RhoA stimulates IEC-6 cell proliferation by increasing polyamine-dependent Cdk2 activity

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Submitted 27 January 2003; accepted in final form 15 June 2003

Guo, Huazhang, Ramesh M. Ray, and Leonard R. Johnson. RhoA stimulates IEC-6 cell proliferation by increasing polyamine-dependent Cdk2 activity. Am J Physiol Gastrointest Liver Physiol 285: G704–G713, 2003.—Although RhoA plays an important role in cell proliferation and in Ras transformation in fibroblasts and mammary epithelial cells, its role in intestinal epithelial cells (IEC) is unknown. In a previous study (Ray RM, Zimmerman BJ, McCormack SA, Patel TB, and Johnson LR. Am J Physiol Cell Physiol 276: C684–C691, 1999), we showed that polyamine depletion [Nα-difluoromethylornithine (DFMO) treatment] strongly inhibits the proliferation of IEC. In this report, we examined the effect of RhoA on IEC-6 cell proliferation and whether polyamine depletion inhibits cell proliferation in the presence of constitutively active RhoA. Constitutively active RhoA and vector-transfected IEC-6 cell lines were grown in the presence or absence of DFMO, which causes polyamine depletion by inhibiting ornithine decarboxylase, the first rate-limiting step in polyamine synthesis. Constitutively active RhoA significantly increased the rate of cell proliferation. These cells also lost contact inhibition and formed conspicuous foci when they were fully confluent. Decreased p21Waf1/Cip1 expression and increased cyclin-dependent kinase (Cdk2) mRNA levels and activity accompanied the increased proliferation. The inhibition of p21Waf1/Cip1 was independent of p53. There was no activation of the Ras-Raf-MEK-ERK pathway in the RhoA-transfected cell line. Polyamine depletion totally prevented the effect of activated RhoA on IEC-6 cell proliferation, focus formation, and Cdk2 expression. The stability of mRNA and protein for Cdk2 and p21Waf1/Cip1 in V14-RhoA cells was not significantly different from that of vector-transfected cells. In conclusion, RhoA activation decreased p21Waf1/Cip1 expression and increased basal and serum-induced ornithine decarboxylase activity, Cdk2 expression, Cdk2 protein, and Cdk2 activity, leading to the stimulation of IEC proliferation and transformation. Polyamine depletion totally prevented RhoA's effect on proliferation by decreasing Cdk2 expression and activity.

Cell proliferation is a fundamental process for the organization and maintenance of the integrity of the mucosa of the small intestine. The cells of the intestinal epithelium are in a constant state of renewal. As mature cells are desquamated into the intestinal lumen, new cells proliferate and migrate up the villi to replace them. The entire epithelium of the small intestine is replaced every 3–6 days, making it one of the fastest-growing tissues of the body. When damaged, the mucosa of the gastrointestinal tract has the ability to repair itself rapidly, almost entirely within 24 h, with cell proliferation playing an important role in this process (39). The regulation of cell proliferation is a complex process, which is primarily regulated by external growth factors, neurotransmitters, and mediators provided by adjacent paracrine cells, neurons, and distant endocrine cells. Extracellular signals, which transduce through intercalated signal transduction pathways, converge on the cell cycle machinery to regulate proliferation (2). Excessive activation in these pathways will cause proliferating disorders: hyperplasia, benign tumorigenesis, and carcinogenesis (6).

RhoA is a member of the Rho subfamily of the Ras-like small G protein superfamily. Like Ras, RhoA functions as a molecular switch by cycling between an active guanosine triphosphate-bound state and an inactive guanosine diphosphate-bound state. Although RhoA was initially described as an important regulator of cytoskeletal reorganization, it has gained attention as a regulator of cell cycle progression and cell transformation. There are two lines of evidence showing that RhoA promotes tumor formation and progression. In vivo studies, such as those by Qiu et al. (29), have shown that RhoA synergistically acted with Ras to promote transformation and that inactivation of RhoA reversed Ras-induced transformation. Another line of evidence is derived from the analysis of clinical tumor specimens. Fritz et al. (11) showed that RhoA expression in tumors from colon, breast, and lung was increased compared with that of the corresponding normal tissue originating from the same patient. Similarly, Kamai et al. (16) showed that the mRNA levels of RhoA were greater in testicular germ cell tumors than in samples from unaffected resected testis. The degree of increase was related to tumor stage. These studies strongly linked RhoA with oncogenesis and tumor progression. However, most of the in vitro studies on the role of RhoA in cell proliferation and transformation were done in fibroblasts. The involve-
ment of RhoA in intestinal epithelial cell (IEC) proliferation and transformation is largely unknown.

