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IGF-I stimulates human intestinal smooth muscle cell growth by regulation of G_1 phase cell cycle proteins

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Kuemmerle, John F., Huiping Zhou, and Jennifer G. Bowers. IGF-I stimulates human intestinal smooth muscle cell growth by regulation of G_1 phase cell cycle proteins. *Am J Physiol Gastrointest Liver Physiol* 286: G412–G419, 2004. First published October 30, 2003; 10.1152/ajpgi.00403.2003.—Autocrine production of insulin-like growth factor-I (IGF-I) regulates growth of human intestinal muscle cells by activation of distinct phosphatidylinositol 3-kinase (PI3-kinase)-dependent and ERK1/2-dependent pathways. The aim of the present study was to determine the mechanisms by which IGF-I regulates the G_1 phase of the cell cycle and muscle cell proliferation. Incubation of quiescent cells with IGF-I stimulated time-dependent cell cycle progression measured by using fluorescence-activated cell sorting analysis and by incorporation of [^{3}H]thymidine. Studies using a microarray-based approach were used initially to identify genes expressed in human intestinal muscle encoding proteins known to participate in the G_1 phase of the cell cycle that were regulated by IGF-I. Incubation of muscle cells for 24 h with IGF-I elicited greater than fivefold increase in the expression of cyclin D1 and greater than twofold increase in retinoblastoma protein (Rb1). IGF-I elicited a time-dependent increase in cyclin D1 protein levels mediated jointly by ERK1/2-dependent and PI3-kinase-dependent mechanisms. Increase in cyclin D1 levels was accompanied by a time-dependent increase in cyclin D1-dependent cyclin-dependent kinase-4 (CDK4) activity. IGF-I also elicited a rapid time-dependent increase in Rb-(Ser807/811) phosphorylation, the specific target of the cyclin D_1-dependent CDK4 kinase, and a slower increase in total Rb protein levels. We conclude that IGF-I stimulates G_1 phase progression, DNA synthesis, and cell proliferation of human intestinal smooth muscle cells. Effects of IGF-I on proliferation are mediated jointly by ERK1/2-dependent and PI3-kinase-dependent pathways that regulate cyclin D1 levels, CDK4 activity, and Rb activity.

retinoblastoma; cyclin D; cyclin-dependent kinase; extracellular signal-regulated kinase 1/2; phosphatidylinositol 3-kinase

DURING THE G_1 PHASE OF the eukaryotic cell division cycle, signals transduced from activated growth factor receptors converge on the nucleus to regulate progression through the cell cycle. These signals can cause cells to either enter the G_1 phase of the cell cycle and divide or to exit from the cell cycle (12, 31). After the restriction point in the G_1 phase is passed, the cell cycle progresses largely independent of growth factors. During the G_1 phase, DNA synthesis and proliferation are initiated by increases in cyclin D and E expression (7, 12, 29, 31). Effects of cyclins D and E are mediated by the concomitant increase in the activity of their specific cyclin-dependent kinase (CDK) partners, CDK4 and CDK6 (cyclin D-specific) and CDK2 (cyclin E-specific). The retinoblastoma (Rb) family of proteins, p105, p107 and p130, act as transcriptional repressors in the quiescent cell by binding and sequestering a variety of transcription factors, of which members of the E2F family are well recognized in this regard (10). Phosphorylation of Rb on specific residues by active cyclin-CDK pairs decreases the affinity of the Rb protein for E2F, releasing bound transcription factors to act on their target genes, many of which regulate cell division (9, 34).

Insulin-like growth factor-I (IGF-I) exerts two complementary effects on cellular proliferation: *1*) it is mitogenic for many cells, and *2*) it promotes the increase in cell size required for cell division to occur. In a variety of muscle types, IGF-I regulates proliferation through its effects on the cell cycle. In skeletal muscle, IGF-I enhances G_1/S phase progression in a phosphatidylinositol 3-kinase (PI3-kinase)-dependent manner (5) and can exert both mitogenic and myogenic effects (6). In cardiac myocytes, IGF-I regulates the G_1 phase cell cycle machinery by increasing cyclin D1 and E expression in an ERK1/2-dependent fashion and increasing CDK activity (30). IGF-I also stimulates DNA synthesis in vascular, airway, and uterine smooth muscle. Although the signaling cascades mediating proliferation of smooth muscle have been identified in some cell types, the mechanisms by which IGF-I regulates the cell cycle are less well understood. In uterine smooth muscle cells, IGF-I acts synergistically with estrogen to regulate cell growth and leiomyoma formation, in part, through its effects on cell cycle progression (1, 3). In vascular smooth muscle cells, IGF-I regulates proliferation and atheroma formation (26).

The important role of endogenous IGF-I in the regulation of visceral and vascular smooth muscle growth is being increasingly appreciated. Although distinct intracellular signaling cascades activated by IGF-I have been identified in vascular smooth muscle and intestinal smooth muscle, the effects of IGF-I on cell cycle proteins in these cell types are less well characterized (8, 18). Overexpression of IGF-I cDNA in transgenic animals results in hyperplasia of vascular and visceral smooth muscle tissues including the muscularis propria of the intestine (27, 37). Upregulated expression of IGF-I has been implicated in the development of uterine leiomyoma and vascular atheromas and may also be involved in the development of intestinal strictures in Crohn's disease (3, 26, 40).

The aim of the present study was to determine the mechanisms by which IGF-I controls the G_1 phase of the cell cycle in human intestinal muscle and thereby regulates muscle cell proliferation. A microarray-based approach was utilized initially to identify the genes encoding G_1-phase cell cycle proteins that were expressed by human intestinal smooth muscle

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and might be regulated by IGF-I. The mechanisms mediating IGF-I-dependent regulation of the G_1 phase of the cell cycle that involved the identified genes were then investigated directly. The ability of IGF-I to elicit cell cycle progression and DNA synthesis was confirmed by using fluorescence-activated cell sorting (FACS) analysis and [^{3}H]thymidine incorporation. The present paper shows that IGF-I increases cyclin D1 mRNA expression and protein levels. The time-dependent increase in cyclin D1 was accompanied by concomitant increases in cyclin D1-dependent CDK4 kinase activity and Rb(Ser807/811) phosphorylation. The increase in cyclin D1 levels and cyclin D1-dependent CDK4 activity was mediated jointly by activation of ERK1/2 and PI3-kinase. The stimulatory effects of IGF-I on the G_1 phase of the cell cycle were accompanied by increased DNA synthesis and cell proliferation that followed a similar time course.

