Insulin-like growth factor-binding protein-5 (IGFBP-5) Stimulates Growth and IGF-I Secretion in Human Intestinal Smooth Muscle by Ras-dependent Activation of p38 MAP Kinase and Erk1/2 Pathways*  

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Insulin-like growth factor-binding protein-5 (IGFBP-5) and insulin-like growth factor-I (IGF-I) are produced by human intestinal smooth muscle cells. Endogenous IGF-I stimulates growth and increases IGFBP-5 secretion. IGFBP-5 augments the effects of IGF-I by facilitating interaction of IGF-I with the IGF-I receptor tyrosine kinase. Andress (Andress, D. L. (1998) Am. J. Physiol. 274, E744–E750) and Berfield et al. (Berfield, A. K., Andress, D. L., and Abrass, C. K. (2000) Kidney Int. 57, 1991–2003) have shown that in osteoblasts and kidney mesangial cells, IGFBP-5 stimulates proliferation and filopodia formation independently of IGF-I, presumably by activating a distinct IGFBP-5 receptor serine kinase. The present study determined whether IGFBP-5 exerts direct effects on growth in human intestinal smooth muscle cells and identified the intracellular signaling pathways involved. IGFBP-5 caused a concentration-dependent increase in [3H]thymidine incorporation and an increase in IGF-I secretion that occurred independently of IGF-I and the IGF-I receptor tyrosine kinase. IGFBP-5-induced phosphorylation of p38 MAP kinase, which was abolished by SB203580, or expression of a dominant negative Ras mutant, Ras(S17N), and phosphorylation of Erk1/2, which was abolished by a Raf1 kinase inhibitor, U1026, or expression of Ras(S17N). IGFBP-5-stimulated [3H]thymidine incorporation and IGF-I secretion were partly inhibited by SB203580 or U1026 and abolished by the combination of the two inhibitors or by expression of Ras(S17N). These data show that IGFBP-5 stimulates growth and IGF-I secretion in human intestinal smooth muscle cells by activation of p38 MAP kinase-dependent and Erk1/2-dependent pathways that are independent of IGF-I. A positive feedback mechanism therefore links IGFBP-5 and IGF-I secretion that reinforces their individual effects on growth. 

Insulin-like growth factor-I (IGF-I) stimulates proliferation of cells and is required for sustained growth of many cells (1); transformation and maintenance of the transformed state also require IGF-I receptor activation in some cells (2); and IGF-I protects cells from apoptosis (3). The central role of IGF-I in the regulation of smooth muscle cell growth in both the normal and pathologic states is manifested by the hyperplasia of intestinal and vascular smooth muscle in transgenic animals overexpressing a human IGF-I cDNA (4, 5). The effects of IGF-I are modulated by IGF-binding proteins. Six IGF-binding proteins (IGFBP-1–6) have been identified that can either augment the effects of IGF-I by facilitating the interaction of IGF-I with its cognate receptor or inhibit the effects of IGF-I by diminishing the interaction of IGF-I with its receptor (6). The presence and effect of each IGF binding protein, however, is both tissue- and species-specific. 

IGFBP-1, IGFBP-3, and IGFBP-5 indirectly influence cell growth by modulating the interaction of IGF-I with the IGF-I receptor and also directly influence cell growth by interacting with distinct cell surface receptors. IGFBP-1 interacts with the α5β1 integrin receptor expressed by placental cells and Chinese hamster ovary cells (7). IGFBP-3 interacts with the Type V TGF-β receptor expressed in T47D breast cancer cells and mink lung epithelial cells (8). Recently, an IGFBP-5-specific receptor has been characterized in mouse osteoblasts and rat kidney mesangial cells (9). IGFBP-5 binds with high affinity to this ~420-kDa membrane-bound receptor protein and elicits autophosphorylation of serine residues (10). One intracellular signaling pathway coupled to this receptor is the small G-protein, Cdc42, through which IGFBP-5-dependent mesangial cell filopodia formation is mediated (11). 

