Coupling of the Insulin-like Growth Factor-I Receptor Tyrosine Kinase to \( \text{G}_{12} \) in Human Intestinal Smooth Muscle

\( \text{G}_{12} \)-Dependent Mitogen-Activated Protein Kinase Activation and Growth*†

Received for publication, December 11, 2000
Published, JBC Papers in Press, December 18, 2000, DOI 10.1074/jbc.M01145200

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Endogenous insulin-like growth factor-1 (IGF-I) stimulates growth of cultured human intestinal smooth muscle by activating distinct mitogen-activated protein (MAP) kinase-dependent and phosphatidylinositol 3-kinase-dependent signaling pathways. In Rat1 and Balb/c3T3 fibroblasts and in neurons the IGF-I receptor is coupled to an inhibitory G protein, \( \text{G}_{0} \), which mediates \( \text{G}_{12} \)-dependent MAP kinase activation. The present study demonstrates whether in normal human intestinal smooth muscle cells the IGF-I receptor activates a heterotrimeric G protein and the role of G protein activation in mediating IGF-I-induced growth. IGF-I elicited IGF-I receptor tyrosine phosphorylation, resulting in the specific activation of \( \text{G}_{12} \). \( \text{G}_{12} \) subunits selectively mediated IGF-I-dependent MAP kinase activation; \( \text{G}_{62} \) subunits selectively mediated IGF-I-dependent inhibition of adenyl cyclase activity. IGF-I-stimulated MAP kinase activation and growth were inhibited by pertussis toxin, an inhibitor of \( \text{G}_{0} \) activation. Cyclic AMP inhibits growth of human intestinal muscle cells. IGF-I inhibited both basal and forskolin-stimulated cAMP levels. This inhibition was attenuated in the presence of pertussis toxin. IGF-I stimulated phosphatidylinositol 3-kinase activation, in contrast to MAP kinase activation, occurred independently of \( \text{G}_{12} \) activation. These data suggest that IGF-I specifically activates \( \text{G}_{0} \), resulting in concurrent \( \text{G}_{0} \)-dependent stimulation of MAP kinase activity and growth, and \( \text{G}_{62} \)-dependent inhibition of cAMP levels resulting in disinhibition of cAMP-mediated growth suppression.

Insulin-like growth factor (IGF)-1 mediates three distinct regulatory effects on cell growth by activation of the IGF-I receptor. IGF-I stimulates proliferation of cells and may be required for optimal growth of these cells (1–4). Transformation and maintenance of the transformed state also require IGF-I receptor activation in some cells (4, 5). IGF-I can also protect cells from apoptosis (6, 7).

Three proteins have been identified which are rapidly recruited to the membrane after IGF-I receptor tyrosine phosphorylation: insulin receptor substrate (IRS)-1/2, Src-homology/collagen (Shc), and CT-10-regulated kinase (8–10). Through these substrates IGF-I mediates activation of two main signaling cascades, the MAP kinase and PI 3-kinase pathways, which can act either in conjunction, in opposition, or individually to mediate the response to IGF-I whether proliferative, transforming, or anti-apoptotic (6, 11, 12).

In Rat1 fibroblasts IGF-I activates a pertussis toxin (PTx)-sensitive heterotrimeric G protein leading to \( \text{G}_{62} \)-mediated, Ras-dependent MAP kinase stimulation (13). The signal is transmitted by \( \text{G}_{12} \) subunits dissociated from an IGF-I-activated inhibitory (PTx-sensitive) G protein. This mechanism of ras-dependent MAP kinase activation is shared by both the IGF-I receptor tyrosine kinases and G protein-coupled receptors, such as the lysophosphatidic acid receptors (13–16). Activation of either receptor tyrosine kinases or the G protein-coupled receptors induces rapid tyrosine phosphorylation of docking proteins, e.g. Shc and Grb2, which function as membrane scaffolds for the recruitment of Ras guanine nucleotide exchange factors, e.g. mSOS, that regulate Ras activity. The regulation of Ras activity by this pathway has further been shown to involve the participation of either Src family non-receptor tyrosine kinases or focal adhesion kinases depending on the ligand and the cell type examined (15, 16).

IGF-II acting through its cognate IGF-II/mannose 6-phosphate receptor stimulates growth and metabolic effects. Coupling of this receptor to the inhibitory heterotrimeric G protein, \( \text{G}_{62} \), has also been described in both membranes derived from mouse Balb/c3T3 fibroblasts and COS cells transfected with IGF-II/Man-6-P receptor cDNA (17–19). Upon stimulation by IGF-II, but not by Man-6-P, the activated IGF-II/Man-6-P receptor interacts with \( \text{G}_{12} \) through the Arg2410-Lys2413 sequence in its C-terminal intracellular domain (19). In contrast to IGF-I-induced IGF-I receptor activation where MAP kinase stimulation is \( \text{G}_{77} \)-dependent, IGF-II-induced activation of the IGF-II/Man-6-P receptor results in \( \text{G}_{62} \)-dependent inhibition of adenylate cyclase activity, an effect only potentiated by the \( \text{G}_{62} \) subunits derived from \( \text{G}_{12} \) activation (19).