The polyamines putrescine, spermidine, and spermine are organic polycations found in virtually all cells of higher eukaryotes. Studies with polyamine-deficient mutants and inhibitors of polyamine synthesis have shown that polyamines are essential for normal cell growth and differentiation (17, 21, 27, 40). Intracellular polyamine levels are highly regulated and are primarily dependent on the activity of ornithine decarboxylase (ODC), which catalyzes the first rate-limiting step in polyamine biosynthesis. An increase in ODC activity is one of the earliest biochemical events associated with the induction of cellular proliferation (27, 40). Depletion of polyamines by DL-α-difluoromethylornithine (DFMO), a specific irreversible inhibitor of ODC, attenuates trophic responses in a number of tissues (22, 27). Thus DFMO has been studied as a candidate chemotherapeutic agent (20, 24, 28, 31, 35).

Materials and Methods

Materials. The IEC-6 cell line (CRL-1592) was obtained from the American Type Culture Collection (Manassas, VA) at passage 13. This line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (30). The cells are nontumorigenic and retain the undifferentiated character of epithelial stem cells. Stable constitutively active RhoA (HA-V14-RhoA) and vector-transfected IEC-6 cells were generated and characterized in a previous study (33). Cell culture medium, trypsin/EDTA, antibiotics, and insulin were obtained from Gibco (Grand Island, NY). Fetal bovine serum (FBS), dialyzed FBS (1,000 mol wt cutoff), propidium iodide, and diethyl pyrocarbonate were from Sigma (St. Louis, MO). Bicinchoninic acid protein assay reagent kit was purchased from Pierce (Rockford, IL). Cdk2, p21Waf1/Cip1, and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-p53, p21Waf1/Cip1, and p53 antibodies were purchased from Cell Signaling Technology (Beverly, MA). [γ-32P]ATP and the enhanced chemiluminescence Western blot detection system were purchased from DuPont-NEN (Boston, MA). SDS sample loading buffer and electrophoresis apparatus were from Bio-Rad (Hercules, CA). DFMO was a gift from ILEX Oncology (San Antonio, TX).

Trizol RNA extraction reagent was from Life Technologies (Gaithersburg, MD). Moloney murine leukemia virus reverse transcriptase, PCR nucleotide mix, and random primers were purchased from Promega (Madison, WI). SYBR green PCR master mix was from Applied Biosystems (Foster City, CA). All other chemicals were of the highest purity commercially available. PCR primers were designed by using Primer 3 software from White Head Institute (Cambridge MA).

Cell culture. Constitutively active RhoA (pcDNA-3-HA-V14-RhoA) or empty vector (pcDNA-3) stably transfected IEC-6 cell lines were maintained in T-150 flasks in a humidified, 37°C incubator in an atmosphere of 10% CO2. The maintaining medium consisted of DMEM with 5% heat-inactivated FBS, 400 µg/ml G418, 10 µg/ml insulin, and 50 µg/ml gentamicin sulfate. The medium was changed twice per week. For real-time PCR, stock cells were plated at 3.33 × 10^4 cells/cm² in DMEM in T25 flasks. For growth studies, cell cycle analysis, Cdk2 activity assays, and Western blots, stock cells were plated at 3.33 × 10^3 cells/cm² in T75 flasks. Control cells were grown in the DMEM-containing medium (5% dialyzed FBS, 10 µg insulin, and 50 µg gentamicin sulfate/ml). Cells treated with DFMO were grown in control medium plus 5 mM DFMO. Cells treated with DFMO and putrescine were grown in control medium plus 5 mM DFMO and 10 µM putrescine. Cells were grown at 37°C in a humidified atmosphere with 10% CO2 and fed every other day.

