The retinoblastoma protein Rb is critical for the regulation of mammalian cell cycle entry. Hypophosphorylated Rb is considered to be the active form and directs G₁ arrest, while hyperphosphorylated Rb permits the transition from G₁ to S phase for cell proliferation. Upon stimulation by various growth factors, Rb appears to be phosphorylated by a cascade of phosphorylation events mediated mainly by kinases associated with cyclins D and E. Here we report that in prototype small intestine crypt stem cells (RIEC-6), stimulation with either epidermal growth factor or fetal bovine serum results in an unexpected rapid and sustained Rb phosphorylation at sites Ser⁷⁸⁰, Ser⁷⁹⁵, and Thr⁸²¹ which precedes cyclin D1 expression, cyclin D1/cdk4 complex formation, and cdk4 kinase activity. Rb phosphorylation at Ser⁷⁸⁰ and Ser⁷⁹⁵ is prevented by MEK, but not phosphatidylinositol 3-kinase, inhibitors. In vitro, Rb is directly phosphorylated by active ERK1/2 as shown by [γ-³²P]ATP labeling. The phosphorylation sites are further directed to Ser⁷⁸⁰ and Ser⁷⁹⁵ by kinase assays using recombined active ERK1/2 or immunoprecipitated phospho-ERK1/2 from mitogen stimulated cells. Pull-down assays revealed that Rb interacts with active ERK1/2 but not their inactive unphosphorylated forms. Upon EGF stimulation, phosphorylated ERK1/2 co-immunoprecipitates together with phosphorylated Rb. Collectively, these results demonstrate a novel rapid Rb phosphorylation at specific sites induced by mitogen stimulation in epithelial cells of the small intestine. These data specifically identify ERK1/2 as the kinase responsible for Rb phosphorylation targeted to sites Ser⁷⁸⁰ and Ser⁷⁹⁵. It appears that ERK1/2 could be an important link between a mitogenic signal directly to Rb, thereby providing a rapid response mechanism between mitogen stimulation and cell cycle machinery.

Following the acute loss of intestinal length, there is an important compensatory response that occurs in the remnant bowel. This response, termed adaptation, is primarily a mitogenic signal to the intestinal crypt cells to grow taller villi and deepen the crypts, thereby augmenting the remaining absorptive and digestive mucosal surface area (1). Epidermal growth factor receptor (EGFR) signaling plays a vital role in intestinal adaptation after small bowel resection (2). It has been shown that inhibition of the EGFR by several experimental conditions significantly blunts the adaptation response (3), while EGF transgenic mice and administration of exogenous EGF result in an enhanced adaptation response (4, 5).

The exact mechanism(s) of intestinal adaptation are not well understood; however, EGFR-mediated increases in crypt cell proliferation are thought to be critically important. Activation of the EGFR initiates a complicated signaling network, which controls a myriad of biological outcomes, including cell proliferation, differentiation, apoptosis, and migration, etc. (6, 7). Mitogen activation of cell cycle machinery in many cells is thought to channel through the Ras/MEK/ERK or Ras/PI3K/ AKT pathway culminating in the induction of cyclin D1 expression (8–14). Expressed cyclin D1 associates with cyclin-dependent kinases (cdk) 4 and 6 causing their activation. Activated cdk4/6 then phosphorylates Rb, which leads to cell cycle progression (15). The exact mechanism for EGFR directed Rb phosphorylation within crypt enterocytes is fundamental for elucidating a more complete understanding of the process of intestinal adaptation after massive small bowel resection.

Rb affects the cell cycle progression by modulation of the activity of E2F transcription factors, which regulate the expression of many genes that are essential for cell cycle progression and S phase entry (16–18). Rb activity itself is regulated by a cascade of phosphorylation events during different phases of the cell cycle that are mediated mainly by kinases associated with cyclins D and E (15, 19–21). The cyclin D-cdk4/6 complex phosphorylates Rb at early G₁ phase and results in its partial activation, thus allowing the transcriptional activation of limited target proteins including cyclin A/E. In late G₁ phase, cyclin E associates with cdk2, and the resulting complex further phosphorylates Rb. The hyperphosphorylation of Rb then leads to disruption of the transcriptional repression complex and allows the expression of essential proteins required for G₁/S transition. During S phase, cyclin A-cdk2 activity is responsible for maintaining Rb phosphorylation (15). Most of this information has been derived from experiments using overexpression systems or in cancer cell lines in which the Rb regulation system may already be altered. It is therefore unclear as to what regulates Rb phosphorylation in vivo following a mitogenic stimulus for normal cell cycling.

Recently, mice have been generated whereby cyclins D1/D2/D3 (22) or cdk 4/6 (23) have been inactivated. Both strains of mice developed until mid/late gestation and then died secondary to defects in hematopoiesis. Despite this, it was noted that the majority of other tissues had developed normally. The quiescent cdk4/6-null or cyclin D1/D2/D3-
null embryonic fibroblast responded to serum stimulation and re-entered the cell cycle normally although with either lower efficiency or increased requirement for mitogenic stimulation. These in vivo results strongly suggest the presence of an alternative mechanism to initiate cell cycle entry besides the cyclin-cdk pathway. Interestingly, it has been shown thatraf-1, which is a MAPKKK, could interact and phosphorylate Rb directly, and disruption of the raf-1/Rb interaction inhibits Rb phosphorylation and cell proliferation (24, 25). Collectively, accumulated data favor the model of an alternative cyclin-independent pathway that could potentially regulate Rb phosphorylation, thereby playing an important role during mitogen induced cell cycle re-entry.