MATERIALS AND METHODS

Culture of smooth muscle cells isolated from normal human jejunum. Muscle cells were isolated and cultured from the circular muscle layer of human jejunum as described previously (16, 19, 20). Briefly, 4- to 5-cm segments of normal jejunum were obtained from patients undergoing surgery for morbid obesity according to a protocol approved by the University Office of Research Subject Protection. After opening the segments along the mesenteric border, the mucosa was dissected away and the remaining muscle layer was cut into 2 × 2-cm strips. Slices were obtained from the circular layer by using a Stadie-Riggs tissue slicer, which allows thin slices to be made with muscle fiber orientation clearly visible. Thus the slices obtained from the longitudinal muscle layer (with subadjacent myenteric plexus) are differentiated and separated from the slices obtained from the circular muscle layer. Slices were incubated overnight at 37°C in 20 ml of DMEM plus 10% fetal bovine serum (DMEM-10) containing 200 U/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamycin, and 2 μg/ml amphotericin B to which was added 0.0375% collagenase (CLS type II) and 0.1% soybean trypsin inhibitor. Muscle cells dispersed from the circular layer were harvested by filtration through 500-μm Nitex mesh and centrifugation at 150 *g* for 5 min. Cells were resuspended and washed twice by centrifugation at 150 *g* for 5 min. After resuspension in DMEM-10 containing the same antibiotics, the cells were plated at a concentration of 5×10^5 cells/ml as determined by counting in a hemocytometer. Cultures were incubated in a 10% CO_2 environment at 37°C. DMEM-10 medium was replaced every three days until the cells reached confluence.

Primary cultures of muscle cells were passaged on reaching confluence by first being washed three times with PBS. After the PBS was removed, cells were treated for 2 min with 0.05% trypsin and 0.53 mM EDTA. The trypsin activity was neutralized by the addition of a fourfold excess of DMEM-10. The resulting cell suspension was centrifuged at 350 *g* for 10 min at 4°C. The pellet was resuspended in DMEM-10 at a concentration of 2.5×10^6 cells/ml and plated in the appropriate cultureware. The medium was changed after 24 h. All subsequent studies were performed in first-passage cultured cells after 7 days, at which time the cells are confluent. We have previously shown that these cells express a phenotype characteristic of intestinal smooth muscle as determined by immunostaining for intestinal smooth muscle markers and expression of γ-enteric actin. Epithelial cells, endothelial cells, neurons, and interstitial cells of Cajal are not detected in these cultures (16, 35).

[^{3}H]thymidine incorporation assay. Proliferation of smooth muscle cells in culture was measured by the incorporation of [^{3}H]thymidine as described previously (16–20). Briefly, the cells were washed free of serum and incubated for 24 h in serum-free DMEM. Quiescent muscle cells were incubated for additional periods of time up to 24 h with a maximally effective concentration of IGF-I (100 nM). During the final 4 h of this incubation period, 1 μCi/ml [^{3}H]thymidine was added to the medium. [^{3}H]Thymidine incorporation into the perchloric acid extractable pool was used as a measure of DNA synthesis.

Western blot analysis. Levels of cyclin D1, CDK4, phospho-Rb(Ser807/811), and total Rb were measured by Western blot analysis using standard methods (4, 18, 20). Briefly, confluent muscle cells were rendered quiescent by incubation for 24 h in serum-free medium. The cells were stimulated with 100 nM IGF-I for additional periods of time up to 24 h. The reaction was terminated by two rapid washes in ice-cold PBS after which nuclear lysates were prepared from the cells. After measurement of total nuclear proteins using the Bio-Rad DC protein assay kit, aliquots containing equal amounts of nuclear protein (10 μg) were separated with SDS-PAGE under denaturing conditions. After the proteins were electrotransferred to nitrocellulose, the membranes were incubated overnight with a 1:1,000–1:2,000 dilution of antibodies recognizing cyclin D1, CDK4, phospho-Rb(Ser807/811), or total Rb. Bands of interest were visualized with enhanced chemiluminescence on a FluorChem 8800 (Alpha Innotech, San Leandro, CA), and the resulting digital images were analyzed by using AlphaEaseFC version 3.1.2 software.

Preparation of RNA and microarray analysis of IGF-I-regulated genes. Confluent muscle cells were rendered quiescent by incubation in serum-free DMEM for 24 h. The cells were stimulated with a maximally effective concentration of IGF-I (100 nM) for an additional 24 h. The cells were washed twice in ice-cold PBS, and total RNA was prepared by using RNAqueous (Ambion, Austin, TX). Biotin-labeled cDNA probes were prepared by using 5 μg of total RNA, biotin-16-dUTP, reverse transcriptase (50 units), and RNase inhibitor. The labeling mixture was annealed at 42°C for 90 min. The labeling reaction was terminated by denaturing at 68°C for 30 min. The array membranes were prehybridized for 1 h at 60°C with heat-denatured salmon sperm DNA (100 μg/ml). The membrane was hybridized with biotin-16-dUTP-labeled cDNA probe overnight at 60°C. The membranes were washed three times for 15 min at 60°C with 2× SSC-1% SDS and then washed three times for 15 min at 60°C with 0.1× SSC-0.5% SDS. The membranes were then incubated with a 1:7,500 dilution of alkaline phosphatase-conjugated streptavidin for 30 min, and spots were detected by chemiluminescence. Digital images were obtained by using a FluoChem 8800 (Alpha Innotech), and the resulting data were analyzed by using GEArray Analyzer version 1.2 software (SuperArray, Frederick, MD). Microarray analysis was performed on three separate paired samples. The potential target genes were identified by a greater than twofold change in response to treatment with IGF-I.