Human intestinal smooth muscle cells produce IGF-I, and three IGF-binding proteins, IGFBP-3, IGFBP-4 and IGFBP-5, each of which plays an autocrine role in the regulation of growth in human intestinal muscle cells (12, 13). Binding of IGF-I to the IGF-I receptor tyrosine kinase activates distinct PI3-kinase-dependent and Erk1/2-dependent pathways that stimulate both proliferation and IGFBP-5 production (13, 14). IGF-I-dependent stimulation of growth in these cells is inhibited by the indirect actions of IGFBP-3 and IGFBP-4 and is augmented by the indirect actions of IGFBP-5 (12, 13). IGF-I and IGFBP-5 expression is increased within the intestinal muscle layer in regions of active inflammation and strictureing in Crohn’s disease and in models of experimental enterocolitis (15, 16). It is not known whether an IGFBP-5-specific receptor is expressed by human intestinal muscle cells or what role this receptor plays in the regulation of growth. 

This study shows that an IGFBP-5 receptor is present in human intestinal smooth muscle cells. Binding of IGFBP-5 to its cognate receptor activates both the p38 MAP kinase and...
Erk1/2 signaling cascades. Activation of these pathways by IGFBP-5 mediates jointly stimulation of growth and secretion of IGF-I. Thus, dual stimulatory pathways link IGF-I and IGFBP-5 secretion, reinforcing their individual abilities to stimulate growth of human intestinal muscle cells.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IGFBP-5, the dominant negative Ras(S17N) mutant in pUSEamp(+) vector, and antibodies to the signaling intermediates, p38 MAP kinase, MKK3/6, Ras, Raf1, MKK1/2, and Erk1/2, were obtained from Upstate Biotechnology (Lake Placid, NY); collagenase and soybean trypsin inhibitor were obtained from Worthington Biochemical Inc (Freehold, NJ); Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Mediatech Inc. (Herndon, VA); fetal bovine serum was obtained from Summit Biotechnologies, Inc. (Fort Collins, CO); [3H]thymidine (specific activity, 6 Ci/mmol) was obtained from Amersham Biosciences; [125I]IGF-I radioimmunoassay kit was obtained from Peninsula Laboratories (San Carlos, CA); Western blotting materials and protein assay kit were obtained from BioRad Laboratories; plastic cultureware was obtained from Corning (Corning, NY); antibodies to the phosphorylated isoforms of Raf1 (Ser259), MKK1/2 (Ser177/Ser211), Erk1/2 (Thr202/Tyr204), MKK3/6 (Ser189/Ser207), and p38 MAP kinase (Thr180/Tyr182) were obtained from Cell Signaling Technology (MA); IGF-I was obtained from Biosource (1000 U/ml); U1026, SB203580 and the Raf1 kinase inhibitor, 5-iodo-(residues 149-149) of Raf1 coupled to glutathione-agarose (20). The immunoprecipitated proteins were washed three times with lysis buffer and resuspended in 2× Laemml sample buffer. The proteins were boiled for 5 min and then separated on 15% agarose gels by SDS-PAGE. The separated proteins were electrotransferred to nitrocellulose membranes. Nitrocellulose membranes were incubated overnight with 1 μg/ml anti-Ras antibody (clone RAS10). The bands of interest corresponding to activated Ras were visualized with enhanced chemiluminescence and quantitated with densitometry.

