Insulin-like Growth Factors Mediate Heterotrimeric G Protein-dependent ERK1/2 Activation by Transactivating Sphingosine 1-Phosphate Receptors

Received for publication, June 5, 2006, and in revised form, July 24, 2006 Published, JBC Papers in Press, August 22, 2006, DOI 10.1074/jbc.M605359200

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Although several studies have shown that a subset of insulin-like growth factor (IGF) signals require the activation of heterotrimeric G proteins, the molecular mechanisms underlying IGF-stimulated G protein signaling remain poorly understood. Here, we have studied the mechanism by which endogenous IGF receptors activate the ERK1/2 mitogen-activated protein kinase cascade in HEK293 cells. In these cells, treatment with pertussis toxin and expression of a Gq/11 subunit receptor tyrosine kinase (SK) 1 from the cytosol to the plasma membrane, increased endogenous SK activity within 30 s of stimulation, and caused a statistically significant increase in intracellular and extracellular sphingosine 1-phosphate (SIP) concentration. Using a GFP-tagged SIP1 receptor as a biological sensor for the generation of physiologically relevant SIP levels, we found that IGF-1 and IGF-2 induced GFP-SIP1 receptor internalization and that the effect was blocked by pretreatment with the SK inhibitor, dimethylsphingosine. Treating cells with dimethylsphingosine, silencing SK1 expression by RNA interference, and blocking endogenous SIP receptors with the competitive antagonist VPC23019 all significantly inhibited IGF-stimulated ERK1/2 activation, indicating that the signal was almost completely G protein-dependent. Treatment with IGF-1 or IGF-2 promoted translocation of green fluorescent protein (GFP)-tagged sphingosine kinase (SK) 1 from the cytosol to the plasma membrane, increased endogenous SK activity within 30 s of stimulation, and caused a statistically significant increase in intracellular and extracellular sphingosine 1-phosphate (SIP) concentration. Using a GFP-tagged SIP1 receptor as a biological sensor for the generation of physiologically relevant SIP levels, we found that IGF-1 and IGF-2 induced GFP-SIP1 receptor internalization and that the effect was blocked by pretreatment with the SK inhibitor, dimethylsphingosine. Treating cells with dimethylsphingosine, silencing SK1 expression by RNA interference, and blocking endogenous SIP receptors with the competitive antagonist VPC23019 all significantly inhibited IGF-stimulated ERK1/2 activation, suggesting that IGFs elicit G protein-dependent ERK1/2 activation by stimulating SK1-dependent transactivation of SIP receptors. Given the ubiquity of SK and SIP receptor expression, SIP receptor transactivation may represent a general mechanism for G protein-dependent signaling by non-G protein-coupled receptors.

The insulin-like growth factors type 1 and 2 (IGF-1 and -2)2 are single chain polypeptides that share structural homology with proinsulin. The actions of IGF-1 and IGF-2 are mediated by binding to two structurally distinct plasma membrane receptors, referred to as the IGF-1 and IGF-2/mannose 6-phosphate (M6P) receptors. Most of the metabolic and mitogenic effects of IGF-1 and IGF-2 are thought to result from binding to the IGF-1 receptor, a receptor tyrosine kinase with structural homology to the insulin receptor. It is composed of two extracellular α subunits and two transmembrane β subunits linked by disulfide bonds (1, 2). Ligand binding to the α subunits activates the intrinsic β subunit receptor tyrosine kinase activity, leading to tyrosine phosphorylation of adapter proteins, such as insulin receptor substrate 1 (IRS-1) and Shc and Gab1 (3–6). Subsequent src homology 2 or protein tyrosine-binding domain-dependent recruitment of enzymes with phospholipase, phosphatase, and protein and lipid kinase activity transmits signals intracellularly, including activation of the mitogenic Ras cascade and initiation of phosphatidylinositol 3-kinase (PI3K)/Akt-dependent cell survival. Adding to the diversity, in some cell types IGF-1 stimulates matrix metalloprotease-dependent shedding of epidermal growth factor (EGF) family peptide ligands, such as heparin-binding EGF (7, 8). Heparin-binding EGF release transactivates ErbB receptors, conferring a mechanism for IGF-1 to produce Ras-dependent signaling in either an autocrine or paracrine manner. In contrast to the IGF-1 receptor, the IGF-2/M6P receptor is a 300-KDa single transmembrane glycoprotein that binds both the nonglycosylated IGF-2 polypeptide and glycosylated M6P-containing ligands (9, 10). It contains a large extracellular domain, a single membrane-spanning region, and a short cytoplasmic tail. The IGF-2/M6P receptor lacks intrinsic catalytic activity. Although it may mediate transmembrane signal transduction by IGF-2 under some circumstances, cell surface IGF-2/M6P receptors are thought to function primarily in the capture and lysosomal degradation or processing of extracellular peptide ligands, including IGF-2, transforming growth factor-β1, and proliferin (11–14).