In our current study, we use RIEC-6 cells, which retain the undifferentiated character of the small intestine crypt stem cell (26) to investigate EGF-induced Rb phosphorylation. We found that EGF stimulation induced a rapid and sustained Rb phosphorylation at Ser780, Ser795, and Thr821. We provide in vitro and in vivo evidence to demonstrate that activated ERK1/2 is responsible for mitogen induced rapid Rb phosphorylation specifically at Ser780 and Ser795. This process occurs independent of cyclin/cdk pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-P-Ser780, anti-P-Ser795, anti-P-Ser807/811, anti-ERK1/2, anti-P-ERK1/2 (both monoclonal and polyclonal), anti-Akt, anti-P-Akt, and anti-cyclin D1 antibodies were from Cell Signaling Technology (Beverly, MA). Anti-cdk4 antibody was from BD Biosciences. Anti-P-Ser449/Thr252, anti-P-Thr821, and anti-P-Thr826 antibodies were from BIOSOURCE International (Camarillo, CA). The MAPK inhibitor U0126 was obtained from Cell Signaling. LY294002 and recombinant human EGF were from Sigma. The EGFR tyrosine kinase inhibitor ZD1839 was obtained from AstraZeneca (Cheshire, England).

**Plasmids and Protein Expression**—The construct GST fusion Rb large pocket domain containing amino acids from 379 to 928 (GST-LP) was a kind gift from Eric Knudsen (Department of Cell Biology, University of Cincinnati). A GST fusion Rb C-terminal region (GST-SP), GST fusion Rb single point mutant of Ser780 to A (S780A) and Ser795 to A (S795A) were made by PCR using GST-LP as the template and containing amino acids from 701 to 928. The PCR fragment was subsequently cloned into a pGEX 6P-1 vector (Amersham Bioscience). The PCR fragment was subsequently cloned into a pGEX 6P-1 vector (Amersham Bioscience). The sequences were confirmed by DNA sequencing and the GST fusion proteins were expressed in Escherichia coli strain DH5α, purified using glutathione-Sepharose beads (Amersham Bioscience) following the manufacturer's protocols.

**Cell Culture and Flow Cytometry**—RIEC-6 cells was obtained from American Type Culture Collection (Manassas, VA), and cells in passage 15 to passage 22 were used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (ATCC) complemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 10 µg/ml insulin. For all experiments, the cells were first starved for 24 h and then stimulated for the indicated time period.

For flow cytometry, RIEC-6 cells were grown on 100-mm culture dishes and serum-starved for 24 h for cell cycle synchronization. Following appropriate treatments, cells were trypsinized and transferred into 15-ml Falcon tubes. After centrifugation at 1000 × g for 5 min, cells were resuspended in 1 ml of phosphate-buffered saline, and 2 ml of 100% EtOH were added under continuous vortex. After 2 h of incubation at 4 °C, cells were pelleted by centrifugation. The pellets were resuspended in 500 µl of 0.2% RNase (Sigma catalog number R6513) and subsequently mixed with 490 µl of phosphate-buffered saline and 10 µl of propidium iodide (Sigma catalog number P4170). The final mixture was incubated for 30 min in the dark before analysis with FACS. Results were analyzed using Cell Quest (BD Biosciences).

**Whole-cell Lysates, Triton X-100 Extract Preparation, Immunoprecipitation, and Western Blotting**—Whole-cell lysates were prepared by directly adding 1× SDS sample buffer to the cells. For Triton X-100 cell extract preparation, the cells were washed with ice-cold phosphate-buffered saline and then lysed for 30 min in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 plus protease inhibitor tablet (Roche Applied Science) and phosphatase inhibitor mixture I/II (Sigma). The lysates were then centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant were saved as Triton X-100 cell extracts. For immunoprecipitation, the protein A plus G beads (Santa Cruz Biotechnology, Santa Cruz, CA) were washed three times with lysis buffer without protease inhibitors and then incubated with cell extracts for 1 h (preclearing) at 4 °C. Following this, the beads were spun down and discarded, and the supernatant was further incubated with protein A plus G beads and appropriate antibody for overnight at 4 °C. After washing in lysis buffer, the absorbed complexes were removed from the beads by heating for 5 min at 100 °C in 1× SDS sample buffer and separated on 10% polyacrylamide gels. Proteins resolved on gels were transferred to nitrocellulose membranes and detected with appropriate antibodies by Western blot.