Human intestinal smooth muscle cells produce IGF-I, which plays a autocrine role in the regulation of growth in culture (20). In these cells IGF-I-stimulated growth is mediated by activation of distinct MAP kinase-dependent and PI 3-kinase-dependent signaling cascades (21). Whether IGF-I-stimulated MAP kinase activation and growth in these cells involve the activation of a heterotrimeric G protein, and the roles of the G...
protein subunits in mediating this effect are not known. The specific G protein activated by IGF-I and the roles of the α and β subunits derived from G protein activation on growth have not been examined.

In the present study we show that IGF-I specifically activates the PTX-sensitive inhibitory G protein, Gβγ Gβ2 activation results in concurrent Gαi2 activation of MAP kinase activity and growth, and Gα2i2-dependent inhibition of adenylate cyclase, cAMP production and results in disinhibition of cAMP-mediated growth suppression.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of Muscle Cells from Human Jejunum**—Muscle cells were isolated from the circular muscle layer of human jejunum as described previously (20, 21). Segments of normal jejunum were obtained from patients undergoing surgery according to a protocol approved by the Institutional Committee on the Conduct of Human Research. Briefly, muscle cells were isolated by enzymatic digestion for 60 min at 31 °C in a medium containing 0.2% collagenase (CLS type II) and 0.1% soybean trypsin inhibitor. The medium consisted of (in mM): 120 NaCl, 4 KCl, 2.6 KH2PO4, 2 CaCl2, 0.6 MgCl2, 25 HEPES, 14 glucose, and 2.1% Eagle’s essential amino acid mixture. Primary cultures of intestinal muscle cells were initiated and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (DMEM-10) and containing 200 units/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamycin, and 2 μg/ml amphotericin B. The cells were plated at a concentration of 5 × 104 cells/ml and incubated in a 10% CO2 environment at 37 °C. All subsequent studies were performed in first passage cultured cells after 7 days, at which time the cells were confluent.

**[3H]Thymidine Proliferation Assay**—Proliferation of smooth muscle cells in culture was measured by the incorporation of [3H]thymidine as described previously (20, 21). Briefly, the cells were washed free of serum and incubated for 24 h in DMEM-0. After a 24-h incubation in the absence of serum, the cells were incubated for an additional 24 h with [3H]thymidine (5 μCi/ml) in the presence and absence of various test agents. During the final 4 h of this incubation period, 1 μCi/ml [3H]thymidine was added to the medium. [3H]Thymidine incorporation into the perchloric acid-extractable pool was used as a measure of DNA synthesis. DNA content was measured fluorometrically using Hoescht 33528 with excitation at 356 nm and emission at 492 nm. Calf thymus DNA was used as a standard.

**Identification of IGF-I-activated G Proteins**—G protein subunits activated by IGF-I were identified by the method of Okamoto et al. (22) as described previously by us (23–25). Confluent human intestinal smooth muscle cells growing in 100-mm plates were scraped off the plate and homogenized in 20 mM HEPES medium (pH 7.4) containing 2 mM MgCl2, 1 mM EDTA, and 2 mM diethiothreitol. After centrifugation at 27,000 rpm for 15 min, the crude membranes were solubilized for 60 min at 4 °C in 20 mM HEPES medium (pH 7.4) containing 2 mM EDTA, 240 mM NaCl, and 1% CHAPS. The membranes were incubated for 20 min with 60 mM [3S]GTPγS in a solution containing 10 mM HEPES (pH 7.4), 100 μM EDTA, and 10 mM MgCl2. The reaction was stopped with 10 volumes of 100 mM Tris-HCl medium (pH 8.0) containing 10 mM MgCl2, 100 mM NaCl, and 20 μM GTPγS, and the mixture was placed in wells precoated with specific G protein antibodies. After incubation for 2 h on ice, the wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20, and radioactivity from each well was counted. Coating with G protein antibodies (1:1,000) was done after the wells were coated with anti-rabbit IgG (1:1,000) for 2 h on ice. The selective IGF-I receptor tyrosine kinase inhibitor, tyrphostin AG 1024 (100 μM) (26), was used to identify to role of IGF-I receptor tyrosine kinase phosphorylation in G protein activation.