Measurement of IGF-I Production by Radioimmunoassay—IGF-I production was measured as described previously (18). Confluent muscle cells growing in 100-mm plates were washed free of serum. Serum-free DMEM was conditioned by incubation for 24 h with the muscle cells. Samples of conditioned medium were subjected to acid-ethanol treatment to remove IGFBP-binding proteins from the secreted IGF-I as described previously (18) according to the method of Daughaday et al. (21). Briefly, aliquots of conditioned medium were added to an acid-ethanol mixture (87.5% ethanol; 1.5% 2 N HCl (v/v)) at a ratio of 1:4. The mixture was incubated for 2 h at 37 °C and centrifuged, and the supernatant was neutralized with 0.855 M Tris base at a ratio of 5:2 and incubated at 4 °C for an additional 2 h. After centrifugation the resultant IGFBP-free supernatants were assayed for immunoreactive IGF-I by radioimmunoassay using a polyclonal antibody raised in rabbits against human IGF-I. This antibody reacts fully with human IGF-I, has <0.02% cross-reactivity with IGF-II, and has no cross-reactivity with insulin. The limit of detection was 10 pg/tube, and the IC50 was 187 pg/tube. IGF-I was measured in duplicate using 100-μl aliquot samples. Production was expressed as pmol of IGF-I/mg of protein/24 h.

Transient Transfection of Human Intestinal Muscle Cells—Substitution of asparagine for serine at residue 17 (S17N) of Ras results in a 4-fold increase in its affinity for GTP without affecting its affinity for GDP (22). When expressed in cells, Ras(S17N) exerts a dominant negative-like effect by sequestering guanine-nucleotide exchange factor for Ras. cDNA for Ras(S17N) in the pUSEamp(+) expression vector was purified, and human intestinal smooth muscle cells were transiently transfected with either pUSEamp(+)Ras(S17N) cDNA or with pUSEamp(+) alone as control using LipofectAMINE PLUS™ Reagent Kit (Invitrogen). Cells are incubated for 3 h at 37 °C with the transfection reagent-DNA complexes. The DNA-containing medium was replaced with DMEM + 10% FCS. After a 48-h incubation, the dominant negative effect of Ras(S17N) was confirmed by assaying IGFBP-5-stimulated Ras activity as described above.

Statistical Analysis—Values given represent the mean ± S.E. 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.

RESULTS

IGFBP-5 Stimulates Growth and IGF-I Secretion Independently of IGF-I—Our previous work has shown that IGFBP-5 augments, in a concentration-dependent fashion, the stimulatory effects of IGF-I on the growth of human intestinal smooth muscle cells in culture (15). In the present study, we hypothesized that, in addition to its IGF-I-dependent effects, IGFBP-5 might exert direct effects on the growth of human intestinal smooth muscle cells. Cells were examined during the post-confluent phase of culture, when the endogenous levels of both IGFBP-5 and IGF-I are lowest, and in the presence of an IGF-I receptor antagonist, IGF-I analog (1 μM) (17, 23). IGF-I analog, an IGF-I receptor antagonist that blocks the affinity of IGF-I to its high-affinity phosphotyrosine-binding domain on the IGF-I receptor tyrosine kinase, was used to eliminate the effects of IGFBP-5 mediated by facilitation of IGF-I binding to its cognate receptor. We have previously shown that in the presence of this antagonist, the ability of IGF-I to cause phosphorylation of its receptor is abolished. Incubation of quiescent muscle cells with IGFBP-5 (50 nm) for 2 min in the presence of the IGF-I antagonist did not Stimulation of Growth and IGF-I Secretion by IGFBP-5
elicit IGF-I receptor phosphorylation (1.03 ± 0.10% of basal). The results of these initial studies implied that effects attributed to IGFBP-5 represented its IGF-I-independent effects, i.e., when the IGF-I-dependent effects of IGFBP-5 were abolished in the presence of the IGF-I receptor antagonist.