**In Vitro Kinase Assay**—The in vitro kinase reaction was carried out in a total volume of 40 µl containing 5 µg of maltose-binding-protein fusion Rb C-terminal region (MBP-Rb) (Cell Signaling), 100 ng of recombinated active ERK1, or 50 ng of recombinated active ERK2 (Upstate Biotechnology, Charlottesville, VA), 100 µM ATP and 10 µg of [γ-32P]ATP (PerkinElmer Life Sciences) in kinase buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 15 mM MgCl2) at 30 °C for 30 min, then the reaction was terminated by adding 40 µl of 2× SDS sample buffer, and the sample was resolved by SDS-PAGE; the gel was then dried, and results were visualized by autoradiography. For antibody-based detection of Rb phosphorylation catalyzed by recombinant ERK1/2, the kinase assay was performed identically, except the same amount of MBP-Rb or GST fusion Rb or their mutants were used as substrate, and no [γ-32P]ATP was involved. For the immunoprecipitation kinase assay, the kinases were immunoprecipitated as indicated above and the final wash was done by using a kinase buffer. The beads were then resuspended in 20 µl of kinase buffer, 5 µg MBP-Rb was added as substrate, and the reaction was initiated by adding 100 µM ATP. After reaction, the samples were separated by SDS-PAGE and analyzed by Western blot using phosphorylation site-specific antibodies.

**Pull-down Assay**—GST or GST-LP was immobilized on the glutathione-Sepharose beads and mixed with unstimulated or EGF-stimulated RIEC-6 cell extracts for 3 h at 4 °C; the beads were collected by centrifugation and washed in triplicate with cell lysis buffer as mentioned above. Bound protein was then eluted from the beads by adding 1× SDS sample buffer and boiled for 5 min; the sample was separated by SDS-PAGE and analyzed by Western blot.

**RESULTS**

EGF and FBS Display a Differential Phosphorylation Pattern of Rb—To study the effect of EGF on Rb phosphorylation, we applied the phosphorylation site-specific antibodies that have been developed recently. As a positive control, 10% FBS was used to stimulate RIEC-6 cells to determine the Rb phosphorylation response. As demonstrated in Fig. 1, both FBS and EGF strongly activated ERK1 (p44) and ERK2 (p42) as shown by the phosphorylation of ERK1/2 detected by a phosphorylation site (Thr359/Tyr364) specific.
Rb Phosphorylation by ERK1/2

**FIGURE 1.** EGF and FBS induce a differential Rb phosphorylation pattern. RIEC-6 cells were serum-starved for 24 h and stimulated with 100 ng/ml EGF or 10% FBS for the indicated time of period. The cells were lysed with 1× SDS sample buffer; Rb phosphorylation was determined by Western blot analysis using phosphorylation site specific antibodies.

Cyclin D1 expression pattern also induced a similar cyclin D1 expression pattern over the time period tested. FBS stimulation for up to 20 h induced Rb phosphorylation at all sites examined, while EGF stimulation did not induce Rb phosphorylation at Ser780/Thr78/Thr821 and Thr826 during this same period. Under FBS stimulation, Rb was slightly phosphorylated at Ser780/Thr78/Thr821 and Ser826 by 8 h and became further phosphorylated by 20 h. The phosphorylation at Ser807/811 occurred between 8 and 20 h.

Rb Phosphorylation at Ser780/Ser795/Thr821 Induced by EGF or FBS Precedes Expression of Cyclin D1, Formation of Cyclin D1/cdk4 Complex, and cdk4 Kinase Activity—Phosphorylation at Ser780, Ser795, and Thr821 was already significant as soon as 8 h with either EGF or FBS stimulation (Fig. 1). These observations directed us to evaluate Rb phosphorylation at these sites at earlier time points. Surprisingly, both EGF and FBS induced a very rapid (within minutes for Ser780/Thr78 and 1 h for Thr821) and sustained Rb phosphorylation at these sites (Fig. 2, A and B). Phosphorylation of Ser780/Thr78 is thought to be mediated by the cyclin D1 associated kinase Cdk4/6, while phosphorylation of Thr821 is felt to be mediated by the cyclin E associated kinase Cdk2 (27, 28). In contrast with these data, Rb phosphorylation preceded cyclin D1 expression at early time points in our experiments. Cyclin D1 expression was not detectable until after 4 h of mitogen stimulation. Moreover, the phosphorylation of Ser780/Thr78 did not seem to be elevated further by the later expression of cyclin D1. In a recent study, Cdk4 has been suggested to be involved in rapid Rb phosphorylation at Ser780 after mitogen stimulation in vascular smooth muscle cells (29). To determine whether cdk4/6 was responsible for the rapid Rb phosphorylation in RIEC-6 cells, we recovered Triton X-100 extracts from stimulated cells. Cdk4/6 were immunoprecipitated and the complexes detected for cyclin D1 incorporation and kinase activity. Cyclin D1 could be detected within cdk4 complexes at 4 h after mitogen stimulation (Fig. 2C) but not in cdk6 complexes even after 8 h (data not shown). The cdk4 kinase activity was then analyzed using MBP-Rb as a substrate and revealed by immunoblotting using site specific phosphorylation antibodies. While cdk4 was presented evenly in the immunoprecipitated complex, its activity only began to appear 2 h after mitogen stimulation as evidenced by its ability to phosphorylate Rb at Ser780 in an in vitro kinase assay (Fig. 2C). Collectively, these data suggesting that mitogen induced rapid Rb phosphorylation at Ser807/Thr821 is very unlikely to be mediated by cyclin D associated kinases in RIEC-6 cells.

