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

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Running title: Polyamines and RhoA in cell proliferation

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ABSTRACT

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 is unknown. In a previous study, we showed that polyamine depletion (DL-alpha-difluoromethylornithine, DFMO, treatment) strongly inhibits the proliferation of intestinal epithelial cells (IEC-6). 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 (ODC), 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, increased 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, increased basal and serum-induced ODC activity, Cdk2 expression, Cdk2 protein and Cdk2 activity, leading to the stimulation of intestinal epithelial cell proliferation and transformation. Polyamine depletion totally prevented RhoA’s effect on proliferation by decreasing Cdk2 expression and activity.
Key words: transformation, p21\textsuperscript{\textit{Waf1/Cip1}}, p53, Erk1/2, putrescine, ornithine decarboxylase inhibition
INTRODUCTION

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 to 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 hours, 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, transducing through intercalated signal transduction pathways, converge on the cell cycle machinery to regulate proliferation (1). Excessive activation in these pathways will cause proliferating disorders: hyperplasia, benign tumorigenesis, and carcinogenesis (6).

RhoA is a member of the Rho subfamily of Ras-like small G protein superfamily. Like Ras, RhoA functions as a molecular switch by cycling between an active GTP-bound state and an inactive GDP-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 tumor tissues 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 involvement of RhoA in intestinal epithelial cell 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-alpha-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). The relationship between RhoA and polyamines has not been examined in the context of cell proliferation and transformation.
In the present study, we used the IEC-6 cell line, a non-transformed putative crypt cell line derived from rat intestine, as an *in vitro* model, to study (1) the effect of RhoA on cell proliferation, contact inhibition, and focus formation and (2) whether polyamines are involved in the effect of RhoA on cell proliferation. We found that constitutively active RhoA inhibited p21$^{Waf1/Cip1}$ expression, stimulated Cdk2 expression and activity, promoted the G1-S transition, and stimulated IEC-6 cell proliferation. We also found that active RhoA attenuated contact inhibition and facilitated focus formation. Polyamine depletion not only inhibited IEC-6 cell proliferation, but also inhibited the proliferation of IEC-6 cells transfected with constitutively active RhoA.
MATERIALS AND METHODS

Materials. The IEC-6 cell line (American Type Culture Collection 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 BRL (Grand Island, NY). Fetal bovine serum (FBS), dialyzed fetal bovine serum (dFBS; 1,000-molecular weight cutoff), propidium iodide, and diethyl pyrocarbonate (DEPC) were from Sigma (St. Louis, MO). BCA 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 (Ser15), phospho-cdk2 (Thr160), Erk1/2, and phospho-Erk1/2 (Thr202/Tyr204) antibodies were 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 BioRad (Hercules, CA). DFMO was a gift from ILEX Oncology Inc (San Antonio, TX). TRIZol RNA extraction reagent was from Life Technologies (Gaithersburg, MD). Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), 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 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 a week. For real-time PCR, stock cells were plated at 3.33 x 10³ cells/cm² in Dulbecco's modified Eagle medium (DMEM) in T25 flasks. For growth studies, cell cycle analysis, Cdk2 activity assays, and western blots, stock cells were plated at 3.33 x10³ cells/cm² in T75 flasks. Control cells were grown in the DMEM containing medium (5% dFBS, 10 µg insulin, and 50 µg gentamicin sulfate/mL). Cells treated with DFMO were grown in control medium plus 5 mmol/L DFMO. Cells treated with DFMO and putrescine were grown in control medium plus 5 mmol/L DFMO and 10 µmol/L 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 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 of propidium iodide and 10 µg of RNase per ml of PBS) was prepared, and the cells were collected by centrifugation. After washing in PBS and centrifugation, one 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 contact inhibition and focus formation, two cell lines were grown for 15 days after plating. The cultures were then photographed with a video camera, 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 3h. Cells were washed with ice-cold Dulbecco’s PBS. To each dish 0.5 ml ODC assay buffer (1.0 mM Tris-Hcl, 1.0 mM EDTA, 0.05 mM pyridoxyl 5-phosphate, and 5 mM dithiothreitol, pH 7.4) were added, and the cells were frozen at –80°C. Cells were thawed on ice, scraped and transferred to microfuge tubes, sonicated, and centrifuged at 12,000 g for 10 min at 4°C. Supernatants were incubated in stopper vials containing 20µl 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 mg protein per hour. The protein assay was carried out by the BCA method.