* This work was supported by National Institutes of Health Grants DK58283 (to L. M. L.) and GM62887 (to L. M. O.) and the Research Service of the Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: IGF, insulin-like growth factor; DMS, dimethylsphingosine; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinases 1 and 2; GPCR, G protein-coupled receptor; G protein, guanine nucleotide-binding protein; GFP, green fluorescent protein; h, human; M6P, mannose 6-phosphate; PDGF, platelet-derived growth factor; PMA, phorbol myristate acetate; siRNA, small interfering RNA; SK, sphingosine kinase; SIP1, sphingosine 1-phosphate.
IGF-mediated Transactivation of S1P Receptors

It has been known for some time that a subset of the cellular signals transmitted by insulin and IGFs involve heterotrimeric G protein activation. In cultured myocytes, insulin-stimulated processing of the inositol phosphate glycan anchors of membrane proteins requires both regulated proteolysis and pertussis toxin-sensitive G protein-dependent activation of a phosphatidylinositol-specific isomerase of phospholipase C (15–18). In Rat1 fibroblasts, IGF-1-stimulated activation of the ERK1/2 mitogen-activated protein kinase cascade is largely pertussis toxin-sensitive and can be blocked by expressing a Gβγ subunit sequesterant peptide derived from the C terminus of G protein-coupled receptor kinase 2 (GRK2ct) (19). Similar pertussis toxin-sensitive ERK1/2 activation has been reported in undifferentiated 3T3-L1 cells (20), human intestinal smooth muscle cells (21), primary rat cerebellar granule neurons, and NG-108 neuronal cells (22). In HIRcB cells and 3T3-E1 adipocytes, treatment with pertussis toxin or microinjection of a GRK2ct-glutathione S-transferase fusion protein blocks the mitogenic effect of IGF-1 but not of insulin or EGF (23). Other selected actions of IGF-1 also involve pertussis toxin-sensitive G proteins. In Balb/c 3T3 cells, activation of the calcium-permeable cation channel CD20 by IGF-1 is blocked by pertussis toxin (24). Insulin stimulated activation of NADPH-dependent H2O2 generation in human adipocyte membranes in vitro is inhibited by guanosine 5′-O-(2-thiodiphosphate), pertussis toxin, and an antibody against the C terminus of Goαs (25). Additional components of classical seven-membrane-spanning G protein-coupled receptor (GPCR) signaling pathways have been implicated in IGF signaling. For example, β-arrestins, adapter proteins that play a key role in GPCR desensitization, endocytosis, and signaling (26, 27), reportedly interact with IGF-1 receptors and promote their internalization (28). β-Arrestins have been reported to play a role in IGF-1-stimulated ERK1/2 activation (23, 28), Akt activation (29), and ubiquitin-dependent IGF-1 receptor down-regulation (30). IGF-2 reportedly mediates pertussis toxin-sensitive activation of a calcium-permeable calcium channel in Balb/c 3T3 cells by binding to the IGF-2/M6P receptor (31).