Identification of cyclin D1 and Rb1 mRNA by RT-PCR. The expression of cyclin D1 and Rb1 mRNA by microarray was confirmed by RT-PCR. RT-PCR was performed by using standard methods as described previously (4). Briefly, 2 μg of total RNA from each preparation were reverse transcribed in a reaction volume of 20 μl containing (in mM) 50 Tris·HCl (pH 8.3), 75 KCl, 3.0 $MgCl_2$, 10 dithiotreitol, and 0.5 dNTP with 2.5 μM random hexamers and 200 U of SuperScript II RT. The reaction was carried out for 10 min at 25°C and for 50 min at 42°C and was terminated by heating to 70°C for 15 min. The reaction mixture was treated with DNase I (5 units) to remove potentially contaminating genomic DNA. The reverse-transcribed cDNA (5 μl) was amplified in a final volume of 50 μl by PCR under standard conditions: 2 mM $MgCl_2$, 125 μM dNTP, and 2.5 U *Taq* DNA polymerase using specific primer pairs for cyclin D1 and Rb1 on the basis of their known sequences and for GAPDH as an internal standard (Table 1). Sense and antisense primers were designed from different exons so that cDNA amplification resulted in PCR products of specific length. For each experiment, a parallel control without RT was processed. The amplified PCR products were separated on agarose gels containing 0.1 μg/ml ethidium bromide. The resulting bands were visualized on a FluorChem 8800 (Alpha Innotech) by using AlphaEaseFC version 3.1.2 software.

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Table 1. *PCR oligonucleotide primers and experimental conditions*

Target	Oligonucleotide	Annealing Temperature, °C	PCR, cycles	Predicted Product Size, bp	Ref. No.
CyclinD1					
Sense	5′-CAGGCTGTGTCCCTCTTCTC-3′	55	35	219	24
Antisense	5′-CAGAAGCTATTCCAATCATCCC-3′				
Rb1					
Sense	5′-CAGATGCAATTGTTTGGGTG-3′	56	30	345	21
Antisense	5′-TGAATGGGCAGTCAATCAAA-3′				
GAPDH					
Sense	5′-GTGAAGGTCGGTGTGAACGGATT-3′	63	25	555	4
Antisense	5′-CACAGTCTTCTGAGTGGCAGTGAT-3′				

RB1, retinoblastoma 1, GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Cell cycle analysis with FACS. Cell cycle analysis was performed by using FACS by adaptation of the method of Freid et al. (11). Briefly, confluent muscle cells were rendered quiescent by incubation in serum-free DMEM for 24 h. The cells were stimulated with a maximally effective concentration of IGF-I (100 nM) for 24 h. The cells were washed twice with ice-cold PBS and scraped into 1 ml of a fluorochrome solution consisting of 3.8 mM Na citrate, 0.5 mg/ml propidium iodide, 0.1% Triton X-100 (vol/vol), and 7,000 units of RNase B (11). The cells were incubated for an additional 30 min to degrade RNA and filtered through 37-μm nylon mesh. Samples were analyzed by FACS on a Coulter Elite ESP (Beckman Coulter, Miami, FL) using standard filter sets. Results were expressed graphically and quantified as the fraction of cells in G_0/G_1, S, or G_2/M phase using Coulter cytologic software.

Measurement of cyclin D1-CDK4 activity by in vitro kinase assay. Cyclin D1-CDK4 kinase activity was measured by the method of Phelps and Xiong (28). Briefly, confluent smooth muscle cells were rendered quiescent by incubation in serum-free DMEM for 24 h. Cells were stimulated with a maximally effective concentration of IGF-I (100 nM) for periods of time from 0 to 24 h. The reaction was terminated by washing cells twice with ice-cold PBS. Cell lysates were prepared in a buffer consisting of (in mM) 50 Tris·HCl (pH 7.5), 150 NaCl, 50 NaF, 1 Na orthovanadate, 1 dithiothreitol, and 1 phenylmethylsulfonyl fluoride with 0.5% Nonidet P-40 to which were added 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin. The resulting lysates were clarified by centrifugation at 14,000 *g* for 10 min at 4°C. Lysates were precleared by incubation with protein A agarose beads for 1 h at 4°C. Samples containing equal amounts of protein (1 mg) were incubated for 2 h at 4°C with 2.5 μg of rabbit anti-CDK4 and for an additional 1 h after the addition of 10 μl of protein A agarose beads. The immune complex-agarose beads were washed twice with ice-cold lysis buffer and twice with ice-cold kinase assay buffer. Kinase assay buffer consisted of (in mM) 10 HEPES (pH 7.0), 10 $MgCl_2$, 5 $MnCl_2$, 1 DTT, and 0.005 ATP. After the final wash, the beads were resuspended in 25 μl of ice-cold kinase assay buffer. Reaction was initiated by the addition of 5 μl of a mixture containing 2 μg of glutathione *S*-transferase (GST)-Rb(792–928) and 5 μCi [γ-^{32}P]ATP (3,000 Ci/mmol) in kinase assay buffer. The reaction continued for 30 min at 30°C and was terminated by the addition of 30 μl of 2× sample buffer to each tube. Samples were boiled for 3 min, after which 20 μl of each sample was loaded onto a 15% polyacrylamide gel and the proteins were separated by SDS-PAGE. Gels were dried and then exposed and quantitated by using a Packard InstantImager phosphorimager (Perkin Elmer, Boston, MA). Results were corrected for nonspecific immunoprecipitation by using preimmune rabbit serum and for endogenous substrates by using GST alone.

Measurement of protein content. The protein content of cell lysates and nuclear lysates was measured by using the Bio-Rad DC protein assay kit according to the manufacturer's directions. Samples were adjusted to provide aliquots of equal protein content before in vitro kinase assay or Western blot analysis.

Statistical analysis. Values given represent the means ± SE of *n* experiments, where *n* represents the number of experiments on cells derived from separate primary cultures. Statistical significance was tested by Student's *t*-test for either paired or unpaired data as appropriate. Analysis of relative densitometric values for Western blots was performed by using AlphaEaseFC version 3.1.2 software. Densitometric values for protein bands were reported in arbitrary units above basal values. Values for pRb(Ser807/811) were normalized to total Rb protein levels after blots were stripped and reblotted.