**Measurement of IGF-I Receptor Phosphorylation**—Binding of IGF-I to the IGF-I receptor results in tyrosine phosphorylation of the IGF-I receptor β subunit (IGF-IRβ). Tyrosine phosphorylation of the IGF-IRβ was measured by immunoprecipitation of the IGF-IRβ and subsequent Western blotting of tyrosine-phosphorylated proteins. Confluent muscle cells growing in 100-mm plates were incubated in serum-free DMEM for 72 h. The cells were stimulated for 2 min with increasing concentrations (1–100 nM) of IGF-I, IGF-II, and del(1–61)IGF-II, a synthetic IGF-II agonist that does not interact with IGF-binding proteins (33). The reaction was terminated by washing with ice-cold phosphate-buffered saline. The cells were lysed in immunoprecipitation buffer consisting of phosphate-buffered saline with added 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM phenylmethylsulfonyl fluoride, 1% (w/v) aprotinin, and 1 mM sodium orthovanadate. Cell lysates containing equal amounts of protein were incubated with 2 μg/ml antibody to the IGF-IRβ for 2 h at 4 °C. 20 μl of protein A/G-agarose was added, and the incubation was continued overnight. Following the immune complex, the samples were collected by centrifugation (13,000 g) and washed four times in immunoprecipitation buffer. The final pellet was resuspended in 40 μl of electrophoresis sample buffer and boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 10% gels under reducing conditions and then transferred to nitrocellulose membranes. The membranes were incubated overnight with 1:1,000 diluted antibodies followed by 1:2,000 dilution of goat anti-mouse IgG-horse radish peroxidase. The bands were visualized with enhanced chemiluminescence and quantitated with densitometry.

**Measurement of MAP Kinase Activity by in Vitro Kinase Assay**—MAP kinase activity was measured as described previously (21). Briefly, confluent muscle cells growing in six-well plates were incubated in serum-free DMEM for 72 h. The cells were stimulated with 100 nM IGF-I for various time periods, 0–240 min, in the presence and absence of test agents. The cells were lysed in a buffer containing (in mM): 10 Tris (pH 7.4), 150 NaCl, 2 EGTA, 2 diethiothreitol, 1 orthovanadate, 1 phenylmethylsulfonyl fluoride, with added 10 μg/ml leupeptin and 10 μg/ml aprotinin. Cellular debris in the lysates was precipitated by centrifugation at 12,000 × g for 30 min at 4 °C and the supernatant was used in duplicate in aliquots of cell lysate containing equal amounts of protein by the incorporation of phosphate from [γ-32P]ATP (1 μCi/30 μl of reaction volume) into a synthetic MAP kinase substrate (Amersham Pharmacia Biotech) for a 30-min incubation at 30 °C. The reaction was terminated, and phosphorylated peptide substrate was separated using phosphocellulose microfuge spin tubes (Pierce). The results are expressed in pmol of phosphate incorporated/min/mg of protein.

**Permeabilization of Muscle Cells**—Confluent muscle cells growing in 100-mm dishes were permeabilized by modification of techniques described previously (23–25, 27). The effects of specific G protein subunits were investigated by immunoneutralization using selective antibodies to the G protein subunits described previously. This technique has not been validated previously and used to identify the signaling mechanisms and functional effects mediated via specific G protein subunits activated by somatostatin, opioid, and muscarinic receptors on intestinal smooth muscle cells (23–25). Briefly, muscle cells were released from the culture plate by treatment with 0.5% (w/v) trypsin containing 0.53 mM EDTA. The cells were washed free of enzymes by centrifugation and resuspended in a cytosol-like buffer containing (in mM): 20 NaCl, 100 KCl, 1 MgSO4, 25 NaHCO3, 1 EGTA, 0.18 Ca2+, and 1% IGF-I-free bovine serum albumin. Cells were permeabilized by incubation with 35 μg/ml saponin at 31 °C for 10 min. The cells were washed free of saponin by centrifugation at 150 × g and resuspended in the same medium with 1.5 mM ATP and ATP-regenerating system (5 mM creatine phosphate and 10 units/ml creatine phosphokinase). The cells were incubated 1 h at neutralizing antibody to Gγ (10 μM) or Gγ (10 μM). The reaction was initiated by addition of 100 nM IGF-I and terminated after 10 min by placing the cells on ice. The cells were rapidly centrifuged at 150 × g and 4 °C and the supernatant removed. The cells were resuspended in MAP kinase lysis buffer (see above), and MAP kinase activity was measured by in vitro kinase assay as described above.