Despite the evidence that heterotrimeric G proteins play significant roles in IGF signaling, the mechanism of G protein activation by such nonclassical G protein-coupled receptors as the IGF-1 and IGF-2 receptors remains poorly understood. Two principal mechanisms have been advanced to account for the phenomenon. The first proposal is that the receptors possess the ability to directly catalyze heterotrimeric G protein guanine nucleotide exchange (32). Consistent with this, several groups have reported that heterotrimeric G protein subunits co-precipitate with the IGF-1 and insulin receptors (22, 23, 25) or have demonstrated that small peptides derived from the insulin and IGF-2 receptors promote a mastoparan-like acceleration of GTP exchange on purified heterotrimeric G proteins in vitro (31, 33–35). The second model advanced to explain heterotrimeric G protein-dependent signaling by non-GPCRs is that the receptors generate some signal that activates an endogenous GPCR, which in turn catalyzes G protein activation. For example, the platelet-derived growth factor (PDGF) receptor has been shown to activate sphingosine kinase (SK) and cause recruitment of β-arrestins to G protein-coupled sphingosine 1-phosphate (S1P) receptors (36, 37). However the evidence that this might represent a general model of GPCR transactivation is presently limited. In this study, we have examined the mechanism whereby endogenous IGF receptors activate the ERK1/2 cascade in HEK293 cells, a system in which IGF-stimulated ERK1/2 activation is almost entirely heterotrimeric G protein-dependent. We found that IGF-1 and IGF-2 stimulated rapid SK activation, S1P production, and S1P receptor activation. Moreover, inhibiting SK activity either pharmacologically or using small interfering RNA (siRNA) against SK1 blocked IGF receptor-mediated S1P receptor and ERK1/2 activation, suggesting that SK-dependent transactivation of endogenous S1P receptors is sufficient to account for G protein-dependent responses to IGF in these cells. Given the structural diversity of the non-GPCRs that have been shown to activate heterotrimeric G proteins and the ubiquity of SK and S1P receptor expression, our data suggest that SK-dependent activation of S1P receptors may represent a general mechanism for G protein-dependent signaling by non-GPCRs.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium, fetal bovine serum, and penicillin/streptomycin were from Invitrogen. FuGENE 6 was from Roche Diagnostics. Double-stranded siRNAs were purchased from Xeragon (Germantown, MD). GeneSilencer™ transfection reagent was from Gene Therapy Systems (San Diego, CA). Primers for real-time PCR were from Integrated DNA Technologies (Coralville, IA). RNeasy kits were from Qiagen Corp. (Valencia, CA), and iScript cDNA synthesis kits and iQ™ SYBR® Green Supermix kits were from Bio-Rad Laboratories. Pertussis toxin was from List Biological Laboratories (Campbell, CA). IGF-1, IGF-2, and phorbol 12-myristate 13-acetate (PMA) were from Sigma. VPC23019 was from Avanti Polar Lipids (Alabaster, AL). d-erythro-Sphingosine 1-phosphate and dimethylsphingosine (DMS) were provided by the MUSC Lipidomics Core (Dept. of Biochemistry, Medical University of South Carolina, Charleston). Rabbit polyclonal anti-ERK1/2 and phosho-ERK1/2 IgG were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was from Amersham Biosciences. Rabbit polyclonal anti-human SK1 antiserum was prepared as described previously (38).

cDNA Constructs—The pEGFP-N1-myc-S1P1R plasmid encoding a myc epitope-tagged S1P1 receptor fused in-frame to the N terminus of green fluorescent protein (GFP) was a generous gift from Dr. Timothy Palmer (Inst. of Biomedical and Life Sciences, University of Glasgow, UK) (39). The pEGFP-N1-ERK2 plasmid encoding GFP-tagged ERK2 was generously provided by Dr. Nigel Bunnett (University of California, San Francisco) (40). The pcDNA3-hSK1 and pEGFP-C3-hSK1 plasmids encoding human SK1 (hSK1) and GFP-tagged hSK1, respectively, were prepared as described previously (38). The pRK5-Gαq (305–359) minigene plasmid encoding the C-terminal 55 amino acids of Gαq/11 was as described (41).