Materials. Recombinant human IGF-I was obtained from Austral Biologicals (San Ramon, CA); collagenase and soybean trypsin inhibitor were obtained from Worthington Biochemical (Freehold, NJ); HEPES was obtained from Research Organics (Cleveland, OH); DMEM was obtained from Mediatech (Herndon, VA); fetal bovine serum was obtained from Summit Biotechnologies (Fort Collins, CO); [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol), and [^{3}H]thymidine (specific activity, 6 Ci/mmol) were obtained from New England Nuclear (Boston, MA); rabbit polyclonal antibodies to cyclin D1 (sc-260) and protein A agarose beads were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal antibody to CDK4 (06-139), mouse monoclonal antibody to phosphor-Rb(Ser801/811) (clone XZ-77) and GST-Rb(792–928) were obtained from Upstate Biotechnology (Lake Placid, NY); rabbit polyclonal antibody to total Rb and anti-rabbit and anti-mouse horseradish peroxidase conjugates were obtained from Cell Signaling Technology (Beverly, MA); microarrays were obtained from SuperArray; the inhibitors LY-294002 and PD-98059 were obtained from Calbiochem (San Diego, CA); Western blot analysis materials and DC protein assay kit were obtained from Bio-Rad (Hercules, CA); and plastic cultureware was obtained from Corning (Corning, NY). All other chemicals were obtained from Sigma (St Louis, MO).

RESULTS

IGF-I stimulates DNA synthesis and cell cycle progression. The effect of IGF-I on the proliferation of human intestinal smooth muscle cells was examined in two complementary ways. First, the ability of IGF-I to elicit a time-dependent increase in the incorporation of [^{3}H]thymidine was used as a measure of DNA synthesis. Second, the ability of IGF-I to stimulate quiescent cells to enter the cell cycle and the resulting changes in distribution of cells in the cell cycle were analyzed by FACS analysis.

Incubation of quiescent muscle cells with 100 nM IGF-I for 0–24 h elicited a time-dependent increase in the incorporation of [^{3}H]thymidine into the perchloric acid soluble (DNA) pool (Fig. 1). IGF-I increased [^{3}H]thymidine incorporation within 4 h by 138 ± 9% above basal and after 24 h by 278 ± 22% above basal (basal, 125 ± 20 counts·min^{-1}·μg protein^{-1}). We have previously shown that IGF-I activates distinct PI3-kinase-

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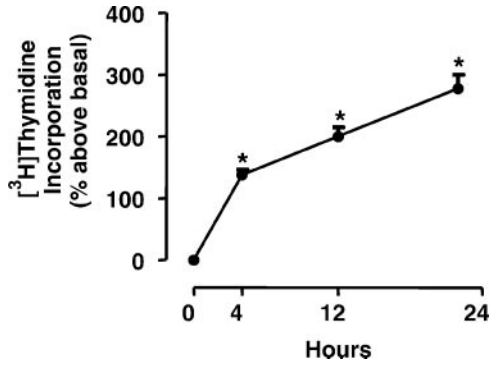

Fig. 1. Insulin-like growth factor (IGF)-I stimulates proliferation of human intestinal muscle cells. Incubation of quiescent muscle cells with 100 nM IGF-I elicits a time-dependent increase in the incorporation of [^{3}H]thymidine that is significant after 4 h and continues for 24 h. Muscle cells rendered quiescent by 24-h incubation in serum-free DMEM were incubated for additional periods of time (0–24 h) with IGF-I. [^{3}H]thymidine (1 μCi) was added for the final 4 h of incubation, and its incorporation into perchloric acid soluble pools was used as a measure of DNA synthesis. Results were expressed as a percent increase over basal values (basal, 125 ± 20 counts·min^{-1}·μg protein^{-1}). Values represent the means ± SE of 4 separate experiments performed in triplicate. *$P < 0.05$ vs. control.

dependent and ERK1/2-dependent pathways that contribute jointly to thymidine incorporation (18).

The ability of IGF-I to cause entry into the cell cycle, stimulation of DNA synthesis, and cell division was confirmed by FACS analysis of the distribution of cells in each phase of the cell cycle. When smooth muscle cells were rendered quiescent by 24-h incubation in serum-free DMEM, 88 ± 6% of cells were in the G_0/G_1 phase, 2 ± 1% were in S phase, and 10 ± 3% were in G2/M phase (Fig. 2*A*). In quiescent muscle cells incubated for a 24-h period with 100 nM IGF-I (the time of maximal [^{3}H]thymidine incorporation), the proportion of cells in G_0/G_1 phase was decreased to 61 ± 3%, the proportion in the S phase was increased to 12 ± 4%, and the proportion in G_2/M phase was increased to 27 ± 5% (Fig. 2*B*).

IGF-I regulates cyclin D1 expression and protein levels. We hypothesized that the effect of IGF-I on muscle cell DNA synthesis and proliferation was mediated, in part, by its effects on the cell cycle machinery of the G_1 phase. This notion was tested by using a gene array-based strategy to identify genes expressed in human intestinal smooth muscle cells encoding proteins known to be involved in regulation of the cell cycle. Quiescent muscle cells and cells incubated with 100 nM IGF-I for 24 h were used to prepare total RNA that was then used in microarray analysis. Several genes involved in the regulation of G_1 phase were identified (Table 2). IGF-I elicited a significant increase in the levels of cyclin D1 mRNA to 510 ± 70% of basal levels ($P < 0.05$). The effect of IGF-I on cyclin expression was specific to the cyclin D1 isoform as the levels of cyclin D2 and cyclin D3 expression were not significantly altered. Expression of cyclin D1 in the microarray analysis was confirmed by using RT-PCR. RT-PCR, by using cyclin D1-specific primers, identified a 219-bp transcript of the predicted size for cyclin D1 (Table 1 and Fig. 3*A*).