In normal human intestinal smooth muscle cells, incubation of quiescent muscle cells with IGFBP-5 for 24 h directly caused concentration-dependent (0.5–50 nM IGFBP-5) increase in [3H]thymidine incorporation (50 nM, 145 ± 9% above basal; basal, 84 ± 3 cpm/mg protein) (Fig. 1). Incubation of human intestinal muscle cells for 24 h with 50 nM IGFBP-5, increased secretion of IGF-I by 90 ± 12% above basal levels (basal, 3.1 ± 0.2; IGFBP-5, 5.7 ± 0.6 pmol/mg protein/24 h, p < 0.05). The ability of IGFBP-5 to stimulate thymidine incorporation and IGF-I secretion in the presence of the IGF-I receptor antagonist implied that these effects were distinct from those mediated via the IGF-I receptor by augmentation of IGF-I binding.

IGFBP-5 Activates the p38 MAP Kinase Signaling Pathway—Activation of p38 MAP kinase was measured using a phospho-specific antibody recognizing the Thr180/Tyr182 phosphorylated (activated) p38 MAP kinase isoform. IGFBP-5 caused rapid, time-dependent phosphorylation of p38 MAP kinase, which attained a maximum within 5 min and declined to lower levels by 60 min (Fig. 2A). The increase in p38 MAP kinase phosphorylation, measured at the 5 min maximum, was concentration-dependent (0.5–50 nM IGFBP-5) (Fig 2B).

IGFBP-5-induced phosphorylation of p38 MAP kinase was abolished by the selective p38 MAP kinase inhibitor, SB203580 (1 μM), but was not affected by either the Raf1 kinase inhibitor, 5-iodo-3-[3,5-dibromo-4-hydroxyphenyl]methylene]-2-indoline (10 nM), or the M KK1/2 inhibitor, U1026 (10 μM) (Fig. 2C) (24).

Activation of the homologs M KK3/6 by IGFBP-5 followed a similar time-course to that of p38 MAP kinase. IGFBP-5 elicited prompt, time-dependent phosphorylation of M KK3/6 (Ser189/Ser207) that was maximal within 5 min and declined to lower levels at 60 min (Fig. 3A). When measured at the 5 min maximum, phosphorylation was also concentration-dependent (0.5–50 nM IGFBP-5) (Fig. 3B). The increase in M KK3/6 phosphorylation induced by IGFBP-5 was not affected by the Raf1 inhibitor, the M KK1/2 inhibitor, or the p38 MAP kinase inhibitor (Fig. 3C).

IGFBP-5 Activates the Erk1/2 Signaling Pathway—Incubation of quiescent muscle cells with IGFBP-5 in the presence of the IGF-I receptor antagonist elicited a prompt, time-dependent phosphorylation of both Erk1/2 isoforms on Thr202/Tyr204. Phosphorylation was maximal within 5 min and declined to lower levels within 60 min (Fig. 4A). When measured at the 5 min peak, IGFBP-5-induced phosphorylation was concentration-dependent (0.5–50 nM IGFBP-5) (Fig. 4B). Erk1/2 phosphorylation was abolished in the presence of either the Raf1 kinase inhibitor (10 nM) or the M KK1/2 inhibitor, U1026 (10 μM), but was not affected by the p38 MAP kinase inhibitor, SB203580 (1 μM) (Fig. 4C).

IGFBP-5 elicited a similar time-dependent increase in (Ser217/Ser221)M KK1/2 phosphorylation that was prompt, attained a maximum within 5 min, and declined to lower levels within 60 min (Fig. 5A). Phosphorylation of M KK1/2 by IGFBP-5 was also concentration-dependent (Fig 5B). The in-
crease in MKK1/2 phosphorylation induced by IGFBP-5 was abolished in the presence of the Raf1 kinase inhibitor (1 nM) and the MKK1/2 inhibitor, U1026 (10 μM), but was not affected by the p38 MAP kinase inhibitor, SB203580 (1 μM), the Raf1 inhibitor (10 nM), or the MKK1/2 inhibitor, U1026 (10 μM). Results are expressed in relative densitometric units. Values represent the means ± S.E. of 3–5 experiments. *, p < 0.05 versus basal levels. A.U., arbitrary units.