**Measurement of PI 3-Kinase Activity by in Vitro Kinase Assay**—PI 3-kinase activity was measured by a modification of the method of Higaki et al. (28) as described previously (21). Briefly, muscle cells grown to confluence in 100-mm dishes were incubated in serum-free DMEM for 72 h. Cells were stimulated for 10 min with 100 nM IGF-I in the presence and absence of various test agents. The cells were lysed in a buffer consisting of (in mM): 50 Tris-HCl (pH 7.8), 150 NaCl, 1 Na2VO3, 2 EDTA, 1 MgCl2, 1 CaCl2, and 30 mM leupeptin, with added 1% (w/v) tryslamol and 1% (w/v) Nonidet P-40. Aliquots of cell lysate containing equal amounts of protein were incubated with 25 μl of anti-phosphotyrosine antibody (PY20) coupled to agarose beads with gentle mixing for 2 h at 4 °C. The beads were collected by centrifugation and washed three times with kinase assay buffer and two times with kinase assay buffer. Kinase assay buffer consisted of (in mM): 50 Tris-HCl (pH 7.8), 50 NaCl, 2 MgCl2, and 0.5 EDTA. After the final washing the beads were resuspended in 30 μl of kinase assay buffer to which 10 μl of sonicated 1 mg/ml phosphatidylinositol was added. The reaction was initiated by the addition of 5 μl of 50 mM ATP containing 0.5 μCi of [γ-32P]ATP and continued for 10 min at 30 °C. The reaction was terminated by the addition of 0.5 ml of 1 × HCl and 2 ml
of chloroform-methanol (2:1, v/v). Phospholipids were recovered from the lower organic phase and dried under \( N_2 \) gas. The dried phospholipids were dissolved in chloroform and spotted on Silica H Gel TLC plates impregnated with 1% potassium oxalate. Chromatograms were developed in chloroform, methanol, 28% \( NH_4 \) water (70:100:15:25, v/v). The plates were air-dried and phospholipids visualized with autoradiography. The spots corresponding to authentic phosphatidylinositol 3,4,5-trisphosphate were scraped off the plates and incorporated \(^{32}\)P quantified by \( \beta \)-scintillation counting. Results are expressed as the increase in \(^{32}\)P incorporation into PI-3-P in cpm above basal values.

**Measurement of Adenylyl Cyclase Activity**—Adenylyl cyclase activity was measured by the method of Salomon et al. (29). Briefly, a 0.1-mg sample of membrane protein was incubated for 15 min at 37 °C in 50 mM Tris-HCl (pH 7.4), 1 mM ATP, 2 mM cAMP, 0.1 mM GTP, 0.1 mM isobutylmethylxanthine, 5 mM MgCl\(_2\), 100 mM NaCl, 5 mM creatine phosphate, 50 units/ml creatine phosphokinase, and 4 \( \times 10^4 \) cpm of \[^{3}H\]ATP. The reaction was terminated by the addition of 2% SDS, 45 mM ATP, and 1.5 mM cAMP. \[^{3}H\]cAMP was separated from \[^{3}H\]ATP by sequential chromatography on Dowex AG50W-4X and alumina columns. The results were expressed as pmol of cAMP/mg of protein/min.

**Measurement of cAMP Production**—Adenylyl cyclase activity was measured by modification of methods described previously (23–25). Briefly, confluent muscle cells growing in six-well plates were incubated in serum-free DMEM for 24 h. Cells were incubated for 10 min in 1 \( \mu \)M isobutylmethylxanthine. cAMP in the cells was stimulated by activation with 10 \( \mu \)M forskolin, and 100 \( \mu \)M IGF-I was added for an additional 5 min. The reaction was terminated using ice-cold 10% trichloroacetic acid in which the cells were incubated for 15 min at 4 °C. The supernatants were extracted three times with water-saturated diethyl ether. The resulting aqueous phase was frozen and lyophilized. The samples were reconstituted for radioimmunoassay in 500 \( \mu \)l of 50 mM sodium acetate (pH 6.2) and acetylated with triethylamine/acetic anhydride (3:1 v/v) for 30 min. cAMP was measured in duplicate using 100-\( \mu \)l aliquots and expressed as pmol/mg of protein.