Growth studies and cell cycle analysis. On days 2, 4, 6, and 8, cell lines in triplicate flasks from three groups (control, DFMO, and DFMO + putrescine) were taken up with trypsin/EDTA. The cells were used for both cell counting and cell cycle analysis. For cell counting, 250 µl of cells were taken out, diluted 40 times, and counted by using a Coulter counter. For cell cycle analysis, the remaining cells were collected by centrifugation for 5 min at 100 g, resuspended in 1 ml of cold PBS, fixed with 4 ml of −20°C absolute ethanol with vortexing, and stored at −20°C. On the day of cell cycle analysis, staining buffer (50 µg propidium iodide and 10 µg RNase/ml PBS) was prepared, and the cells were collected by centrifugation. After the wash in PBS and centrifugation, 1 ml of staining buffer was added to each pellet. The tubes were incubated for 30 min at room temperature and analyzed by flow cytometry. To assess constitutively active RhoA and focus formation, two cell lines were grown for 15 days after transfection. The cultures were then photographed with a video camera and trypsinized, and the cells were counted to evaluate contact inhibition.

Measurement of ODC activity. Cells were grown for 4 days, as described earlier, and serum starved for 24 h. One group of both vector and V14-RhoA cells was stimulated with 5% serum for 3 h. Cells were washed with ice-cold Dulbecco’s PBS. To each dish, 0.5 ml of ODC assay buffer [(in mM) 1.0 Tris-HCl, 1.0 EDTA, 0.05 pyridoxyl-5-phosphate, and 5 di-thiothreitol, pH 7.4] was added, and the cells were frozen at −80°C. Cells were thawed on ice, scraped, transferred to Microfuge tubes, sonicated, and centrifuged at 12,000 g for 10 min at 4°C. Supernatants were incubated in stopper vials containing 20 µl of a 1:5 diluted, labeled ornithine stock for 15 min at 37°C. 14CO₂ was trapped on a filter paper impregnated with NaOH. The reaction was terminated with 10% TCA and further incubated for an additional 10 min to allow complete absorption of CO₂. The trapped 14CO₂ was measured by liquid scintillation counting. Enzyme activity was expressed as picomoles of CO₂ released per milligram of protein per hour. The protein assay was carried out by the bichinchoninic acid method.

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Western blot and Cdk2 activity assays. On day 4, T475 flasks were washed twice with ice-cold PBS. Cells were harvested in ice-cold cell lysis buffer by scraping with a rubber policeman. Cell lysates were centrifuged at 10,000 g for 10 min. The supernatant was used for protein quantification. The amount of protein was determined by following the bicinchoninic acid protein assay reagent manufacturer’s instructions. For Western blotting, 25 μg of total protein were separated on 10–15% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and blotted with antibodies. The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantitated by densitometric scanning. Equal loading of protein was confirmed by staining the membrane with Ponceau S. For the Cdk2 activity assay, 100-μg cell extracts were incubated with 20 μg of anti-Cdk2 antibody. Immune complexes were recovered with protein A/G agarose beads and washed twice with immunoprecipitation buffer (10 mM Tris-HCl at pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 200 μM Na3VO4, 80 μg/ml leupeptin, 40 μg/ml aproamin) and once with kinase assay buffer, which contained 1 mg/ml phosphocellulose (Pharmacia), 1 mM EDTA, and 25 μM ATP. Pellets were resuspended in 40 μl of kinase buffer, which contained 5 μg of histone H1 (Cdk2 substrate) and 10 μCi [γ-32P] ATP, and incubated at 30°C for 30 min. The kinase reaction was terminated by the addition of SDS sample loading buffer. The samples were then heated to 95°C for 5 min and resolved by 10% SDS-PAGE. The gels were dried, and the phosphorylated histone H1 protein sub- strate was visualized by autoradiography.