**Western blot and Cdk2 activity assays.** On day 4, T75 flasks were washed twice with ice-cold PBS. Cells were harvested in ice-cold cell lysis buffer by scraping with a rubber police man. Cell lysates were centrifuged at 10,000 g for 10 min. The supernatant was used for protein quantification. The amount of protein was determined following the BCA Protein Assay Reagent manufacturer's instructions. For western blots, 25 µg of total protein were separated on 10-15 % SDS-PAGE gels and transferred to PVDF 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 pH7.4, 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 aprotinin) and once with kinase assay buffer (25 mM HEPES [pH 7.4], 10 mM MgCl2, 1 mM EDTA, and 25µM ATP). Pellets were resuspended in 40 µl of kinase buffer containing 5 µg 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 substrate was visualized by autoradiography.

RNA extraction, reverse transcription, and real-time PCR. On Day 4, after discarding the growth medium, TRIzol was added to each T25 flask in order 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 260/280 nm. One µg total RNA and 0.5 µg of the random primers were used for reverse transcription with M-MLV RT following the manufacturer's instructions. The resulting cDNA was diluted to 100 µl with DEPC treated water and used as a template for real-time PCR. Briefly, PCR primers were designed with a melting temperature (Tm) 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: p21Waf1/Cip1 Forward primer, 5’-CTGAGAGGCCTGAAGACTCC-3’, Reverse
primer, 5’-CTCTTGCAGAAGACCAATCG-3’; CDK2, forward primer, 5’-
AGATTCTTCTGGG CTGCAA G-3’, Reverse primer, 5’-AGATCCGGAAGAGTTGGTCA-3’;
p53, Forward primer 5’-GCTTCGAGATGTCTCGAGAG-3’, Reverse primer 5’-TTTT
ATGGCGGG ACGTAGAC-3’and Actin, Forward primer, 5’-
CGTGAAAAGATGACCCCAGATCA-3’, Reverse primer, 5’-CCAG CCTGGATGGCTACGT-
3’). PCR reactions were performed using an ABI Prism 7700 Sequence Detection System
(Applied Biosystems). The reaction contained 25 µl SYBR reagent, 5 µl diluted cDNA, and
300-900 nM primers in 50 µl volume. The thermal cycling conditions involved an initial
denaturing step at 95 °C for 15 sec and an anneal-extension step at 60 °C for 1 min. Quantitative
values were obtained from the threshold cycle value (Ct), which is the point where a significant
increase of fluorescence is first detected. The transcript number of rat ß-actin was quantified as
an internal RNA control, and each sample was normalized on the basis of its ß-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 ß-actin and the control group, termed N, were calculated as: N = 2^ΔCt sample -
ΔCt calibrator (http://dorakmt.tripod.com/genetics/realtime.html), where Ct values of the sample and
calibrator were determined by subtracting the average Ct value of a target gene from the
corresponding Ct value of the ß-actin gene.