**IGFBP-5 Activates Ras**—Two methods were used to identify the role of Ras in the signaling pathways activated by IGFBP-5. The first method measured IGFBP-5-induced Ras activation as Ras-GTP using an immunoprecipitation-based assay as described under “Experimental Procedures” (20). The second method identified the requirement for Ras activation in the signaling pathways leading to p38 MAP kinase and Erk1/2 activation by expression of a dominant negative Ras(S17N) mutant in human intestinal muscle cells (22).

Incubation of quiescent human intestinal smooth muscle cells with IGFBP-5 caused time-dependent activation of Ras that was rapid, occurring within 30 s, sustained for up to 2 min, and then declined rapidly to lower levels by 5 min (Fig. 7A). Activation of Ras by IGFBP-5 (0.5–50 nM), measured at the 2
min maximum, was also concentration-dependent (Fig. 7B).

The requirement for Ras activation in IGFBP-5-induced p38 MAP kinase and Erk1/2 activation was examined in cells transiently transfected with a dominant negative Ras(S17N) mutant. In these cells, the ability of 50 nM IGFBP-5 to stimulate p38 MAP kinase phosphorylation was abolished (110 ± 10% inhibition versus vector transfected control, p < 0.05) (Fig. 8A). In cells expressing the dominant negative Ras mutant, the ability of 50 nM IGFBP-5 to stimulate Erk1/2 phosphorylation was also abolished (98 ± 8% inhibition versus vector transfected control, p < 0.05) (Fig. 8B). The dominant negative effect of the Ras(S17N) mutant on Ras activation was confirmed in separate studies. Incubation of quiescent muscle cells transiently transfected with empty vector for 2 min with 50 nM IGFBP-5 elicited an increase in activated, GTP-bound Ras (270 ± 40% above basal), whereas in cells expressing RAS(S17N), the ability of IGFBP-5 to activate Ras was abolished (9 ± 2% above basal, p < 0.05 versus empty vector).

FIG. 5. IGFBP-5 activates MKK1/2. A, incubation of confluent human intestinal muscle cells with 50 nM IGFBP-5 elicits time-dependent phosphorylation of the homologs MKK1/2. Inset, representative Western blot of IGFBP-5-dependent MKK1/2 (Ser217/Ser221) phosphorylation when measured at the 5 min peak. B, IGFBP-5 elicits concentration-dependent MKK1/2 phosphorylation when measured at the 5 min peak. Inset, representative Western blot of IGFBP-5-dependent MKK3/6 phosphorylation. C, Incubation of muscle cells with 50 nM IGFBP-5 for 5 min elicits MKK1/2 phosphorylation, which is not affected by the p38 MAP kinase inhibitor, SB203580 (1 μM), but is abolished by the Raf1 inhibitor (1 μM) or the MKK1/2 inhibitor, U1026 (1 μM). Results are expressed in relative densitometric units. Values represent the means ± S.E. of 3–5 experiments. *, p < 0.05 versus basal levels; **, p < 0.05 versus IGFBP-5 alone. A.U., arbitrary units.

FIG. 6. IGFBP-5 activates Raf1. A, incubation of confluent human intestinal muscle cells with 50 nM IGFBP-5 elicits time-dependent phosphorylation of Raf1. Inset, representative Western blot of IGFBP-5-dependent Raf1 phosphorylation. B, IGFBP-5 elicits concentration-dependent Raf1 phosphorylation when measured at the 5 min peak. Inset, representative Western blot of IGFBP-5-dependent Raf1 phosphorylation. C, incubation of muscle cells with 50 nM IGFBP-5 for 5 min elicits Raf1 phosphorylation, which is not affected by the p38 MAP kinase inhibitor, SB203580 (1 μM), or the MKK1/2 inhibitor, U1026 (10 μM), but is abolished by the Raf1 inhibitor (10 μM). Results are expressed in relative densitometric units. Values represent the means ± S.E. of 3–5 experiments. *, p < 0.05 versus basal levels; **, p < 0.05 versus IGFBP-5 alone. A.U., arbitrary units.
coupled to an increase in $[^3H]$thymidine incorporation. In the presence of the p38 MAP kinase inhibitor, SB203580 (1 µM), the ability of 50 nM IGFBP-5 to stimulate $[^3H]$thymidine incorporation was inhibited 69 ± 5% ($p < 0.05$) (Fig. 9). At the 10 µM concentrations used in the present study, SB203580 selectively inhibits p38 MAP kinase activation without affecting other protein kinases (25, 26).