RNA extraction, reverse transcription, and real-time PCR. On day 4, after the growth medium was discarded, Trizol was added to each T25 flask to harvest the cells. Total RNA was extracted from harvested cells following the manufacturer’s (Trizol) instructions. The yield and quality of total RNA was measured by absorbance at 260 or 280 nm. One microgram of total RNA and 0.5 μg of the random primers were used for reverse transcription with Moloney murine leukemia virus reverse transcriptase following the manufacturer’s instructions. The resulting cDNA was diluted to 100 μl with diethyl pyrocarbonate-treated water and used as a template for real-time PCR. Briefly, PCR primers were designed with a melting temperature of 59–61°C. Amplicon size was 50–150 bases. Forward and reverse primers spanning exon-exon junctions were selected to avoid amplification of genome sequences (primers used were: p21Waf1/Cip1, forward primer, 5'-AGATGCTCTTCTGGGCTGCAAG-3'; reverse primer, 5'-AGATCCGGAGAGTTTGCTGA-3'; Cdk2, forward primer, 5'-CGTAGAGCTTCTGGGCTCAAG-3'; reverse primer, 5'-AGATCCGGAGAGTTTGCTGA-3'); p53, forward primer 5'-GCTTCGAGATTTTCCAGAG-3'; reverse primer, 5'-TTTTATAGCCGAGACGTAAG-3'; and actin, forward primer, 5'-CGTGGAAAAGATCGACCGATCA-3'; and reverse primer, 5'-CCAGCCTGGATGCTACGAT-3'). PCR reactions were performed by using an ABI Prism 7700 sequence detection system (Applied Biosystems). The reaction contained 25 μl of SYBR reagent, 5 μl of diluted cDNA, and 300–900 nM primers in a 50-μl volume. The thermal cycling conditions involved an initial denaturing step at 95°C for 15 s and an anneal-extension step at 60°C for 1 min. Quantitative values were obtained from the threshold cycle value (Ct), which is the cycle at which a significant increase of fluorescence was first detected. The transcript number of rat β-actin was quantified as an internal RNA control, and each sample was normalized on the basis of its β-actin content. The relative gene expression level of each sample was then normalized to the vector-transfected cell line in control medium (calibrator). Final results, expressed as N-fold difference in gene expression relative to β-actin and the control group, termed N, were calculated as: \( N = 2^{\Delta \Delta Ct} \) sample – ΔCt control. \( \Delta \Delta Ct \) was calculated by subtracting the average Ct value of a target gene from the corresponding Ct value of the β-actin gene.

Cdk2 and p21Waf1/Cip1 mRNA stability studies. Cells were grown as described earlier for real-time PCR analysis. On day 4, 15 μg/ml actinomycin-D was added and further incubated for 0–9 h. At 3-h time intervals, cells were washed and RNA was extracted. For RNA isolation and quantification, reverse transcription was carried out as described above. Briefly, 2 μg of RNA from each time point sample of vector- and V14-Rhoa-transfected cells was used for reverse transcription. The resulting cDNA was diluted to 100 μl with diethyl pyrocarbonate-treated water. Ten microliters of diluted cDNA for Cdk2 and actin and 20 μl of p21Waf1/Cip1 were used as a template for PCR by using gene-specific primers. One microliter of biotin-UTP was added to each reaction, and PCR was carried out as described in the previous section. PCR products were separated by gel electrophoresis, transferred to Gene Screen Plus membranes, and detected by using a streptavidin-alkaline phosphatase conjugate by enhanced chemiluminescence detection using the continuous distending pressure star reagent. PCR products were also transferred to membranes by using slot blots and analyzed as above. The intensity of PCR products was quantified and normalized with actin to determine the stability of Cdk2 and p21Waf1/Cip1 mRNA.

Statistics. Numerical data are presented as means ± SE of three experiments. Western blots and Cdk2 activities shown are representative of three experiments. Statistical analysis was performed by using ANOVA and appropriate post hoc testing. \( P \) values of <0.05 were considered significant.

RESULTS

RhoA stimulates cell proliferation, compromises cell contact inhibition, and induces focus formation. HA-V14-Rhoa-transfected IEC-6 cells had a significantly increased rate of proliferation compared with vector-transfected cells (Fig. 1A). Despite this strong stimulation by RhoA, DFMO inhibited not only the growth of vector-transfected IEC-6 cells but also the growth of HA-V14-Rhoa-expressing cells (Fig. 1A). Consistent with this result, the addition of exogenous putrescine restored the proliferation rates of both cell lines almost to control levels (Fig. 1A). In addition, constitutively active Rhoa (V14-Rhoa)-expressing cells had relatively higher basal levels of OD activity compared with vector-transfected serum-starved cells. Interestingly, the stimulation of cells with serum further increased OD activity of V-14 Rhoa cells significantly compared with vector-transfected cells (Fig. 1B).