**FIGURE 2.** Rb phosphorylation at Ser780, Ser795, and Thr821 is rapid and precedes cyclin D1 expression, cyclin D1/cdk4 complex formation and cdk4 kinase activity. RIEC-6 cells were serum-starved for 24 h and stimulated with 100 ng/ml EGF (A) or 10% FBS (B, C) for the indicated time of period. In A and B, the cells were lysed directly with 1× SDS sample buffer, Rb phosphorylation was determined by Western blot (WB) analysis using phosphorylation site specific antibodies, and the other proteins were detected with the corresponding antibodies. In C, the cells were lysed with 1% Triton X-100, and the soluble fraction was utilized to immunoprecipitate cdk4; the immunoprecipitated complex was then either subjected to immunoblotting for cdk4 and cyclin D1 or kinase assay utilizing MBP-Rb as substrate and analyzed by Western blot using phosphorylation site-specific antibody.

**Inhibition of MEK, but Not PI3K, Prevents Rb Phosphorylation at Ser807/Ser795/Thr821—**The temporal activation of ERK1/2 was noted closely parallel Rb phosphorylation of Ser780/Ser795. Sequence analysis revealed that Rb has multiple potential MAPK phosphorylation sites (30, 31), including Ser807/Ser795/Thr821. We therefore next tested the possibility of direct ERK1/2 phosphorylation of Rb. First, we determined the effect of the ERK1/2 upstream kinase MEK inhibitors. As expected, ERK1/2 activation was abolished by pretreating RIEC-6 cells with either PD98059 (data not shown) or U0126 (Fig. 3, A and B). Rb phosphorylation at Ser780 was totally blocked by administration of U0126; phosphorylation at Ser795 was prominently reduced, while phosphorylation at Thr821 was only slightly affected by the drug under conditions of either EGF or FBS stimulation. The EGFR inhibitor ZD1839 totally abolished EGF induced signals such as ERK1/2 and Rb phosphorylation (data not shown). However, this inhibitor did not significantly affect either serum stimulated Rb phosphorylation or ERK1/2 phosphorylation until a later time point (6 h). These results suggest that alternative pathways for activation of the downstream kinase ERK1/2 may contribute to EGF stimulated Rb phosphorylation at these sites. We also noticed that in these cells, inhibition of ERK1/2 activity only partially reduced cyclin D1 expression. This contrasts with a previous study in which EGF stimulation did not induce Rb phosphorylation at Ser780, Ser795, and Thr826 during this same period. Under FBS stimulation, Rb was slightly phosphorylated at Ser780/Thr78/Thr821 and became further phosphorylated by 20 h. The phosphorylation at Ser807/811 occurred between 8 and 20 h.

**Inhibition of MEK, but Not PI3K, Prevents Rb Phosphorylation at Ser807/Ser795/Thr821**
which sustained ERK1 activation was required for cyclin D1 expression in CHO cells (32).

In addition to the ERK pathway, the PI3K pathway is also known to be activated by EGFR stimulation (7) and has been considered to play multiple roles during cell cycle progression (33). We therefore interrogated the role for PI3K as a possible contributor toward Rb phosphorylation at Ser780/Ser795/Thr821 in our cells. As shown in Fig. 3, C and D, treatment of the cells with the PI3K inhibitor LY294002 abolished most of the kinase activity as evidenced by the disappearance of phosphorylated Akt, the conventional effector of PI3K. The expression of inactive Akt remained the same under all conditions. Rb phosphorylation at Ser780, Ser795, or Thr821 was unaffected, despite PI3K activity inhibition. These data endorse the notion that PI3K is unlikely to be involved in mitogen-stimulated Rb phosphorylation at these 3 sites. It is also important to point out that PI3K inhibition had no effect on activation of ERK1/2, while cyclin D1 expression was totally diminished. These observations are in agreement with other investigators (34). Collectively, these results indicate that ERK1/2, but not PI3K is involved in mitogen-induced rapid Rb phosphorylation at sites Ser780/Ser795/Thr821.

**ERK1/2 Phosphorylates Rb at Ser780/Ser795**—We next sought to determine whether Rb could be a potential substrate for ERK1/2 directly. To test this, MBP-Rb was incubated with a recombined constitutive active GST fusion ERK1/2 and [γ-32P]ATP in vitro. As shown in Fig. 4A, MBP-Rb was strongly phosphorylated by both active ERK1 and ERK2, while the carrier protein MBP or active ERK1/2 itself only showed background radiolabeling under the reaction conditions. This result indicates that Rb is a substrate of ERK1/2.