**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 was appropriate.

**Materials**—Recombinant human IGF-I and IGF-II were from Australs Biologicals (San Ramon, CA). Collagenase and soybean trypsin inhibitor were from Worthington. HEPES was from Research Organics (Cleveland, OH). DMEM was from Mediatech Inc. (Herndon, VA). Fetal bovine serum was from Summit Biotechnologies, Inc. (Fort Collins, CO). The MAP kinase assay kit, [\( ^{32}P \)]ATP (specific activity 3,000 Ci/mmol), \[^{3}H\]thymidine (specific activity 6 Ci/mmol), \[^{3}H\]ATP (specific activity 26.5 Ci/mmol), and \[^{32}P\]ATP (specific activity 2,000 Ci/mmol) were from Amersham Pharmacia Biotech. [\(^{32}\)S\)]GTP\( \gamma \)S (specific activity 1,250 Ci/mmol) was from PerkinElmer Life Sciences. Western blotting materials and the protein assay kit were from Bio-Rad. Anti-phosphotyrosine PY20-agarose beads were from Transduction Laboratories (Lexington, KY). Phosphocellulose spin columns were from Pierce. Thin layer chromatography plates were from Analtech (Newark, DE). Plastic cultureware was from Corning (Corning, NY). Antibodies to G\(_{i3}\), G\(_{i2}\), G\(_{i1}\), and G\(_{s}\) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to G\(_{i2}\) was from Chemicon (Temecula, CA). Anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY).

### RESULTS

**Effect of PTx on IGF-I-induced Growth—IGF-I increased \[^{3}H\]thymidine incorporation by 289 ± 9% above basal (basal, 34 ± 2 cpm/ng DNA) (Fig. 1). The growth elicited by IGF-I was partially inhibited, 35 ± 5%, in the presence of 200 ng/ml PTx. We have shown previously that IGF-I-induced growth is mediated jointly by activation of distinct MAP kinase-dependent, PI 3-kinase-independent and a MAP kinase-independent, PI 3-kinase-dependent pathways (21). To determine whether PTx-sensitive growth elicited by IGF-I was mediated by activation of the MAP kinase-dependent or the PI 3-kinase-dependent pathways, cells were incubated with the MAP kinase kinase inhibitor PD98059 (10 \( \mu \)M) or the PI 3-kinase inhibitor LY294002 (10 \( \mu \)M). We have shown previously that at the concentrations used these inhibitors selectively block activation of MAP kinase and PI 3-kinase, respectively, in these cells (21). Thymidine incorporation in response to 100 \( \mu \)M IGF-I was partly inhibited (35 ± 5%) by PTx (Fig. 1) (21, 30, 31). The MAP kinase kinase inhibitor had only a minor additive effect to that of PTx (MAP kinase kinase inhibitor + PTx: 42 ± 10% inhibition versus PTx alone: 35 ± 5% inhibition, \( p < 0.01 \) versus IGF-I + PTx). The results suggest that the inhibition of IGF-I-induced growth mediated by activation of the MAP kinase pathway was sensitive to PTx, whereas the portion mediated by activation of the PI 3-kinase pathway was insensitive to PTx.

**IGF-II and insulin were also examined for their ability to stimulate growth of these cells. 100 nM IGF-II increased \[^{3}H\]thymidine incorporation by 40 ± 25% above basal, and 100 nM insulin increased \[^{3}H\]thymidine incorporation by 35 ± 15% above basal values. The increase in \[^{3}H\]thymidine incorpora-
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**Fig. 2. IGF-I causes concentration-dependent tyrosine phosphorylation of the IGF-I receptor.** Panel A, representative anti-phosphotyrosine Western blot of IGF-IRβ immunoprecipitates stimulated with IGF-I, IGF-II, or del(1–6)IGF-II. Panel B, IGF-I (closed circles) caused concentration-dependent tyrosine phosphorylation of the IGF-IRβ subunit. IGF-II (closed squares) and del(1–6)IGF-II (open circles) did not cause tyrosine phosphorylation of the IGF-IRβ subunit. Quiescent muscle cells were incubated with 1–100 nM IGF-I, IGF-II, or del(1–6)IGF-II for 2 min. IGF-IRβ tyrosine phosphorylation was measured in anti-phosphotyrosine Western blots of IGF-IRβ immunoprecipitates as described under “Experimental Procedures.” Results are expressed as a percent above basal phosphorylation in relative densitometric units. Values represent the mean ± S.E. of three or four separate experiments. ** denotes $p < 0.01$ versus basal.

**Fig. 3. PTx inhibits IGF-I-stimulated MAP kinase activation.** IGF-I (○) elicited time-dependent MAP kinase activation. In the presence of 200 ng/ml PTx (○), IGF-I-induced MAP kinase stimulation was strongly inhibited at 2 and 10 min and was abolished (returned to basal unstimulated levels) at longer time periods. MAP kinase activity was measured by an in vitro kinase assay as the incorporation of $[^{32}P]ATP$ into synthetic MAP kinase substrate as described under “Experimental Procedures.” Results are expressed as the increase in MAP kinase activity above basal (basal, 1.77 ± 0.35 pmol of P/min/mg of protein). Values represent the mean ± S.E. of three separate experiments. * denotes $p < 0.05$ versus IGF-I alone.