Under our experimental conditions, both vector- and HA-V14-Rhoa-transfected cells reached confluence at day 10. After 15 days of incubation, the number of cells in the HA-V14-Rhoa-transfected cell line was significantly more than in the vector-transfected cell line (Fig. 2). This implies that constitutively active Rhoa compromised contact inhibition and that the cells continued to proliferate. In addition, fully confluent Rhoa- transfected IEC-6 cells formed conspicuous foci (Fig. 3D) compared with vector-transfected cells (Fig. 3A).
These observations suggest that sustained activation of RhoA transformed IEC-6 cells, leading to focus formation. DFMO, however, not only inhibited proliferation but also prevented focus formation in constitutively active RhoA-expressing cells (Fig. 3E). Exogenous putrescine restored focus formation in HA-V14-RhoA-transfected cells (Fig. 3F).

**Cell cycle analysis.** By day 4, vector- and HA-V14-RhoA-transfected cells were in the initial rapid growth phase in control and DFMO + putrescine groups (Fig. 1, A and C). At this time, cells were also polyamine depleted by DFMO (32). Thus we used day 4 cells for cell cycle analysis, Western blots, Cdk2 activity assays, and real-time PCR.

The more rapidly growing HA-V14-RhoA cells had fewer cells in the G1 phase of the cell cycle (Fig. 4A), indicating that constitutively active RhoA stimulated cell proliferation by promoting the transition from G1 to S and from S to G2/M. DFMO, however, arrested a significant proportion of cells in the G1 phase in both cell lines (Fig. 4B). Exogenous putrescine prevented G1 arrest in both cell lines, with the HA-V14-RhoA cell line having fewer cells in the G1 phase (Fig. 4C).

**RhoA decreases ERK1/2 activities.** Activation of the Ras-Raf-MEK-ERK signal pathway stimulates cell proliferation (5, 8–10, 12, 23, 26). Given the stimulating effect of constitutively active RhoA on IEC-6 cell proliferation, we determined the activity of ERK1/2 in transfected IEC-6 cell lines. HA-V14-RhoA significantly decreased phospho-ERK1/2 proteins (P < 0.05),
the active forms of ERK1/2 (Fig. 5), without affecting the total amount of protein. This implies that RhoA-mediated stimulation of cell proliferation is independent of the Ras-Raf-MEK-ERK pathway.

RhoA inhibits p21Waf1/Cip1 protein, increases Cdk2 protein, and increases Cdk2 activity. Because RhoA promoted the G1/S transition in IEC-6 cells without activating Ras-Raf-MEK-ERK, we examined the downstream cell cycle regulatory proteins Cdk2 and p21Waf1/Cip1. Figure 6A shows that, compared with vector-transfected cells, HA-V14-RhoA-transfected cells have a decreased basal level of p21Waf1/Cip1. Consistent with previous findings (17, 34), DFMO treatment increased p21Waf1/Cip1 expression in vector-transfected IEC-6 cells. In contrast, DFMO treatment did not change p21Waf1/Cip1 expression in HA-V14-RhoA-transfected cells. HA-V14-RhoA-transfected cells had a higher basal level of Cdk2 compared with

Fig. 3. Focus formation in HA-V14-RhoA-transfected IEC-6 cells. Cells were plated at $3.33 \times 10^5$ cells/cm² and grew for 15 days. In control and DL-α-difluoromethylornithine (DFMO)/putrescine (Put), vector-transfected IEC-6 cells did not form foci (A and C). HA-V14-RhoA-transfected IEC-6 cells formed conspicuous foci (D and F). In DFMO medium, neither cell line reached confluence; therefore, no focus formation was observed (B and E).