Cell Culture and Transfection—HEK293 cells were obtained from the American Type Culture Collection and maintained in minimum essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Prior to experimen-
tation, subclones were isolated and screened for pertussis toxin-sensitive ERK1/2 activation in response to IGF-1. Cells plated at low density in 15-cm dishes were grown for 7 days after which colonies representing the progeny of single cells were collected by sterile pipetting and seeded into individual wells of a 96-well culture plate for expansion. Subclones were tested for pertussis toxin-sensitive ERK1/2 activation as described below. Sensitivity ranged from 30 to 50% for individual subclones, and the most sensitive isolates were employed in all subsequent experiments. Transient transfection of 50–60% confluent cultures of HEK293 cells was performed in 10-cm dishes using a ratio of 3 μl of FuGENE 6/μg of plasmid DNA according to the manufacturer’s protocol. Empty pcDNA3.1 vector DNA was added to each transfection as needed to keep the mass of DNA constant. Transfected cells, split into multiple plates as appropriate, were serum-deprived overnight in growth medium supplemented with 0.5% fetal bovine serum and 10 mM HEPES, pH 7.4, prior to stimulation. All assays using transiently transfected cells were performed 48 h after transfection.

siRNA Down-regulation of Sphingosine Kinase 1 Expression—SK1 expression was down-regulated using 200 nM hSK1 sequence-specific siRNAs (5′-GAGCUAGCAAGCCCUU-GCCdTdTd-T3′ and 5′-GGCAAGCCCUUUGACGCUC-dTdTdT-3′). Scrambled siRNA sequences (5′-ACGUACAG-UUCGGAGAAAdTdT-T3′ and 5′-UUUCGGAACGAUGCUC-GUdTdTdT-3′) were used as negative controls. HEK293 cells were seeded in collagen coated 10-cm dishes at a density of 2 × 10^5 cells/dish 24 h before transfection. Cells were transfected using Gene Silencer® siRNA transfection reagent according to the manufacturer’s protocol. The efficiency of the knockdown was determined by quantitative real-time PCR for hSK1 mRNA and immunoblotting for hSK1 protein 48 h after transfection.

Quantitative Real-time PCR—Total cellular RNA was isolated using the RNeasy kit according to manufacturer’s instructions. cDNA was prepared from 1 μg of total RNA with A260/A280 > 1.8 using the iScript cDNA synthesis kit per the manufacturer’s instructions. Quantitative real-time PCR was performed with an iCycler 1Q Real-Time Detection System using the iQ™ SYBR® Green Supermix kit. Reactions were performed using hSK1-specific primers (forward primer 5′-CAGACATGACCACCCAG-3′; reverse primer 5′-ATCTTCAAGTCTATG-3′) and β-actin-specific primers (forward primer 5′-ATTGGCAATGAGCGTCC-3′; reverse primer 5′-GGTATGTTCCGTTGATGC-3′). Real-time PCR results were analyzed using Softmax® Pro software (Molecular Devices Corp.). hSK1 expression data were normalized to expression of β-actin as an endogenous control.

Protein Immunoblotting— Appropriately transfected HEK293 cells were split into poly-D-lysine-coated 12-well plates, serum-deprived overnight, and preincubated in the presence or absence of inhibitors as described in the figure legend (Figs. 1, 5, 6, and 8). Agonist stimulations were for 10 min after which monolayers were washed once in 4°C phosphate-buffered saline and lysed in 200 μl of Laemmli sample buffer. For the determination of ERK1/2 phosphorylation, samples containing 20 μg of cell protein were resolved by SDS-PAGE, and ERK1/2 and phospho-ERK1/2 were detected by protein immunoblotting using rabbit polyclonal anti-phospho-ERK1/2 IgG with horseradish peroxidase-conjugated polyclonal donkey anti-rabbit IgG as the secondary antibody. Immune complexes were visualized by enzyme-linked chemiluminescence and quantified using a Fluor-S Multimager. In each experiment, equal loading of ERK1/2 protein was confirmed by probing parallel immunoblots using anti-ERK1/2 antisera.