The effects of IGF-I in these cells were mediated by activation of the IGF-IR, whereas those mediated by IGF-II were not.

**Effect of PTx on IGF-I-mediated Activation of MAP Kinase**—We have shown previously that activation of MAP kinase by IGF-I is concentration-dependent and time-dependent (21). MAP kinase activation in response to a maximally effective concentration of IGF-I (100 nM) is rapid, occurring within 2 min, attains a maximum at 10 min, and declines to basal levels within 240 min (21). In the presence of 200 ng/ml PTx, activation of MAP kinase by 100 nM IGF-I is inhibited at 2 and 10 min (64 ± 9% and 48 ± 16% inhibition from untreated control, respectively, $p < 0.05$) (Fig. 3). MAP kinase activation by IGF-I at longer time points (30–240 min) was abolished, i.e. returned to basal unstimulated levels. The results implied that the early activation of MAP kinase by IGF-I is partially dependent on activation of an inhibitory G protein. Sustained MAP kinase activation is fully dependent on activation of an inhibitory G protein.

**Identification of the Inhibitory G Protein Activated by IGF-I**—The ability of PTx to inhibit both IGF-I-induced MAP kinase activation and growth implied that an inhibitory G protein is activated by IGF-I in human intestinal muscle cells. The identity of the G protein that accounts for the sensitivity to PTx was measured by the binding of $[^{35}S]$GTPγS to G protein subunits in membranes prepared from these cells. 100 nM IGF-I selectively increased the activity of $G_{a_{12}}$, 252 ± 17% above basal (basal, 7.020 ± 118 cpm/mg of protein) (Fig. 4). IGF-I did not affect the activity of $G_{a_{11}}$, $G_{a_{13}}$, or $G_{a_{oq/11}}$. In the presence of a selective inhibitor of the IGF-I receptor tyrosine kinase, tyrphostin AG 1024 (100 μM), IGF-I-induced $G_{a_{12}}$ activation was inhibited 78 ± 4% ($p < 0.01$). The results implied that the activation of $G_{a_{12}}$ by the IGF-I receptor occurred as a result of IGF-I receptor tyrosine kinase autophosphorylation.

Coupling of the IGF-II/Man-6-P receptor to a PTx-sensitive inhibitory G protein has been described in Balb/3T3 fibroblasts (18). In human intestinal muscle cells, IGF-II did not increase the activity of $G_{o_{11}}$, $G_{o_{12}}$, $G_{o_{13}}$, or $G_{o_{oq/11}}$ (Fig. 4).

**Functional Role of $G_{o_{12}}$ in IGF-I-mediated MAP Kinase Activation**—Permeabilized muscle cells were used to determine which of the G protein subunits activated by the IGF-I receptor mediated IGF-I-induced MAP kinase activation. Activation of $G_{a_{12}}$ by IGF-I implied the concomitant activation of $G_{o_{12}}$. Therefore, the individual roles of the $G_{o_{12}}$ and the $G_{o_{oq}}$ subunits in IGF-I-mediated MAP kinase activation were examined by in-
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IGF-I selectively activates $G_{i2}$. Panel A, 100 nM IGF-I caused a significant increase in the binding of [35S]GTP-$S_i$ complexes to wells precoated with $G_{i2}$ antibody but not to wells precoated with antibody to $G_{i3}$, $G_{o2}$, or $G_{ao2}$. Panel B, 100 nM IGF-II did not increase the binding of [35S]GTP-$S_i$ complexes. Membranes isolated from human intestinal muscle cells were solubilized in CHAPS and incubated with [35S]GTP-$S_i$ in the presence and absence of IGF-I or IGF-II for 20 min as described under “Experimental Procedures.” Results are expressed as the increase in bound radioactivity in cpm/mg of protein. Values represent the mean ± S.E. of five separate experiments. ** denotes $p < 0.01$ versus GTP$S_i$ alone.

Inhibition of cAMP Production by IGF-I—The activation of $G_{i2}$ by IGF-I suggested that IGF-I may inhibit the accumulation of cAMP. This notion was tested in two complementary ways. In the first, the role of the IGF-I-activated $G_{i2}$ subunit in regulating adenylyl cyclase activity was determined. In the second, the effect of IGF-I on cAMP levels was measured.