The Erk1/2 pathway was also activated by IGFBP-5 and led to an increase in $[^3H]$thymidine incorporation. In the presence of the MKK1/2 inhibitor, U1026 (10 µM) (26), the increase in $[^3H]$thymidine incorporation induced by 50 nM IGFBP-5 was also partly inhibited, 40 ± 6%, ($p < 0.05$) (Fig. 9). At the 10 µM concentrations used in the present study, U1026 has been previously shown to be highly selective for MKK1/2 inhibition without affecting other protein kinases. In cells transfected with the dominant negative Ras(S17N) mutant, the ability of 50 nM IGFBP-5 to stimulate $[^3H]$thymidine incorporation was also abolished (vector, 158 ± 6% above basal; Ras(S17N), 10 ± 8% above basal).

In the presence of the combination of the p38 inhibitor, SB203580 (1 µM), and the MKK1/2 inhibitor, U1026 (10 µM), the ability of 50 nM IGFBP-5 to stimulate $[^3H]$thymidine incorporation in human intestinal smooth muscle cells was nearly abolished at 89 ± 2% inhibition ($p < 0.01$) (Fig. 9). The results suggest that activation of these two Ras-dependent pathways, p38 MAP kinase and Erk1/2, in human intestinal smooth muscle cells...
**Stimulation of Growth and IGF-I Secretion by IGFBP-5**

**Fig. 10. IGFBP-5-induced IGF-I secretion is mediated jointly by p38 MAP kinase and Erk1/2 activation.** Incubation of human intestinal muscle cells incubated with 50 nM IGFBP-5 for 24 h elicits an increase in the secretion of IGF-I that is partly inhibited by the p38 MAP kinase inhibitor, SB203580 (1 μM), or the MKK1/2 inhibitor, U1026 (10 μM). In the presence of the combination of the two inhibitors, the increase induced by IGFBP-5 was abolished, and basal levels of IGF-I secretion were slightly inhibited. The secretion of IGF-I was measured by radioimmunoassay as described under "Experimental Procedures." Results are expressed as the increase in IGF-I secretion in pmol of IGF-I/24 h/mg of total cell protein above basal level (basal = 3.1 pmol/24 h/mg of protein). Values represent the mean ± S.E. of 4–6 experiments. *p < 0.05 versus IGFBP-5-induced secretion.

The present study provides the first evidence that IGFBP-5 has direct, IGF-I-independent actions of IGFBP-5. IGFBP-5 Stimulates IGF-I Secretion by Activation of p38 MAP Kinase and Erk1/2—We have previously shown that IGFBP-5 stimulates IGFBP-5 production (13) by activation of the same signaling pathways, Erk1/2 and PI3-kinase, that mediate the growth stimulatory effects of IGF-I on human intestinal muscle cells. In the present study, the possibility that the increase in IGF-I secretion mediated by IGFBP-5 might be regulated by the same pathways, p38 MAP kinase and Erk1/2, was also examined. The increase in IGF-I secretion induced by IGFBP-5 was partially inhibited by the p38 MAP kinase inhibitor, SB203580 (1 μM) (63 ± 8% inhibition), or the MKK1/2 inhibitor, U1026 (10 μM) (56 ± 12% inhibition). In the presence of the combination of the two inhibitors, the increase induced by IGFBP-5 was abolished, and basal levels of IGF-I secretion were slightly inhibited (Fig. 10).