Assay of Sphingosine Kinase Activity—SK activity was determined as described previously with minor modifications (42). After stimulation, cells were collected by centrifugation for 5 min at 3000 × g and resuspended in ice-cold 0.1 M phosphate buffer containing 20 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.5 mM deoxyribonucleoside, 15 mM NaF, 1 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 0.4 mM phenylmethylsulfonyl fluoride, 20% glycerol, 0.1% Triton X-100, and protease inhibitor mixture. After sonication and assay of protein concentration, equal amounts of lysate protein were incubated for 30 min at 37°C with 50 μM sphingosine (delivered in 4 mg/ml fatty acid-free bovine serum albumin) and [γ-32P]ATP (5 μCi; 50 μM final concentration). The reaction was terminated by the addition of 20 μl of 1 N HCl and 800 μl of chloroform/methanol/HCl (100:200:1) and allowed to stand at room temperature for 10 min. Subsequently, 240 μl of chloroform and 240 μl of 2 M potassium chloride were added, and samples were centrifuged at 3000 × g for 5 min. The aqueous layer was aspirated, and 250 μl of the organic layer was transferred to new glass tubes. The samples were dried and resuspended in chloroform/methanol/HCl (100:200:1). Lipids were resolved on silica thin layer chromatography plates using 1-butanol/methanol/acetic acid/water (80:20:10:20) as the solvent system. Labeled S1P was visualized by autoradiography, and radioactivity corresponding to sphingosine phosphate bands was scraped and counted in a scintillation counter.

In Vivo Assay of Sphingosine 1-Phosphate Formation—Serum-deprived HEK293 cells in 2 ml of medium/60-mm dish were pulsed with 300 nM [3H]sphingosine (2.5 μCi) in the presence or absence of stimuli as described in the legend to Fig. 4. Cells were harvested by the addition of 2.5 volumes of chloroform-butanol/HCl (50:50:1), and the organic layer was dried under nitrogen. Lipids were separated using thin layer chromatography in chloroform/methanol/15% calcium chloride (60:35:8). Labeled S1P was visualized by autoradiography. Bands corresponding to S1P standards were scraped and incorporated radioactivity measured by scintillation counting.

Confocal Fluorescence Microscopy—For visualization of GFP-SK1 and myc-S1P1–GFP receptor, appropriately transfected HEK293 cells were plated in collagen-coated 35-mm glass-bottom dishes, serum-deprived overnight, preincubated with or without inhibitors, and stimulated as described in the figure legends (Figs. 2 and 7). After stimulation, cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 30 min at room temperature, and rewarshed with phosphate-buffered saline before examination. Confocal microscopy was performed using a Zeiss LSM 510 laser-scanning microscope with 488 nm excitation and 516–560 nm emission filter sets.

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**Protein Immunoblotting**—Appropriately transfected HEK293 cells were plated in collagen-coated 35-mm glass-bottom dishes, serum-deprived overnight, preincubated with or without inhibitors, and stimulated as described in the figure legends (Figs. 2 and 7). After stimulation, cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 30 min at room temperature, and rewarshed with phosphate-buffered saline before examination. Confocal microscopy was performed using a Zeiss LSM 510 laser-scanning microscope with 488 nm excitation and 516–560 nm emission filter sets.
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RESULTS

Insulin-like Growth Factors Activate ERK1/2 through a Heterotrimeric G Protein-dependent Pathway in HEK293 Cells—