Table 2. *Insulin-like growth factor affects the expression of genes encoding G_1 phase cell cycle proteins*

Symbol	GenBank No.	UniGene No.	Ratio	Description
CCND1	M64349	Hs.82932	5.1±0.7*	Cyclin D1
CCND2	X68452	Hs.75586	1.3±0.1	Cyclin D2
CCND3	M90814	Hs.83173	1.4±0.2	Cyclin D3
CDK4	M14055	Hs.95577	1.1±0.3	Cdk4
CDK6	X66365	Hs.38481	1.4±0.5	Cdk6
RB1	M15400	Hs.75770	2.1±0.3*	Retinoblastoma 1 (Rb, p105)
RBL1	NM_002895	Hs.87	1.1±0.1	Retinoblastoma-like 1 (p107)
RBL2	NM_005611	Hs.79362	1.4±0.2	Retinoblastoma-like 2 (p130)

Values represent the means ± SE of 3 separate experiments. Results are expressed as the ratio of expression in cells treated with 100 nM IGF-I for 24 h to untreated quiescent muscle cells. CCN, cyclin; CDK, cyclin-dependent kinase; RBL, RB-like. *$P < 0.05$.

Changes in cyclin D1 mRNA expression measured by using microarray analysis were investigated further by measuring the effect of IGF-I on cyclin D1 protein levels using Western blot analysis. Incubation of quiescent muscle cells with 100 nM IGF-I caused a time-dependent increase in the levels of cyclin D1 protein (Fig. 3*B*). After 24-h incubation with IGF-I, cyclin D1 protein levels had increased by 224 ± 8% above basal.

Pathways mediating the IGF-I-induced increase in cyclin D1 levels were examined by using a selective inhibitor of the ERK1/2 pathway, PD-98059 (2), and of the PI3-kinase pathways, LY-294002 (36). Our previous work had shown that the ability of IGF-I to stimulate proliferation of human intestinal smooth muscle cells is mediated jointly by activation of these two signaling pathways and that these pathways, as well as the effects of the inhibitors, are distinct in these cells (18). The

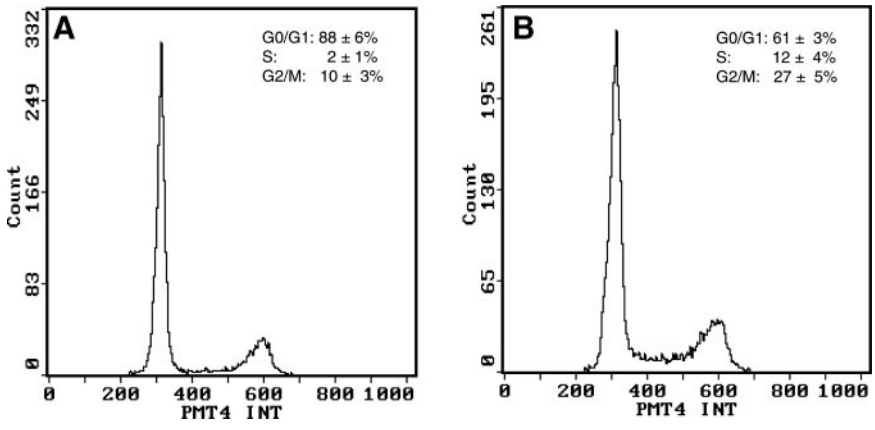

Fig. 2. Fluorescence-activated cell sorting (FACS) analysis of IGF-I-stimulated cell cycle progression. *A*: representative FACS analysis of muscle cells rendered quiescent (G_0/G_1 phase) by incubation for 24 h in serum-free DMEM. *B*: representative FACS analysis of quiescent muscle cells incubated with 100 nM IGF-I for 24 h and resulting increase in the proportion of cells in S and G_2/M phases. Results obtained in quiescent and IGF-I-treated cells were expressed as the percentage of total cells in G_0/G_1, S, or G_2/M phase of the cell cycle. Tabulated values represent the means ± SE of 3 separate experiments. PMT, photomultimer tube; INT, integrated.

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MAPK kinase 1/2 inhibitor PD-98059 (10 μM) inhibited by 65 ± 7% the IGF-I-induced increase in cyclin D1 protein levels (Fig. 3*B*). The PI3-kinase inhibitor, LY-204002 (10 μM), also inhibited by 81 ± 12% the IGF-I-induced increase in cyclin D1 protein (Fig. 3*C*).

IGF-I regulates the activity of cyclin D1-CDK4. Effects of D-type cyclins are mediated in conjunction with their binding partners, CDK4 and CDK6. In their bound form, cyclins and CDKs provide the regulatory and catalytic domains, respectively, of a functional "holoenzyme" (29, 31). The kinase activity of the cyclin D1-CDK4 pair increases during the early G_1 phase as a result of the increasing cyclin D1 levels. Cyclin D1-dependent CDK4 activity is therefore not typically regulated by changes in the levels of CDK4 protein (29, 31). This notion was confirmed, however, before determination of the effect of IGF-I on cyclin D1-dependent CDK4 activity.

In our initial gene array experiments, treatment with IGF-I (100 nM) for 24 h did not significantly affect CDK4 mRNA levels (110 ± 30% of basal levels) (Table 2). Similarly, incubation of cells with 100 nM IGF-I for increasing periods of time (0–24 h) did not significantly alter the levels of CDK4 protein (Fig. 4*A*). The ability of IGF-I to regulate cyclin D1-dependent CDK4 activity was then examined by using the in vitro kinase assay described in MATERIALS AND METHODS. Incubation of muscle cells with 100 nM IGF-I for increasing periods of time elicited a time-dependent increase cyclin D1-dependent CDK4 activity that increased to 240 ± 40% above basal after 24 h (Fig. 4*B*).

IGF-I stimulates Rb phosphorylation. The microarray-based studies showed that IGF-I (100 nM) caused a significant increase in the expression of the mRNA encoding for the 105-kDa isoform Rb1. After incubation for 24 h with 100 nM IGF-I, the levels of Rb1 mRNA increased to 210 ± 30% of basal levels (Table 2) ($P < 0.05$). Similar changes in the mRNA for the 107 kDa Rb-like-1 (RBL-1) and for the 130 kDa Rb-like-2 (RBL-2) were not observed. Expression of Rb1 by human intestinal smooth muscle cells was confirmed by using RT-PCR (Table 1 and Fig. 5*A*).

A key mechanism by which cell cycle progression is influenced by the cyclin D1-CDK4 pair is their ability to regulate Rb phosphorylation and thereby influence transcription factor availability. Cyclin D1-CDK4 complexes regulate the availability of transcription factors by virtue of their ability to act as kinases for Rb protein phosphorylation (15, 39). In its basal state, Rb is phosphorylated but can be hyperphosphorylated on distinct residues by specific cyclin-CDK pairs. Phosphorylation of Rb releases bound transcription factors such as E2F1–3.