Since mitogen-stimulated Rb phosphorylation at Ser780/Ser795 was most affected by MEK inhibition, we next tested whether ERK1/2 could specifically phosphorylate Rb at Ser780 or Ser795. A GST fusion Rb construct containing amino acids 701–928 (GST-SP) was generated. In addition, point mutations of Ser780 to Ala (S780A) or Ser795 to Ala (S795A) were also produced. The constructs were then expressed in E. coli, and the GST fusion proteins were purified from Triton X-100 lysates of bacteria by glutathione-affinity purification. The protein was then incubated with or without active ERK1/2, and the reaction results were analyzed by Western blot using phosphorylation site specific antibodies. In the complete absence of kinases (Fig. 4B) or the presence of an inactive ERK2 (data not shown), no phosphorylation at Ser780 could be detected. Application of either active ERK1 or ERK2 resulted in a robust phosphorylation signal at Ser780 with GST-SP and S795A, but not S780A, indicating the specific Rb phosphorylation at Ser780 by active ERK1/2. Although the antibody P-S795 showed a slight background for all the GST fusion proteins even without kinase involvement, ERK1 application resulted in a significant increase in the phosphorylation signal with GST-SP and S780A but not S795A. These results indicate a specific Rb phosphorylation at Ser780 by active ERK1 (Fig. 4B). Further experiments indicate that even though ERK1 could phosphorylate Rb at both Ser780 and Ser795, the efficiency of phosphorylation was different. We found high phosphorylation efficiency at Ser780 and low efficiency at Ser795 (see supplemental Fig. 1). When ERK2 was used in the kinase assay, no more than background signal was detected by antibody P-S795 with all the fusion proteins (Fig. 4B). As a control, the same amount of protein was present in all the reactions as shown by the Poceau S staining of the same membrane utilized for Western blot (Fig. 4B).

To further confirm ERK1/2 phosphorylation of Rb, RIEC-6 cells were stimulated and Triton X-100 cell extracts were recovered. Phospho-ERK1/2 was immunoprecipitated followed by a kinase assay. As shown in Fig. 4C, the immunoprecipitated phospho-ERK1/2 could phosphorylate Rb at Ser780 and Ser795 under the conditions tested. As a control, similar levels of phospho-ERK1/2 were measured in this assay. Collectively these results clearly demonstrate that active ERK1/2 can phosphorylate Rb specifically at Ser780/Ser795.

**Phosphorylated ERK Interacts Directly with Phosphorylated Rb in Vivo and in Vitro**—To determine whether ERK1/2 could physically interact with Rb, we first performed a pull down assay. We used GST-LP, which contained the large pocket domain of Rb as a bait for the pull down. As expected EGF stimulation resulted in robust ERK1/2 phosphorylation in RIEC-6 cells (Fig. 5A). In contrast, the total unphosphorylated ERK1/2 was maintained at a similar level. The Triton X-100 cell extracts were generated from the stimulated and unstimulated cells, which were incubated together with either GST alone or GST-LP, which was immobilized on the glutathione-Sepharose beads. After extensive washing, the bound protein was eluted by 1× SDS sample buffer.
buffer and presented for Western blotting. As shown in Fig. 5A, GST-LP only pulled down the phosphorylated form of ERK1/2 but not the unphosphorylated form. As a control, GST itself did not pull down anything from the cell extracts. These results indicate that the large pocket domain could interact with the active form of ERK1/2. The same Triton X-100 cell extracts were utilized to perform pull-down assay. Equal amounts of fusion protein used for all reactions were confirmed by Ponceau S staining of the same membrane after Western blot. C, RIEC-6 cells were stimulated as indicated, then lysed with 1% Triton X-100, and the soluble fraction was utilized to immunoprecipitate (IP) phospho-ERK1/2. The immunoprecipitated complexes were then either subjected to immunoblotting for the presence of phospho-ERK1/2 or kinase assay (KA) utilizing MBP-Rb as substrate and analyzed by Western blot (WB) using phosphorylation site-specific antibodies.

FIGURE 4. ERK1/2 phosphorylates Rb in vitro. A, kinase assay was performed using MBP-Rb as a substrate and incubated with recombined active ERK1/2 in the presence of [γ-32P]ATP. Proteins were resolved on 10% SDS-PAGE, and Rb phosphorylation was detected by autoradiography. B, kinase assay was performed using purified GST-SP (lanes 1 and 4) or mutant S780A (lanes 2 and 5) or mutant S795A (lanes 3 and 6) as substrates. Reactions were carried out in the absence or presence of recombinant active ERK1/2. Protein was resolved by 10% SDS-PAGE and Rb phosphorylation was detected by Western blot analysis using phosphorylation site-specific antibodies. Equal amounts of fusion protein used for all reactions were confirmed by Ponceau S staining of the same membrane after Western blotting. B, RIEC-6 cells were serum-starved for 24 h and stimulated with EGF (100 ng/ml) for 15 min. The cells were then lysed with 1% Triton X-100, and the soluble fraction was utilized to do the pull-down assay. GST or GST-LP was immobilized to glutathione-Sepharose 4B beads and used as bait. The GST and GST-LP were visualized by Ponceau S staining of the same membrane after Western blotting. B, RIEC-6 cells were stimulated with EGF (100 ng/ml) for 15 min, the cell extracts were prepared as in described for A, and immunoprecipitation was performed using a polyclonal anti-phospho-ERK1/2 antibody, anti-P-Ser780 antibody, or control antibody. The immunocomplexes were recovered, and the bound protein was eluted and separated, and the binding of ERK1/2 to the GST-LP was verified by Western blot (WB) analysis using anti-ERK1/2 or anti-phospho-ERK1/2 antibodies. The GST and GST-LP were visualized by Ponceau S staining of the same membrane after Western blotting. B, RIEC-6 cells were serum-starved for 24 h and stimulated with EGF (100 ng/ml) for 15 min, the cell extracts were prepared as in described for A, and immunoprecipitation was performed using a monoclonal anti-phospho-ERK1/2 antibody.