The activity of adenylyl cyclase was measured in membranes prepared from human intestinal muscle cells as the conversion of [3H]ATP into cAMP. Incubation of membranes with 10 μM forskolin increased adenylyl cyclase activity by 362 ± 44% above basal (basal, 6.8 ± 0.5 pmol of cAMP/mg of protein/min) (Fig. 6). Forskolin-stimulated adenylyl cyclase activity was inhibited 68 ± 2% in the presence of 100 nM IGF-I (Fig. 6). Inhibition of forskolin-stimulated adenylyl cyclase activity was attenuated in the presence of the selective $G_{i2}$-neutralizing antibody but not affected by the common $G_o$-neutralizing antibody (Fig. 6).

Intact human intestinal smooth muscle cells were incubated with IGF-I alone, to determine its effects on basal cAMP levels, and with IGF-I in the presence of forskolin, to determine the effect of IGF-I on forskolin-stimulated cAMP levels. 100 nM IGF-I by itself was capable of decreasing basal cAMP levels by 30 ± 4% (basal, 23.5 ± 1.4 pmol/mg of protein) (Fig. 7A). The increase in cAMP stimulated by 10 μM forskolin (144 ± 4 pmol/mg of protein above basal) was strongly inhibited, 89 ± 1%, in the presence of 100 μM IGF-I (Fig. 7B). The inhibition of forskolin-stimulated cAMP production by IGF-I was attenuated, 58 ± 2%, by pretreatment with PTx (Fig. 7B).

Functional Role of cAMP in IGF-I-mediated Inhibition Growth—Activation of adenylyl cyclase and the resulting increase in cAMP levels are generally accompanied by inhibition of growth in smooth muscle cells (34, 35). The effect of adenylyl cyclase activation on growth of human intestinal smooth muscle has not been examined previously. Accordingly, it was investigated by the addition of forskolin to activate adenylyl
IGF-I inhibits cAMP production. Panel A, 100 nM IGF-I inhibits basal cAMP levels by 30 ± 4%. Panel B, 10 μM forskolin-stimulated cAMP production is inhibited 89 ± 1% by 100 nM IGF-I. In the presence of 200 ng/ml PTx, the ability of IGF-I to inhibit forskolin-stimulated cAMP production is decreased by 58 ± 2%. Human intestinal muscle cells were incubated with IGF-I for 10 min, and cAMP levels were measured in trichloroacetic acid extracts by radioimmunoassay as described under “Experimental Procedures.” Results are expressed as pmol of cAMP/mg of protein (basal, 23.5 ± 1.4 pmol of cAMP/mg of protein). Values represent the mean ± S.E. of three separate experiments. * denotes p < 0.01 versus basal levels; ** denotes p < 0.01 versus forskolin alone; + + denotes p < 0.01 versus forskolin + IGF-I.

The evidence supporting the activation of G<sub>i2</sub> by IGF-I and distinct signaling pathways coupled to stimulation of growth: a MAP kinase-dependent, PI 3-kinase-independent and a MAP kinase-independent, PI 3-kinase dependent pathway (21). These two pathways jointly mediate growth in response to IGF-I. The present paper shows that activation of MAP kinase by IGF-I occurs through the activation of G<sub>i2</sub> by the tyrosine-phosphorylated IGF-I receptor and is mediated by the G<sub>bg</sub> subunits. The G<sub>bg</sub> subunits derived from G<sub>bg</sub> activation may also participate in the regulation of growth by IGF-I by attenuating the levels of growth inhibitory cAMP.

IGF-I-mediated Activation of PI 3-Kinase Occurs Independently of a PTx-sensitive G Protein—The independence of IGF-I-induced PI 3-kinase activation from the PTx-sensitive G protein was examined directly by measurement of PI 3-kinase activation in the presence of PTx. Preincubation of muscle cells with 200 ng/ml PTx did not alter maximal IGF-I-induced increase in PI 3-kinase activation (IGF-I, 112 ± 8% above basal; IGF-I + PTx, 132 ± 9% above basal) (Fig. 9). The results confirmed the G<sub>bg</sub>-independent nature of IGF-I-mediated PI 3-kinase activation and PI 3-kinase-dependent growth in these cells.

DISCUSSION

In human intestinal muscle cells IGF-I activates two distinct signaling pathways coupled to stimulation of growth: a MAP kinase-dependent, PI 3-kinase-independent and a MAP kinase-independent, PI 3-kinase dependent pathway (21). These two pathways jointly mediate growth in response to IGF-I. The present paper shows that activation of MAP kinase by IGF-I occurs through the activation of G<sub>i2</sub> by the tyrosine-phosphorylated IGF-I receptor and is mediated by the G<sub>bg</sub> subunits. The G<sub>bg</sub> subunits derived from G<sub>bg</sub> activation may also participate in the regulation of growth by IGF-I by attenuating the levels of growth inhibitory cAMP.

