the iQ5 RT-PCR Detection System (Bio-Rad). All reactions were run in triplicate and contained SYBR PrimeScript RT-PCR Kit (Takara) according to the manufacturer’s instruction, which run in triplicate and contained SYBR PrimeScript RT-PCR iQ5 RT-PCR Detection System (Bio-Rad). All reactions were analyzed for the expected product. RT-PCR was done using the checked by running a virtual PCR, and the amplifications were run in triplicate and contained SYBR PrimeScript RT-PCR Kit (Takara) according to the manufacturer’s instruction, which fluoresces when intercalated with DNA, with GAPDH used as the internal standard. The reactions were carried out in a 25 μL reaction volume. The thermal profile for all SYBR Green PCRs consisted of an initial incubation of 10 s at 95°C followed by 45 cycles of 5 s at 95°C, 25 s at 54°C, and 15 s at 72°C. After the completion of the 45 cycles, there were two additional incubations of 1 min at 95°C and 1 min at 55°C. Next, a melting curve for the temperatures between 55°C and 95°C with 0.5°C increments was recorded using the samples. Amplification, detection, and data analysis were done with iCycler IQ5 Optical System software (Bio-Rad). The expression of each mRNA was normalized to that of GAPDH and analyzed by the comparative threshold cycle (Ct) method.

Cell Cycle Assay

Cells were harvested by trypsinization, washed three times with ice-cold PBS, and fixed with 70% ethanol solution at 4°C. The fixed cells were rehydrated in PBS, resuspended in protease RNase (125 units/mL; Calbiochem) in PBS, and incubated for 30 min at 37°C. DNA was then stained with propidium iodide (50 μg/mL; Sigma-Aldrich) for 30 min at room temperature in the dark. Samples were analyzed with a FACScan (Beckman Coulter; EPICS ELITE ESP model). The percentage of cells in each phase of the cell cycle was estimated using ELITE software.

Cell Proliferation Assay

The cells were cultured in 96-well plates under proper stimuli. After 1, 2, 3, or 4 day growth, cell medium was changed with fresh medium containing 10 μL Cell Counting Kit-8 reagent (WST-8; (2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt; DOJINDO Lab) for 2 h at 37°C according to the manufacturer’s instructions. After incubation with Cell Counting Kit-8, the formazan dye generated by the activity of dehydrogenases in the cells was proportional to the number of living cells and the absorbance was measured using a microplate reader at 490 nm with a reference at 630 nm. Absorances were converted to percentages by comparisons with the untreated control. Cell numbers were determined from a standard plot of known cell numbers versus the corresponding absorbance density.

Immunofluorescence Microscopy

For analysis of the intracellular location of p21Waf1/Cip1 and p27Kip1 proteins, AGS cells were cultured in 6-well plates with coverslips and stimulated as described previously. Forty-eight hours later, the cells grown on coverslips were fixed with 4% paraformaldehyde and 4% sucrose for 30 min and then washed with PBS. Cells were then permeabilized in PBS containing 0.25% Triton X-100, preblocked for 90 min with 3% bovine serum albumin in PBS, and incubated with the primary antibodies of p21Waf1/Cip1 and p27Kip1 for 90 min at room temperature. After washing, cells were incubated with secondary antibodies conjugated to Alexa Fluor 594 (red) dyes (Invitrogen) for 90 min. The coverslips were washed and then mounted on slides using ProLong Gold antifade with 4′,6-diamidino-2-phenyindole (Invitrogen). Cells were visualized with an Olympus IX81 microscope (60× objectives).

Statistical Analysis

Experimental results were expressed as mean ± SD (n = 3) from data of at least three independent experiments. Statistical significance was evaluated using a one-way ANOVA test. P < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Juan Huang, Jitao Zhou, Yajie Zhu, and Xiangzheng Chen for technical assistance and Des. Bo Xiao, Xiaoxia Du, and Yan Li for helpful discussions.

References

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