FIGURE 5. ERK1/2 interacts directly with Rb. A, RIEC-6 cells were serum-starved for 24 h and stimulated with or without EGF (100 ng/ml) for 15 min. The cells were then lysed with 1% Triton X-100, and the soluble fraction was utilized to do the pull-down assay. GST or GST-LP was immobilized to glutathione-Sepharose 4B beads and used as bait. The bound protein was eluted and separated, and the binding of ERK1/2 to the GST-LP was verified by Western blot (WB) analysis using anti-ERK1/2 or anti-phospho-ERK1/2 antibodies. The GST and GST-LP were visualized by Ponceau S staining of the same membrane after Western blotting. B, RIEC-6 cells were stimulated with EGF (100 ng/ml) for 15 min, the cell extracts were prepared as in described for A, and immunoprecipitation was performed using a polyclonal anti-phospho-ERK1/2 antibody, anti-P-Ser780 antibody, or control antibody. The immunocomplexes were recovered, and the bound protein was eluted and separated, and the binding of ERK1/2 to the GST-LP was verified by Western blot (WB) analysis using anti-ERK1/2 or anti-phospho-ERK1/2 antibodies.

FIGURE 6. EGF alone does not induce S-phase entry. RIEC-6 cells were serum-starved and stimulated with EGF (100 ng/ml) or 10% FBS for 20 h. The percentage of cells in the S phase was determined by staining cells with the propidium iodide followed by FACS. The data are mean values ± S.E. representing four independent experiments.

in 95% of the cells arresting in G0 phase. Following 20 h of serum stimulation, roughly 20% of the cells were in S phase. However, under EGF stimulation, only about 7% of the cells were in S phase despite the findings that Ser780/Ser795/Thr821 are all phosphorylated during this time. These results suggest that phosphorylation of Rb at Ser780/Ser795/Thr821 is not enough to cause cell cycle re-entry after serum deprivation alone. Phosphorylation of Rb at additional sites is therefore likely to be required to fully inactivate the cell cycle arrest function of Rb.

DISCUSSION
Most of our current knowledge about the function of Rb phosphorylation is derived from studies using an over expression system or in vitro...
cell free analysis. The detection of phosphorylated Rb is often either based on the slow migration band detected by a general Rb antibody or [γ-32P]ATP incorporation (21, 24, 27, 28, 35). While these studies have provided us with very useful information about the function of Rb phosphorylation, the site-specific or temporal regulation of Rb phosphorylation was not fully elucidated. In the present study, we use phosphorylation site(s)-specific antibodies to detect mitogen-induced Rb phosphorylation in a prototypical undifferentiated small intestine epithelial cell line (26). We observed a very rapid and sustained Rb phosphorylation occurring at sites Ser780/Ser795/Thr821 by FBS or EGF stimulation. Phosphorylation at other sites Ser249/Thr252/Ser807/811/Thr826 could only be induced by FBS stimulation at later time points. This is an important observation, since traditional detection methods based on different migration velocities between the hypophosphorylated and hyperphosphorylated forms of Rb would not be possible to identify. Furthermore, the temporal and site specific differences in Rb phosphorylation in response to FBS and EGF stimulation would be impossible to distinguish.

The significance of Rb phosphorylation at different sites is suggested by the observations that Rb function may be modulated according to the location of its phosphorylation. For example, Ser780, Ser795, and Thr821/826 appear to be involved in the disruption of E2F binding to Rb (35). Alternatively, phosphorylation of Ser807 may disrupt the binding of c-Abl to Rb (21). In addition, it has been presented that Thr821/Thr826 phosphorylation is required for releasing the LXCXE motif containing protein from Rb repression complex (21, 27). In vascular smooth muscle cells, mitogen-induced rapid Rb phosphorylation at Ser795 by cdk4 may dissociate E2F binding from Rb (29). In SAOS-2 cells, Rb phosphorylation at Ser795 by cdk4 is required for activation of the Rb cell cycle arrest function (28). Phosphorylation at Thr826 is felt to be required for pp32 binding to Rb and inhibits pp32 mediated apoptosis (36). It has also been reported that Rb phosphorylation at Ser807 by cyclin E-cdk2 is critical for disruption of the pocket A and B interface and preventing Rb from binding and inactivating E2F (20).