Previous studies have demonstrated that IGF-1 activation of ERK1/2 can occur by diverse mechanisms, only some of which are heterotrimeric G protein-dependent (7, 8, 19). To obtain an optimal model for studying the role of G proteins in IGF signaling, we initially screened subclones of HEK293 cells for pertussis toxin-sensitive IGF-stimulated ERK1/2 activation. As shown in Fig. 1A, pretreatment with pertussis toxin inhibited IGF-1- and IGF-2-stimulated ERK1/2 phosphorylation by 47 and 66%, respectively, in the most sensitive clone examined. In contrast, EGF-stimulated ERK1/2 activation was not significantly affected. To test whether activation of pertussis toxin-insensitive G<sub>q/11</sub> family G proteins contributed to the residual IGF-induced ERK1/2 signal in these cells, we employed a minigene plasmid encoding a 55-amino acid polypeptide derived from the C terminus of G<sub>q</sub> (G<sub>q</sub>-(305–359)) that impairs signaling by blocking the receptor-G protein interface. We have previously shown that expressing G<sub>q</sub>-(305–359) selectively inhibits G<sub>q/11</sub>-coupled receptor signaling in vitro and in vivo (41). In these experiments we coexpressed GFP-tagged ERK2 (40) to could monitor GFP-ERK2 phosphorylation in the transfected cell population without interference from signal generated by endogenous IGF receptors in cells that failed to express the minigene plasmid. As shown in Fig. 1B, IGF-stimulated GFP-ERK2 phosphorylation in cells expressing G<sub>q</sub>-(305–359) was inhibited by 50–70% compared with vector-transfected controls. Both IGF-1- and IGF-2-stimulated GFP-ERK2 phosphorylation was almost abolished when these cells were also treated with pertussis toxin, suggesting that G protein activation accounted for virtually all of the IGF-stimulated ERK activation in these cells.

IGF-1 and IGF-2 Stimulation Leads to Membrane Translocation and Activation of Sphingosine Kinase—

The finding that pertussis toxin and G<sub>q</sub>-(305–359) additively inhibited IGF-stimulated ERK activation suggested that both G<sub>q</sub>/o and G<sub>q/11</sub> family G proteins were involved in the response. One mechanism that could account for this would be IGF-induced transactivation of an endogenous GPCR. We considered the endothelial differentiation gene (Edg) family S1P receptors as likely candidates. S1P receptors are ubiquitously expressed, couple to both G<sub>q</sub>/o and G<sub>q/11</sub>, and are known to be involved in diverse cellular responses including cell growth, survival, and migration (43, 44). Native HEK293 cells have been reported to express S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub> receptors (45) or only S1P<sub>2</sub> and S1P<sub>3</sub> receptors (46, 47). Northern blot analysis revealed that our cells expressed mRNA for the S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub> receptors (data not shown). The ligand for S1P receptors, S1P, is a bioactive sphingolipid metabolite formed through the action of the sphingosine kinases SK1 and SK2 (48). Previous work has demonstrated that external stimuli, such as phorbol ester-dependent protein kinase C activation, can promote membrane translocation and activation of SK1 (39, 49), suggesting that regulated production of S1P might provide a mechanism for transactivating S1P receptors.

To test whether IGF stimulation activates SK1, we initially employed confocal fluorescence microscopy to determine whether IGF treatment affected the cellular distribution of transiently expressed GFP-tagged human SK1. As shown in Fig. 2, GFP-hSK1 in non-stimulated HEK293 cells was diffusely distributed throughout the cell nucleus, cytosol, and plasma membrane. Consistent with published reports, treatment with PMA...
caused GFP-hSK1 to translocate to the plasma membrane within 10 min. Exposure to either IGF-1 or IGF-2 produced a similar redistribution of GFP fluorescence. We next tested whether IGF treatment activated SK by measuring SK1 activity in stimulated HEK293 cell lysates. As shown in Fig. 3A, a 10-min exposure to IGF-1 or IGF-2 led to a 3–4-fold increase in SK activity (60.1 ± 3.6 and 78.4 ± 3.8 pmol/min/mg protein, respectively, compared with basal activity of 19.1 ± 2.1 pmol/min/mg protein), a response that was similar in magnitude to that seen in PMA-treated cells (data not shown). As IGF-stimulated ERK1/2 activation is detectable within 2 min of stimulation and maximal within 5–10 min (19), we measured the time course of IGF-stimulated SK activation to determine whether it was rapid enough to play a role in the initial phase of ERK1/2 activation. As shown in Fig. 3B, maximal IGF-1 and IGF-2-stimulated SK activity was achieved within 30 s of stimulation and persisted for at least 30 min in the continued presence of ligand.