**Cdk2 and p21^Waf1/Cip1 mRNA stability studies.** Cells were grown as described earlier for real time
PCR analysis. On day 4, 15µg/ml actinomycin-D was added and further incubated for 0-9h. At
3h time intervals cells were washed and RNA was extracted. RNA isolation and quantification,
reverse transcription (RT) was carried out as described above. Briefly, 2µg RNA from each time
point samples of vector and V14-RhoA-transfected cells was used for RT. The resulting cDNA
was diluted to 100 µl with DEPC treated water. 10µl of diluted cDNA for Cdk2 and actin and 20µl for p21Waf/Cip was used as a template for PCR using gene specific primers. 1µl 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 using a streptavidin-alkaline phosphatase conjugate by ECL detection using the CDP star reagent. PCR products were also transferred to membranes 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 p21Waf/Cip 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 using ANOVA and appropriate post-hoc testing. P values < 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 to vector-transfected cells (fig.1A). In spite of 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 ornithine decarboxylase (ODC) activity compared to vector transfected serum starved cells. Interestingly, the stimulation of cells with serum further increased ODC activity of V-14 RhoA cells significantly compared to 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 the vector transfected cell line (fig.2). This implies that constitutively active RhoA compromised contact inhibition and 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.1A and fig.1C). 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 (fig4A), indicating that constitutively active RhoA stimulated cell proliferation by promoting the transition from G1 to S and 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 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 to vector transfected cells, HA-V14-RhoA transfected cells have a decreased basal level of p21\textsuperscript{Waf1/Cip1}. Consistent with previous findings (17, 34), DFMO treatment increased p21\textsuperscript{Waf1/Cip1} expression in vector transfected IEC-6 cells. In contrast, DFMO treatment did not change p21\textsuperscript{Waf1/Cip1} expression in HA-V14-RhoA transfected cells. HA-V14-RhoA transfected cells had a higher basal level of Cdk2 compared to vector transfected cells and DFMO decreased Cdk2 expression in both cell lines (Fig.6B). Phospho-Cdk2 (Thr160), the active form of Cdk2, was not increased relative to total Cdk2 (Fig.6C and 6B). Using the Cdk2 substrate histone H1, we found that constitutively active RhoA increased Cdk2 activity (Fig.6E). Polyamine depletion (DFMO) strongly inhibited Cdk2 activity in both cell lines. The data in figure 6 indicate that exogenous putrescine prevented the effect of DFMO on Cdk2 expression and activity.

**RhoA inhibits p21\textsuperscript{Waf1/Cip1} and increases Cdk2 by regulating transcription.** Since the levels of p21\textsuperscript{Waf1/Cip1} and Cdk2 protein expression were changed by constitutively active RhoA and polyamine depletion, we further examined p21\textsuperscript{Waf1/Cip1} and Cdk2 at the mRNA level in both the cell lines. Real-time PCR showed that HA-V14-RhoA dramatically decreased p21\textsuperscript{Waf1/Cip1} mRNA (fig.7A). DFMO increased p21\textsuperscript{Waf1/Cip1} mRNA in HA-V14-RhoA transfected cells but not in vector transfected cells. Exogenous putrescine restored p21\textsuperscript{Waf1/Cip1} mRNA in HA-V14-RhoA transfected cells. As shown in figure 7B, HA-V14-RhoA increased Cdk2 mRNA by 2-fold. DFMO decreased Cdk2 mRNA, and exogenous putrescine restored Cdk2 mRNAs in both cell lines. Observed changes in the steady state RNA and protein levels may result from changes in the stabilities of these molecules. Therefore, we examined the stability of Cdk2 and p21\textsuperscript{Waf1/Cip1} mRNA and protein. Vector and V14-RhoA transfected confluent IEC-6 cells were treated with actinomycin-D and cycloheximide for 0-9h to determine the stability of mRNA and protein.
respectively for the Cdk2 and p21\textsuperscript{Waf1/Cip1}. Southern blotting revealed that levels of Cdk2 mRNA were significantly increased and those of p21\textsuperscript{Waf1/Cip1} were barely detectable in V14-RhoA cells compared to vector-transfected cells at 0h. Due to the low steady state levels of p21\textsuperscript{Waf1/Cip1} mRNA in the V14-RhoA cells compared to 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 p21\textsuperscript{Waf1/Cip1} mRNA decreased significantly after 6h exposure to actinomycin-D in vector-transfected cells. The low levels of p21\textsuperscript{Waf1/Cip1} mRNA in V14-RhoA cells decreased little with actinomycin-D treatment. The stabilities of Cdk2 and p21\textsuperscript{Waf1/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 p21\textsuperscript{Waf1/Cip1} and Cdk2 expression are mediated through the regulation of transcription.