In our study, we found EGF stimulation induces a rapid and sustained Rb phosphorylation at Ser795, Ser795, and Thr252, but it is not enough to induce cell cycle re-entry in small intestine epithelial cells. FBS stimulation induces a normal cell cycle re-entry and Rb is phosphorylated at other sites including Ser249/Thr252, Ser807/811, and Thr826. These findings would suggest that phosphorylation of Rb at these sites are required for cell cycle re-entry. However, whether early Rb phosphorylation at Ser780/Ser795/Thr821 is required for later Rb phosphorylation at Ser249/Thr252, Ser807/811, or Thr826 is currently unclear. When the MEK inhibitor U0126 was used to block early Rb phosphorylation at Ser780/Ser795, it also significantly abolished FBS-induced later Rb phosphorylation at Ser249/Thr252, Ser807/811, and Thr826 (see supplemental Fig. 2). Therefore, at this time point we cannot determine whether sequential Rb phosphorylation at Ser780/Ser795, ERK1/2 kinase activity, or both are required for the later Rb phosphorylation at Ser249/Thr252, Ser807/811, and Thr826. Despite this, the differential Rb phosphorylation pattern at specific sites at early versus later stages following mitogenic stimulation discovered in this study provides further mechanistic insight into the function of Rb phosphorylation in the control of cell cycle machinery.

Mitogen-regulated Rb phosphorylation is thought to be initiated early through cyclin D-ckd4/6 and later via cyclin A/E-ckd2. Mitogen stimulation activates the Ras-Raf-MEK-ERK pathway, which will activate the cyclin D1 promoter resulting in the synthesis of cyclin D1 (9, 33, 37). Simultaneously, mitogen stimulation activates the Ras-PISK-AKT pathway, which triggers a cascade of signals resulting in the stabilizing of cyclin D1 (33). Cyclin D1 then binds to cdk4/6 and initiates Rb phosphorylation by ERK1/2.

In the present study the early phosphorylation of Rb at Ser780/Ser795/Thr821 induced by mitogen stimulation could not be due to cyclin D-ckd4/6 activity, since there was no detectable cyclin D1 expression or cyclin D1/ckd4 kinase activity at the early stimulation time points. Furthermore, even though cyclin D1 expression was fully activated by both FBS and EGF at later time points, only FBS induced a strong Rb phosphorylation at Ser249/Thr252/Ser807/811/Thr826. These observations suggest that cyclin D1 is insufficient by itself to induce Rb phosphorylation, at least at Ser249/Thr252/Ser807/811/Thr826. Pretreating cells with LY294002, a PI3K inhibitor, totally abolished cyclin D1 synthesis while not affecting Rb phosphorylation at Ser780/Ser795/Thr821. These results strongly suggest an alternative mechanism for Rb phosphorylation by mitogen stimulation that is cdk-independent. Similarly, in quiescent U937 cells, serum stimulation results in Rb phosphorylation before the appearance of cyclin D or cdk kinase activity, but this early Rb phosphorylation is found to be mediated by Raf-1 and is independent of its downstream MAPK pathway (24). In mouse embryo fibroblast cells, ERK activity is found not required for Rb-null cell proliferation which indicates the importance of ERK in regulating Rb function (38). Our current study provides strong evidence that ERK1/2 is capable of physically binding to Rb to cause phosphorylation in a rapid fashion in response to mitogen stimulation. These results represent the finding of a novel, alternative way to regulate Rb phosphorylation via the MAPK pathway and to directly link mitogen stimulation and cell cycle machinery that is independent of the traditional cyclin-associated kinase pathway.

The findings in this study involved a prototypical rat intestinal crypt epithelial cell line (26). These cells differ in several respects from crypt stem cells found in the intestine of adult rats and therefore have inherent limitations. First, these cells are derived from fetal rat intestine. They do not show features of terminal differentiation, analogous to what occurs as cells emerge from the crypt/villus junction. Therefore, the effects of factors controlling cell cycle withdrawal and differentiation soon after proliferation cannot be studied. Furthermore, depending on the culture conditions, tight junctions in RIEC-6 cells are not the same as what is encountered in vivo. As it is established that cell adhesion plays a critical role in the control of cell proliferation (39), the contribution of this factor to our results cannot be tested.

Despite the above-mentioned limitations, site-specific phosphorylation of Rb within intestinal crypt cells offers important mechanistic insight into the process of signaling for enterocyte proliferation. This mechanism is especially relevant to the adaptation response of the intestine to massive intestinal loss, which is largely a mitogenic signal to the proliferative crypt region of the bowel mucosa. Since insufficient adaptation commits patients to a lifelong need for supplemental nutrition by vein, a thorough understanding of this mechanism is fundamental toward the development of clinical therapy designed to enhance this important response.