At least two regions of the COOH terminus of Rb are phosphorylated solely by cyclin D1-CDK4 and not by other cyclin-CDK pairs Rb(Ser780) and Rb(Ser807/811) (39). Rb(Ser807/811) phosphorylation was used as a measure of cyclin D1-dependent, CDK4-dependent Rb phosphorylation. Incubation of quiescent smooth muscle cells with 100 nM IGF-I for periods of time from 0–24 h resulted in time-dependent Rb(Ser807/811) phosphorylation. Rb(Ser807/811)

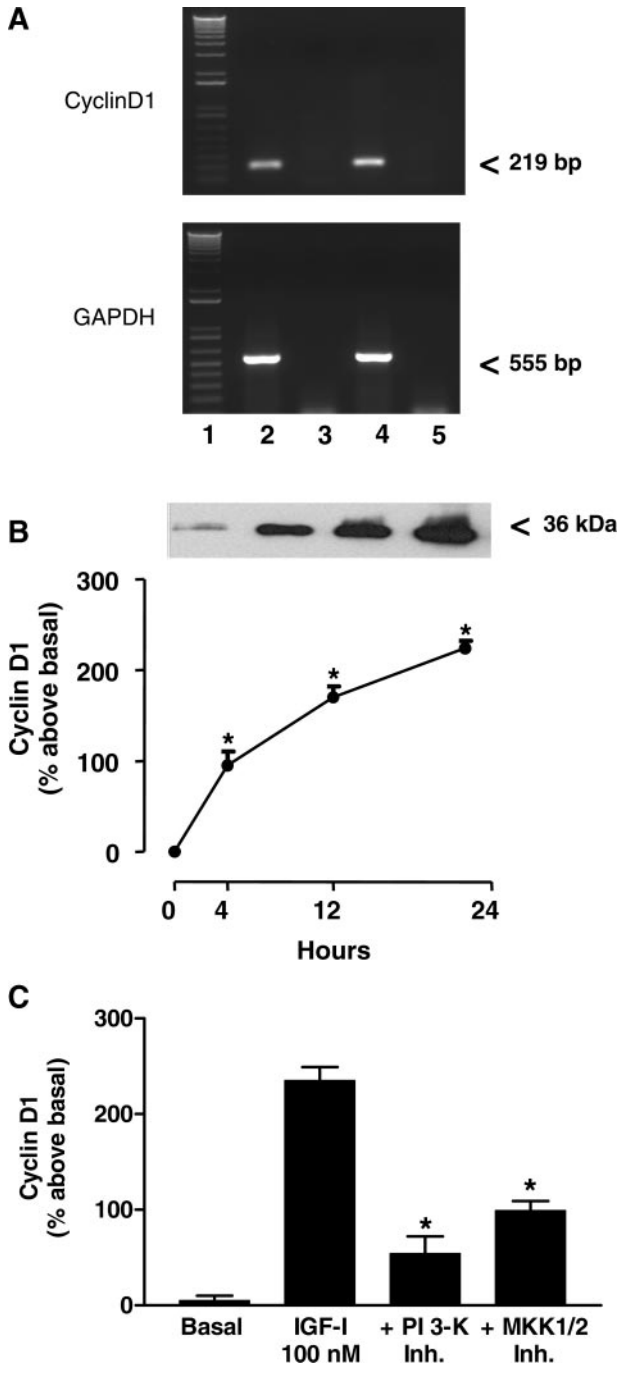

Fig. 3. Effect of IGF-I on cyclin D1 levels. *A*: representative RT-PCR analysis confirming that cyclin D1 was expressed by human intestinal smooth muscle cells. Total RNA harvested from quiescent muscle cells incubated for 24 h with 100 nM IGF-I was reverse transcribed, and the resulting cDNAs were amplified by PCR. RT-PCR amplified a single product of 219-bp size using cyclin D1-specific primers (*top*) and a single product of 555-bp size using GAPDH specific primers (*bottom*) (Table 1). *Lane 1*, 100-bp DNA ladder; *lane 2*, quiescent cells (+RT); *lane 3*, quiescent cells (−RT); *lane 4*, 100 nM IGF-I for 24 h (+RT); *lane 5*, 100 nM IGF-I for 24 h (−RT). No products were observed in the absence of RT (−RT). *B*: IGF-I elicits a time-dependent increase in cyclin D1 protein levels. Incubation of quiescent muscle cells with 100 nM IGF-I for increasing periods of 0–24 h caused an increase in cyclin D1 levels that was significant after 4 h. Cyclin D1 levels were measured by Western blot analysis in nuclear lysates. *Inset*: representative Western blot of time-dependent increase in cyclin D1 levels. *C*: increase in cyclin D1 protein levels induced by IGF-I were inhibited by the phosphoinositol 3-kinase (PI3-kinase) inhibitor, LY-294002 (10 μM), and by the MAPK kinase 1/2 (MKK1/2) inhibitor, PD-98059 (10 μM). Cyclin D1 levels were measured by Western blot analysis in nuclear lysates. Results were expressed in relative densitometric units from Western blot analyses of cyclin D1. Values represent the means ± SE of 4 separate experiments. *$P < 0.05$ vs. IGF-I alone.