\textbf{RhoA inhibits p21\textsuperscript{Waf1/Cip1} through a p53 independent pathway.} To explore the possible mechanism underlying the inhibitory effect of RhoA on p21\textsuperscript{Waf1/Cip1}, we monitored p53, the best known regulator of p21\textsuperscript{Waf1/Cip1}. HA-V14-RhoA increased p53 mRNA, protein, and phosphoprotein levels (Fig.10A, 10B, and 10C, respectively). Thus, the decreased expression of p21\textsuperscript{Waf1/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 signals 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-p21^{Waf1/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 p21^{Waf1/Cip1}, phosphorylation at Thr-160, and dephosphorylation at Thr-14 or Thr-15 (1). Here, we showed that transfection of IEC-6 cells with RhoA decreased p21^{Waf1/Cip1} mRNA (fig.7A) and protein levels (fig.6A). The RhoA mediated decrease of p21^{Waf1/Cip1} was originally reported by Olson et al (25) in fibroblasts, and subsequently confirmed in mammary epithelial cells (18) and mesangial cells (7). Normally p53 increases p21^{Waf1/Cip1} by binding to DNA and increasing the transcription of p21^{Waf1/Cip1} mRNA. A mutation leading to the inactivation of p53 then leads to the decreased production of p21^{Waf1/Cip1}, increased cell proliferation, and frequently induced transformation. UV light and hepatitis B virus X protein also regulate expression of p21 Waf/Cip independently of P53 (2,14). Furthermore, the extent of
histone acetylation of the promoter (proximal promoter) of p21 and the amount of interaction with p300/CREB-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 proximal promoter dictates the induction level of the target gene. Thus, the level of p53 does not necessarily directly correlate with p21Waf/Cip 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 decreased 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 Cdk activating kinase, which activates Cdk2 by phosphorylating Thr-160. In addition to post-translational modification, Cdk2 activity is regulated at the transcriptional level. Stimulation with phorbol ester (15) or serum (13) increase 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 NIH3T3 cells, but later they showed that cells over expressing 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 NIH3T3 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 (fig.1B and 4B). Furthermore, in normal control cells, polyamine depletion increased the level of p21^{Waf1/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 p21^{Waf1/Cip1} (fig.6A). While
polyamine depletion increased the level of \( p21^{\text{Waf1/Cip1}} \) mRNA in the cells transfected with RhoA, the absolute level of mRNA remained several-fold below that in the vector transfected controls (fig. 7A). The low levels of \( p21^{\text{Waf1/Cip1}} \) were not due to decreased stability of its mRNA or protein (fig. 8.9). Thus, it appears that the changes in Cdk2 activity and growth rate, which occurred in the RhoA transfected cells, were independent of \( p21^{\text{Waf1/Cip1}} \). 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 \( p21^{\text{Waf1/Cip1}} \). Furthermore, supplementation of exogenous putrescine along with DFMO prevented the changes in the level of Cdk2 protein and activity, indicating that these effects were due to the depletion of polyamines and not to the DFMO. Increased basal and serum-induced ODC activity in V14-RhoA also indicates that RhoA-mediated proliferation and focus formation 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 MAP kinase and p53. Polyamine depletion decreased Cdk2 activity and inhibited the stimulating effect of RhoA on IEC-6 cell proliferation.
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REFERENCES


Figure Legends

Figure 1. Growth curves of vector and HA-V14-RhoA transfected IEC-6 cells. (A) Cells were plated at 3.33 X10³ cells/cm² and grew for 2, 4, 6, and 8 days before harvesting and counting. In control and DFMO/putrescine, active RhoA transfection stimulated IEC-6 cell proliferation. In DFMO, cell proliferation of both vector and HA-V14-RhoA transfected cell lines were strongly inhibited. (B) Basal and serum stimulated levels of ODC activity of vector and HA-V14-RhoA transfected IEC-6 cells. Mean ± SE, n = 3. *Significant difference compared with corresponding group in vector transfected cells (p < 0.05).

Figure 2. Cell densities of post-confluent vector and HA-V14-RhoA transfected IEC-6 cell lines. Cells were plated at 3.33X10³ cells/cm² and grown for 15 days. Cells were collected by trypsinization, counted by coulter counter to determine cell density. Mean ± SE, n = 3. *Significant difference compared with vector-transfected cells (p < 0.05).

Figure 3. Focus formation in HA-V14-RhoA transfected IEC-6 cells. Cells were plated at 3.33 X10³ cells/cm² and grew for 15 days. In control and DFMO/putrescine, 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).

Figure 4. Cell cycle analysis of vector and HA-V14-RhoA transfected IEC-6 cell lines. Cells were plated at 3.33X 10³ 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. Means ± SE, n = 3. *Significant difference compared with
corresponding cell cycle phase in vector transfected cells.