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REFERENCES
1. O’Brien, D. P., Nelson, L. A., Huang, F. S., and Warner, B. W. (2001) Semin. Pediatr. Surg. 10, 56 – 64
2. Chart, M. S., Arya, G., Ziegler, M. M., and Warner, B. W. (1994) J. Pediatr. Surg. 29, 1035–1038
3. Helmrath, M. A., Erwin, C. R., and Warner, B. W. (1997) J. Surg. Res. 69, 76 – 80
4. Erwin, C. R., Helmrath, M. A., Shin, C. E., Falcone, R. A., Jr., Stern, L. E., and Warner, B. W. (1999) Am. J. Physiol. 277, G533–G540
| Reference                                                                                      |
|------------------------------------------------------------------------------------------------|
| 5. Helmrath, M. A., Shin, C. E., Erwin, C. R., and Warner, B. W. (1998) *J. Pediatr. Surg.* 33, 229–234 |
| 6. Yarden, Y., and Sliwkowski, M. X. (2001) *Nat. Rev. Mol. Cell. Biol.* 2, 127–137             |
| 7. Jorissen, R. N., Walker, F., Pouliot, N., Garrett, T. P., Ward, C. W., and Burgess, A. W. (2003) *Exp. Cell Res.* 286, 31–53 |
| 8. Lavoie, J. N., L’Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996) *J. Biol. Chem.* 271, 20668–20676 |
| 9. Aktas, H., Cai, H., and Cooper, G. M. (1997) *Mol. Cell. Biol.* 17, 3850–3857               |
| 10. Cheng, M., Sexl, V., Sherr, C. J., and Roussel, M. F. (1998) *Cell* 118, 477–491             |
| 11. Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. (1998) *Genes Dev.* 12, 3499–3511 |
| 12. Roovers, K., and Assoian, R. K. (2000) *BioEssays* 22, 818–826                             |
| 13. Rivard, N., Boucher, M. J., Asselin, C., and L’Allemain, G. (1999) *Am. J. Physiol.* 277, C652–C664 |
| 14. Wilkinson, M. G., and Millar, J. B. (2000) *FASEB J.* 14, 2147–2157                      |
| 15. Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* 13, 1501–1512                         |
| 16. Frolov, M. V., and Dyson, N. J. (2004) *J. Cell Sci.* 117, 2173–2181                      |
| 17. Dyson, N. (1994) *J. Cell Sci. Suppl.* 18, 81–87                                        |
| 18. Dyson, N. (1998) *Genes Dev.* 12, 2245–2262                                              |
| 19. Lundberg, A. S., and Weinberg, R. A. (1998) *Mol. Cell. Biol.* 18, 753–761                |
| 20. Harbour, J. W., Luo, R. X., Dei, S. A., Postigo, A. A., and Dean, D. C. (1999) *Cell* 98, 859–869 |
| 21. Knudsen, E. S., and Wang, J. Y. (1996) *J. Biol. Chem.* 271, 8313–8320                   |
| 22. Malumbres, M., Sotillo, R., Santamaria, D., Galan, J., Cerezo, A., Ortega, S., Dubus, P., and Barbacid, M. (2004) *Cell* 118, 493–504 |

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23. Kozar, K., Cieśnierz, M. A., Rebel, V. I., Shigematsu, H., Zaggozdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R. T., Akashi, K., and Sicinski, P. (2004) *Cell* 118, 477–491

24. Dasgupta, P., Sun, J., Wang, S., Fusaro, G., Betts, V., Padmanabhan, J., Sebti, S. M., and Chellappan, S. P. (2004) *Mol. Cell. Biol.* 24, 9527–9541

25. Wang, S., Ghosh, R. N., and Chellappan, S. P. (1998) *Mol. Cell. Biol.* 18, 7487–7498

26. Quaroni, A., Wands, J., Trelstad, R. L., and Isselbacher, K. J. (1979) *J. Cell Biol.* 80, 248–265

27. Zarkowska, T., and Mittnacht, S. (1997) *J. Biol. Chem.* 272, 12738–12746

28. Connell-Crowley, L., Harper, J. W., and Goodrich, D. W. (1997) *Mol. Biol. Cell* 8, 287–301

29. Garnovskaya, M. N., Mukhin, Y. V., Vlasova, T. M., Grewal, J. S., Ullian, M. E., Tho-lanikunnel, B. G., and Raymond, J. R. (2004) *J. Biol. Chem.* 279, 24999–24905

30. Buckley, C. T., Sekiya, F., Kim, Y. J., Ilbey, S. G., and Caldwell, K. K. (2004) *J. Biol. Chem.* 279, 41807–41814

31. Davis, R. J. (1993) *J. Biol. Chem.* 268, 14553–14556

32. Weber, J. D., Raben, D. M., Phillips, P. J., and Baldassare, J. J. (1997) *Biochem. J.* 326, 61–68

33. Liang, J., and Slingerland, J. M. (2003) *Cell Cycle* 2, 339–345

34. Sheng, H., Shao, J., Townsend, C. M., Jr., and Evers, B. M. (2003) *Gut* 52, 1472–1478

35. Knudsen, E. S., and Wang, J. Y. (1997) *Mol. Cell. Biol.* 17, 5771–5783

36. Malumbres, M., and Pellicer, A. (1998) *Front. Biosci.* 3, d887–d912

37. D’Abaco, G. M., Hooper, S., Paterson, H., and Marshall, C. J. (2002) *J. Cell Sci.* 115, 4607–4616

38. Crossin, K. L. (2002) *Ann. N. Y. Acad. Sci.* 961, 159–160