Fig. 4. Cell cycle analysis of vector- and HA-V14-RhoA-transfected IEC-6 cell lines. Cells were plated at $3.33 \times 10^5$ cells/cm² and grown for 4 days in control, DFMO, or DFMO + putrescine. In control (A) and DFMO + putrescine (C), HA-V14-RhoA transfection facilitated the G1/S transition. DFMO (B) (polyamine depletion) caused both vector- and HA-V14-RhoA-transfected cells to arrest in the G1 phase. Values are means ± SE; n = 3. * Significant difference compared with corresponding cell cycle phase in vector-transfected cells ($P < 0.05$).
Polyamines and RhoA in Cell Proliferation

Erk1 Erk2

Phospho-Erk1 Phospho-Erk2

Actin

Vector HA-V14-RhoA

Fig. 5. Constitutively active RhoA decreased phospho-extracellular regulated kinase (ERK) 1/2 without changing total ERK1/2. Vector- and HA-V14-RhoA-transfected IEC-6 cells were plated at 3.33 × 10^3 cells/cm^2 and grown for 4 days. Cell extracts were prepared and then analyzed by Western blot analysis. Blot shown is representative of 3 experiments.

A p21

B Cdk2

C Phospho-Cdk2

D Actin

E Cdk2 activity

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Fig. 6. Effect of constitutively active RhoA expression and polyamine depletion on cell cycle regulators. Vector- and HA-V14-RhoA-transfected IEC-6 cells were plated at 3.33 × 10^3 cells/cm^2 and grown for 4 days under control conditions or in presence of DFMO or DFMO + putrescine. Cell extracts were prepared. p21Waf1/Cip1 (A), cyclin-dependent kinase (Cdk) 2 (B), phospho-Cdk2 (C), and actin (D) protein levels were determined by Western blot analysis. A representative of 3 experiments in each cell line is shown. Cdk2 activity (E) was determined by using histone H1 substrate as described in MATERIALS AND METHODS and is represented as means ± SE from 3 experiments. *Significant difference compared with corresponding group in vector-transfected cells (P < 0.05).

A p21

B Cdk2

Fig. 7. Real-time PCR determination of the levels of p21Waf1/Cip1 and Cdk2 mRNA expression. Constitutively active RhoA decreased p21Waf1/Cip1 (A) and increased Cdk2 (B) mRNAs. Vector- and HA-V14-RhoA-transfected confluent IEC-6 cells were treated with actinomycin-D and cycloheximide for 0–9 h to determine the stability of mRNA and protein, respectively, for the Cdk2 and p21Waf1/Cip1. Southern blotting revealed that levels of Cdk2 mRNA were decreased by 10.220.33.3 on April 13, 2017 http://ajpgi.physiology.org/ Downloaded from

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significantly increased and those of p21Waf1/Cip1 were barely detectable in V14-RhoA cells compared with vector-transfected cells at 0 h. Because of the low steady-state levels of p21Waf1/Cip1, mRNA in the V14-RhoA cells compared with vector-transfected cells, we used slot blots to determine RNA stability. Cdk2 mRNA stability was not significantly different in either vector or V14-RhoA cells (Fig. 8). The stability of Cdk2 mRNA in V14-RhoA cells was comparatively less than in vector cells. Levels of p21Waf1/Cip1 mRNA decreased significantly after 6 h of exposure to actinomycin-D in vector-transfected cells. The low levels of p21Waf1/Cip1 mRNA in V14-RhoA cells decreased little with actinomycin-D treatment. The stabilities of Cdk2 and p21Waf1/Cip1 proteins were not significantly altered in either the V14-RhoA- or vector-transfected cells (Fig. 9). These results imply that the effects of constitutively active RhoA on p21Waf1/Cip1 and Cdk2 expression are mediated through the regulation of transcription.

RhoA inhibits p21Waf1/Cip1 through a p53-independent pathway. To explore the possible mechanism underlying the inhibitory effect of RhoA on p21Waf1/Cip1, we monitored p53, the best-known regulator of p21Waf1/Cip1. HA-V14-RhoA increased p53 mRNA, protein, and phosphoprotein levels (Fig. 10, A–C, respectively). Thus the decreased expression of p21Waf1/Cip1 in the constitutively active RhoA cell line does not appear to be mediated by p53.