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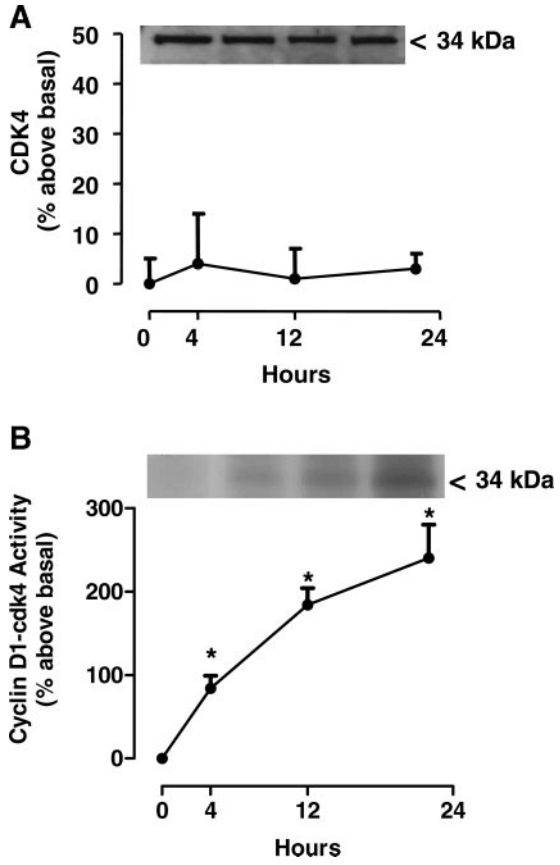

Fig. 4. Effect of IGF-I on cyclin-dependent kinase-4 (CDK4) levels and on cyclin D1-dependent CDK4 activity. *A*: IGF-I does not alter CDK4 protein levels. Quiescent muscle cells were treated for increasing periods of time (0–24 h) with 100 nM IGF-I, and CDK4 levels were analyzed by Western blot. *Inset*: representative Western blot of CDK4 levels in nuclear lysates. *B*: IGF-I causes time-dependent increase in cyclin D1-dependent CDK4 activity. Quiescent muscle cells were treated for increasing periods of time (0–24 h) with 100 nM IGF-I. Cyclin D1-CDK4 activity was measured by in vitro kinase assay as described in MATERIALS AND METHODS. *Inset*: representative results from in vitro kinase assay. Results were expressed in relative densitometric units. Values represent the means ± SE of 3–5 separate experiments. $*P <$ 0.05 vs. control values.

phosphorylation induced by IGF-I was significant within 4 h (138 ± 12% above basal, $P < 0.05$ vs. basal) and by 24 h was 291 ± 39% above basal levels (Fig. 5*C*). IGF-I also elicited a time-dependent increase in total Rb protein levels that were significant after 12 h (100 ± 20% above basal) and continued for up to 24 h (190 ± 25% above basal) (Fig. 5, *B* and *C*).

DISCUSSION

IGF-I generates signals emanating from the activated IGF-I receptor that converge on the nucleus to regulate entry into and progression through the cell cycle. Once the restriction point at the G_1 and S phase boundary is passed, the cell is committed to DNA synthesis and the cell cycle proceeds largely independent of growth factors. Levels and activity of stimulatory cyclins and CDKs are regulated by growth factors during progression through the G_1 phase and control the phosphorylation state of the Rb family of tumor suppressor proteins. The Rb family [Rb1, p107 (RBL-1), and p130 (RBL-2)], regulates the availability of various transcription factors. Notably, during the G_1 phase, Rb regulates the availability of several members of the E2F family of transcription factors that control transcription from a number of proliferation-associated genes (33, 38).

The present paper shows that IGF-I regulates the G_1 phase of the cell cycle and stimulates proliferation of human intestinal

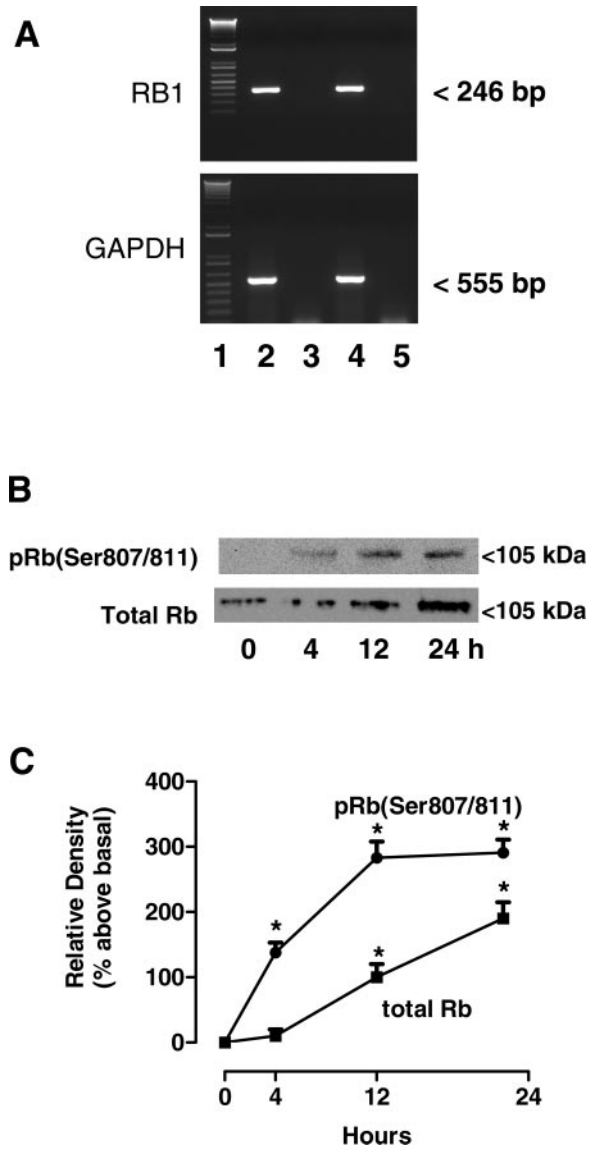

Fig. 5. Effects of IGF-I on the retinoblastoma (Rb) protein. *A*: representative RT-PCR analysis confirming the expression of Rb1 by human intestinal smooth muscle cells. Total RNA harvested from quiescent muscle cells incubated for 24 h with 100 nM IGF-I was reverse transcribed, and the resulting cDNAs were amplified by PCR. RT-PCR amplified a single transcript of 345-bp size using Rb1-specific primers (*top*) and a single transcript of 555-bp size using GAPDH-specific primers (*bottom*). *Lane 1*, 100 bp DNA ladder; *lane 2*, quiescent cells (+RT); *lane 3*, quiescent cells (−RT); *lane 4*, 100 nM IGF-I for 24 h (+RT); *lane 5*, 100 nM IGF-I for 24 h (−RT). No products were observed in the absence of RT (−RT). *B*: representative Western blot analysis of the effect of IGF-I on Rb(Ser807/811) phosphorylation and on total Rb levels. Quiescent muscle cells were incubated for increasing periods of time (0–24 h) with 100 nM IGF-I. Rb(Ser807/811) phosphorylation and total Rb levels were measured by Western blot analysis. *C*: IGF-I (100 nM) elicited a time-dependent increase in Rb(Ser807/811) phosphorylation that preceded a lesser time-dependent increase in total Rb levels. Results are expressed in relative densitometric units. Values represent the means ± SE of 5 separate experiments. $*P < 0.05$ vs. control values.