**DISCUSSION**

The present study provides the first evidence that IGFBP-5 has direct, IGF-I-receptor-independent effects in normal human intestinal smooth muscle cells. The results show that in human intestinal smooth muscle cells, IGFBP-5 acts independently of the IGF-I receptor causes Ras-dependent activation of both p38 MAP kinase and Erk1/2, which jointly stimulate both muscle cell proliferation and IGF-I secretion. One explanation for the IGF-I-independent effects of IGFBP-5 on growth was provided by Andress and colleagues (9, 11), whose work suggested first in murine bone cells and later in rat glomerular mesangial cells the presence of an ~420-kDa IGFBP-5-specific receptor. In cultured neonatal mouse osteoblasts, the receptor undergoes autophosphorylation on serine residues in response to IGFBP-5 (10). In rat glomerular mesangial cells, IGFBP-5 mediates Cdc42-dependent reorganization of the actin cytoskeleton and formation of filopodia (11). The evidence supporting the operation of IGFBP-5-dependent, IGF-I-independent effects in human intestinal smooth muscle cells can be summarized as follows.

1. Incubation of muscle cells with IGFBP-5 causes time- and concentration-dependent activation of both the p38 MAP kinase and the Erk1/2 signaling pathways.
2. Incubation of muscle cells with IGFBP-5 causes concentration-dependent stimulation of proliferation.
3. Incubation of muscle cells with IGFBP-5 does not elicit activation of PI3-kinase as would occur if IGF-I/IGF-I receptor activation were involved.
4. Incubation of muscle cells with IGFBP-5 causes a p38 MAP kinase- and Erk1/2-dependent increase in IGF-I secretion.
5. In the presence of the IGF-I receptor antagonist, IGFBP-5 does not elicit IGF-I receptor phosphorylation.

IGFBP-5 has been shown to stimulate [3H]thymidine incorporation in osteoblast and bone cells independently of IGF-I and the IGF-I receptor (27). The mechanisms mediating the direct, proliferative effects of IGFBP-5 were not delineated. On the basis of the current study and previous work, two potential mechanisms have been identified. The first involves a membrane-bound, IGFBP-5-specific receptor first characterized by Andress in osteoblast cells (9–11), and the second involves nuclear transport of IGFBP-5 and direct nuclear effects mediated in the fashion of a ligand-dependent transcription factor. The latter mechanism was identified in T47D breast cancer cells by Schedlich and colleagues (28, 29). The carboxyl terminus of IGFBP-5 (and IGFBP-3, which also possesses IGF-I-independent effects (30, 31)) share a common nuclear localization sequence, IGFBP-3(215–232) and IGFBP-5(201–218). This sequence has been shown to be required for the importin-β-dependent nuclear translocation of both IGFBP-5 and IGFBP-3 (8, 29). Although this portion of IGFBP-5 also has been shown to participate in binding to the IGFBP-5 receptor, deletion studies have demonstrated that this sequence of the IGFBP-5 peptide is not required for activation of its cell surface receptor (27). Nuclear transport of IGFBP-5 was not specifically addressed in the current study; however, the ability of the p38 and MKK1/2 inhibitors in combination to abolish both proliferation and IGF-I secretion in response to IGFBP-5 suggests that if nuclear transport of IGFBP-5 does occur in these cells, it is not required for IGFBP-5-stimulated proliferation or IGF-I secretion.