**DISCUSSION**

Cdk2 is a member of the Cdk protein kinase family, an important part of the cell cycle control system. Regulated oscillation of Cdk activity leads to progression through the cell cycle: DNA replication, mitosis, and cytokinesis. Extracellular and intracellular sig-

![V14- RhoA](image1)

**Fig. 8.** Stability of mRNA for Cdk2 and p21Waf1/Cip1. Slot blotting of biotin-UTP labeled PCR amplified products by using gene-specific primers as described in METHODS AND MATERIALS was carried out. The amounts of PCR products were detected by using streptavidin-AP conjugate followed by enhanced chemiluminescence detection. Cdk2 (A), p21Waf1/Cip1 (B), and actin (C) from vector and V14-RhoA cells are shown. Similar results were also obtained with Southern blot analysis. A representative slot blot is shown.

![Actin](image2)

**Fig. 9.** Stability of Cdk2 and p21Waf1/Cip1 proteins. Vector and V14-RhoA cells were grown for 3 days and serum starved for 24 h before treatment with cycloheximide for 0–9 h. Cell extracts were analyzed by Western blot for Cdk2, p21Waf1/Cip1, and actin protein by using specific antibodies. Blot shown is representative of 3 experiments.

**Fig. 10.** Constitutively active RhoA increased p53 mRNA (A), p53 protein (B), and phospho-p53 protein levels (C). Vector- and HA-V14-RhoA-transfected IEC-6 cells were plated at $3.33 \times 10^5$ cells/cm$^2$ and grown for 4 days. RNA extracts were analyzed by real-time PCR. Protein extracts were prepared and then analyzed by Western blot analysis. Blot shown is representative of 3 experiments. Values are means ± SE; n = 3. *Significant difference compared with vector-transfected cells (P < 0.05).
nals converge on Cdk2 to determine whether a cell commits to DNA synthesis. Extracellular signals, such as growth factors, activate receptor tyrosine kinases to activate the Ras-Raf-MEK-ERK pathway, which eventually activates Cdk2 and promotes cell cycle progression (38). Intracellular signals, such as DNA damage, activate the p53-p21Waf1/Cip1 pathway to inhibit Cdk2 activity, which leads to cell cycle arrest. In this study, we found that, by increasing Cdk2 activity, constitutively active RhoA promoted the G1/S transition, stimulated cell proliferation, and induced focus formation in IEC-6 cells.

Because Cdk2 plays an important role in the cell cycle, it is one of the most highly regulated proteins in mammalian cells. Cdk2 activity is increased by association with cyclin E, dissociation from p21Waf1/Cip1, phosphorylation at Thr160, and dephosphorylation at Thr14 or Thr15 (2). In this study, we showed that transfection of IEC-6 cells with RhoA decreased p21Waf1/Cip1 mRNA (Fig. 7A) and protein levels (Fig. 6A). The RhoA-mediated decrease of p21Waf1/Cip1 was originally reported by Olson et al. (25) in fibroblasts and subsequently confirmed in mammalian epithelial cells (18) and mesangial cells (7). Normally, p53 increases p21Waf1/Cip1 by binding to DNA and increasing the transcription of p21Waf1/Cip1 mRNA. A mutation leading to the inactivation of p53 then leads to the decreased production of p21Waf1/Cip1, increased cell proliferation, and frequently induced transformation. Ultraviolet light and hepatitis B virus X protein also regulate expression of p21Waf1/Cip1 independently of p53 (1, 14). Furthermore, the extent of histone acetylation of the promoter (proximal promoter) of p21 and the amount of interaction with p300/cAMP-response element binding protein, which contains histone acetyltransferase activity, have been shown to determine the inducibility of p21 by p53 (19). These observations indicate that when p53 binds to responsive element(s) of a target gene, its ability to interact with histone acetyltransferase-containing proteins and subsequent acetylation of histones bound to a proximal promoter dictates the induction level of the target gene. Thus the level of p53 does not necessarily directly correlate with p21Waf1/Cip1 transcription. In the case of our cells transfected with RhoA, however, there was actually a significant increase in both p53 protein and mRNA levels (Fig. 10). Thus the increase in proliferation (Fig. 1) and decrease in p21Waf1/Cip1 levels that we observed were independent of p53. Additionally, in the cell line expressing active RhoA, there was no increase in phospho-Cdk2 relative to total Cdk2 protein. This implies that RhoA did not stimulate Cdk2-activating kinase, which activates Cdk2 by phosphorylating Thr-160. In addition to posttranslational modification, Cdk2 activity is regulated at the transcriptional level. Stimulation with phorbol ester (15) or serum (13) increases Cdk2 mRNA in HL60, human keratinocytes, and human lung fibroblasts. We found a significant increase in Cdk2 mRNA and protein in the RhoA-transfected cells, without significant changes in the stabilities of Cdk2 mRNA and protein, indicating that RhoA increases Cdk2 activity by increasing the transcription and subsequent expression of the protein.