**Figure 5.** Constitutively active RhoA decreased phospho-Erk1/2 without changing total Erk1/2. Vector and HA-V14-RhoA transfected IEC-6 cells were plated at 3.33 X 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.

**Figure 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 X 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. p21^{Waf1/Cip1} (A), Cdk2 (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 using histone H1 substrate as described in MATERIALS AND METHODS and is represented as the means ± SE from 3 experiments. *Significant difference compared with corresponding group in vector transfected cells (p < 0.05).

**Figure 7.** Real-time PCR determination of the levels of p21^{Waf1/Cip1} and Cdk2 mRNA expression. Constitutively active RhoA decreased p21^{Waf1/Cip1} (A), and increased Cdk2 (B) mRNAs. Vector and HA-V14-RhoA transfected IEC-6 cells were plated at 3.33 X 10^3 cells/cm^2 and grown for 4 days in control, DFMO, DFMO/putrescine cultures. RNA extraction, reverse transcription, and real-time PCR were performed as described in MATERIALS AND METHODS. The expression level of each group is relative to that of the vector transfected cell line in control medium. Mean ± SE, n = 3. *Significant difference compared with corresponding group in vector-transfected cells (p < 0.05).
Figure 8. Stability of mRNA for Cdk2 and p21\textsuperscript{Waf/Cip}. Slot blotting of biotin-UTP labeled PCR amplified products using gene specific primers as described in methods was carried out. The amounts of PCR products were detected using streptavidin-AP conjugate followed by ECL detection. (A) CDK2 (B) p21\textsuperscript{Waf/Cip}, and (C) actin from vector and V14-RhoA cells are shown. Similar results were also obtained with Southern blot analysis. A representative slot blot is shown.

Figure 9. Stability of Cdk2 and p21\textsuperscript{Waf/Cip} proteins. Vector and V14-RhoA cells were grown for 3 days and serum starved for 24h before treatment with cycloheximide for 0-9h. Cell extracts were analyzed by western blot for Cdk2, p21\textsuperscript{Waf/Cip}, and actin protein using specific antibodies. Blot shown is representative of 3 experiments.

Figure 10. Constitutively active RhoA increased p53 mRNA (A), p53 protein (B), phospho-p53 protein levels (C). Vector and HA-V14-RhoA transfected IEC-6 cells were plated at 3.33 \texttimes 10^3 cells/cm\textsuperscript{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. Mean ± SE, n = 3. *Significant difference compared with vector-transfected cells (p < 0.05).
**Fig. 1**

### A

**Control**
- Vector
- HA-V14-RhoA

**DFMO**
- Vector
- HA-V14-RhoA

**DFMO+putrescine**
- Vector
- HA-V14-RhoA

*Note: The asterisk (*) indicates statistical significance.*
Fig. 1

B

ODC Activity (pmol CO2/hr/mg protein)

Vector

HA-V14-RhoA

FBS

0

Vector

HA-V14-RhoA

**

*
Cell number/Flask (x400)

Fig. 2

- Vector
- HA-V14-RhoA

* Indicates a significant difference.
Fig. 4

A. Control

B. DFMO

C. DFMO+putrescine

- G1
- S
- G2/M

Percent Cells Analyzed

Vector HA-V14-RhoA

*
Fig. 6

A p21

B Cdk2

C Phospho-Cdk2

D Actin

E Cdk2 activity

C Dp

Vector

HA-V14-RhoA
Fig. 7

A  p21

B  Cdk2

Relative mRNA amount

- Control
- DFMO
- DFMO+putrescine

Vector  HA-V14-RhoA  Vector  HA-V14-RhoA

1.5  2.0  2.5
1.0  0.5  1.0
0.0  0.5  0.0

*
Fig. 9

V14- RhoA

Vector

CDK2
p21
Actin

Cycloheximide Treatment (hour)
Fig. 10

A

Relative mRNA amount

0.0  0.5  1.0  1.5  2.0  2.5

Vector HA-V14-RhoA

100%  118±2.4% *

B

p53

100%  118±2.4% *

C

Phospho-p53

100%  130±2.8% *

D

Actin

Vector HA-V14-RhoA