The intracellular signaling cascades coupled to activation of the IGFBP-5 receptor are largely unknown but appear to begin following the autophosphorylation of serine residues of the receptor (10). In rat glomerular mesangial cells, activation of the IGFBP-5 receptor results in Cdc42-GTPase-activating protein aggregation, reorganization of the actin cytoskeleton, and filopodia formation (11). Whether this signaling pathway is involved in the proliferative responses to IGFBP-5 is unknown. Growth factors utilize distinct small GTPases to mediate specific cytoskeletal reorganizations such as lamellipodium formation (mediated via Rac), stress fiber formation (mediated via Rho), or filopodia formation (mediated via Cdc42). The small G-proteins Rho and Cdc42 are expressed by intestinal smooth muscle cells and mediate neurotransmitter-induced contraction (32). In human intestinal smooth muscle and other muscle cell types, the proliferative effects of IGFBP-5 are mediated by the activation of distinct Raf-Erk1/2 and p38 MAP kinase pathways. In COS-7 and HeLa cells, the small GTPases Rac, Rho, and Cdc42 are requisite cofactors in Ras-dependent Raf activation and subsequent activation of Erk1/2 (33–35). These monomeric G-proteins have also been shown to play a role in the activation of MKK3/6 leading to p38 MAP kinase activation (36, 37). It remains to be determined whether IGFBP-5-induced growth mediated by the Raf-Erk1/2 or p38 MAP kinase pathways involves the participation of the GTPases Rho, Rac, and Cdc42.

Based on our previous work and the results of this current study we propose the following model whereby a positive feedback mechanism links IGF-I and IGFBP-5 (Fig. 11). IGF-I, acting via its cognate receptor and facilitated by the presence of IGFBP-5, activates the PI3-kinase and Erk1/2 pathways that jointly mediate increased proliferation and secretion of IGFBP-5 (14, 17). IGFBP-5, in turn, acting via its cognate receptor, activates the p38 MAP kinase and Erk1/2 pathways...
that jointly mediate increased proliferation and further secretion of IGF-I.

The concomitant presence of IGF-I and IGFBP-5 and the resultant interplay between their signaling pathways may be important factors in mediating their individual effects. We and others (13, 38) have shown that IGFBP-5 protein levels are highly sensitive to protein synthesis inhibitors. Inhibition of RNA stabilizing factor translation by inhibitors such as cycloheximide leads to rapid IGFBP-5 mRNA degradation and results in IGFBP-5 levels falling rapidly to undetectable levels. The 3′-untranslated region of IGFBP-5 mRNA contains several adenosine-uridine-rich elements (38), which confer instability to IGFBP-5 mRNA but can be stabilized by binding cytoplasmic proteins. Hou and colleagues (38) have suggested that IGF-I might stimulate the production of AU-binding proteins that stabilize IGFBP-5 mRNA. In NIH 3T3 cells, the regulation of mRNA abundance is a dynamic process in which stabilizing and destabilizing AU-binding proteins compete (39). In these cells, PI3-kinase and p38 MAP kinase function by activating pathway specific stabilizing AU-binding proteins that regulate interleukin-3 mRNA levels (39). We have previously shown that in human intestinal muscle cells, IGF-I increases IGFBP-5 mRNA levels (39). We have previously shown that IGFBP-5 protein levels are increased to include Crohn’s disease for a number of years (15), and this observation has recently been extended to include Crohn’s disease in humans (16). The expression of both IGF-I and IGFBP-5 is increased in parallel regions of active inflammation and stRICTure formation. In addition to muscle proliferation induced by IGFBP-5 and IGF-I, IGFBP-5 also stimulates collagen secretion (44). The mechanisms responsible for initiating this response have yet to be fully elucidated, but the resulting muscle hyperplasia and extracellular matrix production may be responsible, in part, for luminal narrowing and striCTure formation in the intestine.

In summary, the present paper shows that IGFBP-5 exerts direct, IGF-1-receptor-dependent effects in human intestinal smooth muscle cells. By jointly activating p38 MAP kinase and Erk1/2, IGFBP-5 stimulates both muscle cell proliferation and IGF-I secretion. A positive feedback mechanism linking IGFBP-5 and IGF-I secretion in these cells reinforces their individual ability to cause intestinal smooth muscle cell growth.

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