Although synergy of RhoA with the Ras-Raf-MEK-ERK pathway in cell transformation is well established (29, 36), whether RhoA by itself induces transformation is uncertain. Avraham and Weinberg (4) first reported that human RhoA did not produce foci in Rat-1 or NIH/3T3 cells, but later they showed that cells overexpressing RhoA displayed an altered pattern of growth and formed tumors in nude mice (3). Although Self et al. (37) demonstrated that RhoA induced focus formation in NIH/3T3 cells, they also found that RhoA-transfected cells did not grow significantly faster than the parental cells. We found that transfection with constitutively active RhoA stimulated proliferation, compromised contact inhibition, and induced focus formation in IEC-6 cells. Thus sustained activation of RhoA appeared to transform IEC-6 cells. In addition, we found no increase in phospho-ERK1/2 in the RhoA-transfected cell line, suggesting that constitutively active RhoA did not activate the Ras-Raf-MEK-ERK pathway to achieve its stimulating effect on cell growth and transformation. Oncogenesis is a progressive process, which involves initial hyperplasia, progressive benign tumor formation, and eventually invasive malignancy. In this process, successive activation of oncogenes and inactivation of tumor suppressor genes play major roles. Based on our findings in this study, we propose that RhoA can act as an oncogene in gastrointestinal epithelia. This hypothesis is supported by the finding that RhoA protein is increased in some colonic tumors (11).

We were then interested in whether the effect of RhoA on IEC-6 cell proliferation and transformation was polyamine dependent. Although the effects of polyamines on cell proliferation have been described earlier in various cell lines, to our knowledge, there are no reports examining the effect of polyamine depletion on stimulated cell proliferation by RhoA. We found that polyamine depletion inhibited the G1/S transition in the cell cycle and cell proliferation in both vector- and HA-V14-RhoA-transfected IEC-6 cells to the same level (Figs. 1B and 4B). Furthermore, in normal control cells, polyamine depletion increased the level of p21Waf1/Cip1 and inhibited Cdk2 expression and activation, which explained their decreased rate of growth. However, polyamine depletion of cells transfected with RhoA caused a profound inhibition of Cdk2 expression and activation without altering p21Waf1/Cip1 (Fig. 6A). Although polyamine depletion increased the level of p21Waf1/Cip1 mRNA in the cells transfected with RhoA, the absolute level of mRNA remained several times below that in the vector-transfected controls (Fig. 7A). The low levels of p21Waf1/Cip1 were not due to decreased stability of its mRNA or protein (Figs. 8 and 9). Thus it appears that the changes in Cdk2 activity and growth rate, which occurred in the RhoA-transfected cells, were independent of p21Waf1/Cip1. This result is reminiscent of our findings in Caco-2 cells, a line of transformed human...
colon cells (32), where polyamine depletion inhibited proliferation independently of p21Waf1/Cip1. Furthermore, supplementation of exogenous putrescine along with DFMO prevented the changes in the level of Cdk2 activity and cell proliferation are polyamine-dependent effects (Fig. 1B). Thus the effects of RhoA on Cdk2 expression, Cdk2 activity, and cell proliferation are polyamine dependent.

In conclusion, activation of RhoA stimulated IEC-6 cell proliferation, inhibited contact inhibition, and induced focus formation. The increased proliferation was due to a stimulation of Cdk2 expression that was independent of mitogen-activated protein kinase and p53. Polyamine depletion decreased Cdk2 activity and inhibited the stimulating effect of RhoA on IEC-6 cell proliferation.

We thank Mary Jane Viar, Claire Covington, Dr. Wenlin Deng, and Dr. Sujoy Bhattacharya for technical support.

DISCLOSURES
This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-16505 and the Thomas A. Gerwin Endowment.

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