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smooth muscle cells. Effects of IGF-I on smooth muscle cell proliferation are mediated jointly by the activation of PI3-kinase and ERK1/2-dependent pathways that regulate cyclin D1 expression and thus cyclin D1-CDK4 activity and the transcriptional repressor Rb. Evidence that IGF-I regulates the G_1-phase cell cycle machinery and causes human intestinal smooth muscle cells to proliferate can be summarized as follows: *1*) IGF-I increases cyclin D1 mRNA and protein levels; *2*) IGF-I increases cyclin D1-dependent CDK4 activity; *3*) IGF-I stimulates phosphorylation (inactivation) of Rb(Ser807/811); *4*) IGF-I causes quiescent cells to incorporate [^{3}H]thymidine into DNA; and *5*) IGF-I causes cell cycle progression in quiescent cells.

We (18) have previously shown that in human intestinal smooth muscle cells, IGF-I stimulates the activation of distinct ERK1/2 and PI3-kinase pathways that jointly regulate proliferation. Although activation of p70S6 kinase by IGF-I occurs downstream of PI3-kinase, p70S6 kinase activation is required for proliferation to occur in response to either PI3-kinase or ERK1/2 activation, because p70S6 kinase mediates phosphorylation of the 40S ribosomal protein S6 (14, 17). In turn, S6 regulates the translation of 5′-terminal oligopyrimidine tract mRNAs that encode for numerous components of the protein synthesis machinery, i.e., ribosomes and elongation factors (14). In addition to the effects of IGF-I on p70S6 kinase activity and the ribosomal machinery needed for proliferation to occur, the present paper shows that IGF-I also affects cell division directly by regulating the machinery of the cell cycle in G_1 phase.

Three isoforms of cyclin D have been identified: cyclins D1, D2, and D3, which serve similar functions during the G_1 phase of the cell cycle (7, 12, 31). Cyclin D levels are low in quiescent cells and rise progressively during early G_1 phase in response to stimulation by growth factors. In human intestinal smooth muscle cells, IGF-I preferentially increases the mRNA abundance and protein levels of the cyclin D1 isoform with a time course that parallels the effects of IGF-I on [^{3}H]thymidine incorporation. Growth factors stimulate a PI3-kinase-dependent increase in translation of cyclin D mRNA that precedes their effect on increased cyclin D gene transcription (25). Specific binding partners of D-type cyclins, CDK4 and CDK6, act together with D-type cyclins to regulate the phosphorylation state of the Rb protein family of proteins (7, 10). In human intestinal smooth muscle cells, IGF-I increases cyclin D1 levels jointly through PI3-kinase-dependent and ERK1/2-dependent mechanisms. Cyclin D1-dependent CDK4 activity is regulated by the effects of IGF-I on cyclin D1 levels and not by alterations in CDK4 mRNA or protein levels. These findings are consistent with the view that growth factors regulate the activity of cyclin D-CDK4 holoenzyme pairs by influencing the levels of cyclin D rather than altering the levels of CDK4 (7, 10).

The Rb tumor suppressor protein family consists of three homologous proteins: pRb (105-kDa isoform), p107, and p130 (21–23). pRb is differentially phosphorylated on distinct residues by cell cycle-regulated cyclin-CDK pairs: cyclin D-CDK4, cyclin D-CDK6, cyclin E-CDK2, and cyclin A-CDK2 during the G_1 and S phases (10). Within the COOH terminus of Rb are two regions uniquely phosphorylated by cyclin D-dependent CDK4: Ser780 and Ser807/811. Other regions are uniquely phosphorylated by other cyclin-CDK pairs. In human intestinal smooth muscle cells, IGF-I elicits time-dependent phosphorylation of at least one of these regions, pRb(Ser807/811). The time course of Rb(Ser807/811) phosphorylation preceded that of increased levels of total Rb. This suggests that IGF-I might exert two distinct effects on Rb: a prompt inactivation via cyclin D1-CDK4-dependent phosphorylation and a slower, more modest increase in total Rb levels. Alternatively, the increased levels of Rb phosphorylation after 12–24 h might reflect only the increased levels of total Rb protein and not solely an increased proportion of phosphorylated Rb. Nevertheless, IGF-I-induced pRb phosphorylation, like that induced by other mitogens, results in pRb inactivation and regulates the release of transcription factors bound to Rb in the quiescent cell (34).

The ability of IGF-I and other growth factors to regulate Rb activity is therefore important because of the resulting effect on transcription factor availability (34). One important binding partner of Rb during the early G_1 phase is the E2F family of transcription factors. E2F1, E2F2, and E2F3a belong to a subclass of E2F factors that act as transcriptional activators during progression through the G_1 phase of the cell cycle (38). They associate preferentially with Rb and have lower affinity for other members of the Rb family, p107 and p130. Other E2F family members, e.g., E2F3b, E2F4, and E2F5, are present throughout the cell cycle and regulate the transcription of E2F-dependent genes in quiescent cells and in early G_1 phase before the activation of E2F1, E2F2, and E2F3a (9). Increase in E2F transcription factor availability during early G_1 phase allows for transcription of a number of proteins that are critical in DNA synthesis, e.g., dihydofolate reductase and DNA polymerase, and in cell cycle control, e.g., cyclins D and E, Rb, and p107 (13, 32, 33).

In summary, this study shows that IGF-I stimulates human intestinal smooth muscle cells to progress through the G_1 phase of the cell cycle, synthesize DNA, and divide. IGF-I exerts its effects on the G_1 phase by increasing cyclin D1 levels and stimulating cyclin D1-dependent CDK4 activity via ERK1/2-dependent and PI3-kinase-dependent pathways. In turn, increased cyclin D1-dependent CDK4 activity regulates the transcriptional repressor, Rb, and thus the availability of transcription factors that further control DNA synthesis and cell proliferation.

GRANTS

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