The evidence supporting the activation of G<sub>i2</sub> by IGF-I and distinct roles for G<sub>bg</sub> subunits in the stimulation of MAP kinase leading to growth, and for G<sub>bg</sub> subunits in the inhibition of adenyl cyclase and cAMP leading to growth can be summarized as follows. 1) IGF-I induced specific activation of G<sub>i2</sub> which was sensitive to the IGF-I tyrosine kinase inhibitor, tyrphostin AG 1024. 2) IGF-I-induced MAP kinase activation was inhibited by PTx and by immunoneutralization of G<sub>bg</sub> subunits but not by immunoneutralization of G<sub>bg</sub> subunits. 3)
IGF-I-induced inhibition of forskolin-stimulated adenyl cyclase activity was sensitive to immunoneutralization of G_{i2} but not G_{s} subunits. 4) IGF-I inhibited both basal cAMP production and forskolin-stimulated cAMP production, and the inhibition was attenuated in the presence of PTx. 5) IGF-I-induced growth was partially inhibited by PTx, by a MAP kinase kinase inhibitor, or the PI 3-kinase inhibitor (21); the effects of PTx and the PI 3-kinase inhibitor were additive, whereas those of PTx and the MAP kinase kinase inhibitor were not additive. 6) IGF-I-induced growth was inhibited by forskolin.

IGF-I receptor tyrosine phosphorylation on residues Tyr-1158, Tyr-1162, and Tyr-1163 is required for the mitogenic response mediated by IGF-I receptor activation (8). Several studies have provided evidence that following IGF-I receptor autophosphorylation both IRS-1 and Shc are activated (8, 9). IRS-1 associates with PI 3-kinase (36, 37), Nck (38), Grb2 (38, 39), and PTP1D (40) through an SH2 interaction, whereas Shc interacts with Grb2 (39). The Grb2-docking protein provides a scaffold for recruitment of the Ras guanine nucleotide exchange factor, mSOS, to the plasma membrane and results in p21caax activation. Activation of Ras initiates the RAF/MAP kinase cascade, a key signaling pathway in IGF-I-induced growth.

Although evidence exists linking IGF-I receptor phosphorylation to IRS-1 and Shc activation, in contrast to IRS-1, Shc does not associate directly with either the IGF-I receptor or with IRS-1 (9). Recent evidence demonstrates that not only do receptor tyrosine kinases like the IGF-I receptor but also some G protein-coupled receptors activate Shc (14, 15). In the case of G protein-coupled receptors, Shc activation is mediated by G_{a} subunits derived from G_{q}-coupled receptors and by G_{b} subunits derived from G_{i2}-coupled receptors (15). Both the receptor tyrosine kinase and G protein-coupled receptor signaling cascades involve the participation of Src family nonreceptor tyrosine kinases and lead to the activation of p21caax through the Shc-Grb2-mSOS pathway (15, 16, 41). Thus, Ras-dependent MAP kinase activation is a signaling pathway shared by both receptor tyrosine kinases and G protein-coupled receptors. The participation of G_{b} subunits in IGF-I-induced growth is a signaling pathway shared by both receptor tyrosine kinases and G protein-coupled receptors. The participation of G_{a} subunits in IGF-I-induced growth is a signaling pathway shared by both receptor tyrosine kinases and G protein-coupled receptors.

Therefore, IGF-I receptor tyrosine phosphorylation on IRS-1 activates MAP kinase and PI 3-kinase activity. IRS-1 associates with PI 3-kinase (36, 37), Nck (38), Grb2 (39), and PTP1D (40) through an SH2 interaction, whereas Shc interacts with Grb2 (39). The Grb2-docking protein provides a scaffold for recruitment of the Ras guanine nucleotide exchange factor, mSOS, to the plasma membrane and results in p21caax activation. Activation of Ras initiates the RAF/MAP kinase cascade, a key signaling pathway in IGF-I-induced growth.

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In the case of G protein-coupled receptors, Shc activation is mediated by G_{a} subunits derived from G_{q}-coupled receptors and by G_{b} subunits derived from G_{i2}-coupled receptors (15). Both the receptor tyrosine kinase and G protein-coupled receptor signaling cascades involve the participation of Src family nonreceptor tyrosine kinases and lead to the activation of p21caax through the Shc-Grb2-mSOS pathway (15, 16, 41). Thus, Ras-dependent MAP kinase activation is a signaling pathway shared by both receptor tyrosine kinases and G protein-coupled receptors. The participation of G_{b} subunits in IGF-I-induced growth is a signaling pathway shared by both receptor tyrosine kinases and G protein-coupled receptors. The participation of G_{a} subunits in IGF-I-induced growth is a signaling pathway shared by both receptor tyrosine kinases and G protein-coupled receptors.
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