To test whether IGF stimulation produced a net increase in S1P abundance in vivo, we measured S1P levels in HEK293 cells after pulse labeling with D-[erythro-3H]sphingosine. As shown in Fig. 4, a 10-min exposure to IGF-1, IGF-2, or PMA significantly increased [3H]S1P levels in both whole cell lysates and the extracellular medium, although only 15–20% of the total amount of [3H]S1P recovered was detected in the extracellular medium under either basal or stimulated conditions.

**IGF-1- and IGF-2-stimulated ERK1/2 Activation Requires Sphingosine Kinase Activity**—To determine whether SK was involved in IGF-stimulated ERK1/2 activation, we first employed the SK inhibitor DMS. As shown in Fig. 5, preincubation with DMS abolished IGF-1- and IGF-2-stimulated ERK1/2 activation. The response to PMA was unaffected despite the fact that PMA stimulates SK activity. This SK-independent effect of PMA probably reflects direct activation of c-Raf1 by PMA-sensitive protein kinase C isoforms (50), which would be expected to bypass the requirement for SK activity. Similarly, ERK1/2 activation by exogenously applied S1P was insensitive to DMS, indicating that the DMS-sensitive step in the IGF signaling pathway was upstream of endogenous S1P receptors.

To further test the involvement of SK in the IGF response we employed RNA interference to down-regulate endogenous SK1 expression. As shown in Fig. 6, A and B, simultaneous transfection of two hSK1-specific siRNAs reduced endogenous hSK1 mRNA and protein abundance by 78 and 74%, respectively,
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**FIGURE 4.** IGF-1 and IGF-2 increase levels of both intracellular and extracellular sphingosine 1-phosphate. Serum-deprived HEK293 cells were pulse-labeled with 300 nM [erythrose-3H]sphingosine for 10 min in the presence of 10 nM IGF-1, 10 nM IGF-2, or 500 nM PMA. Lipids were extracted from the cells and media and resolved by thin layer chromatography. [3H]S1P bands were scraped, and incorporated radioactivity was quantified by scintillation counting. A representative autoradiograph of resolved [3H]S1P bands is shown above the graph depicting the mean ± S.D. of duplicate determinations in two different experiments. * (for cells) and † (for medium), greater than in non-stimulated (NS) cells; p < 0.05.

**FIGURE 5.** Activation of ERK1/2 by IGF-1 and IGF-2 is blocked by inhibiting sphingosine kinase activity. Serum-deprived HEK293 cells were pretreated with 20 μM DMS for 30 min prior to stimulation for 10 min with 10 nM IGF-1, 10 nM IGF-2, 100 nM PMA, or 5 nM S1P. Basal (NS, non-stimulated), IGF-1-, IGF-2-, PMA-, and S1P-stimulated ERK1/2 phosphorylation in whole cell lysate samples was determined as described. A representative phospho-ERK1/2 immunoblot is shown above a bar graph depicting the mean ± S.D. of three independent experiments. The change in ERK1/2 phosphorylation is expressed as the fold increase above the basal level in non-stimulated cells not exposed to DMS. *, less than in vehicle-treated cells; p < 0.05.

IGF-1 and IGF-2 Stimulate S1P Receptors and Produce S1P Receptor-dependent ERK1/2 Activation—Because our data indicated that IGF-stimulated ERK1/2 activation in HEK293 cells was heterotrimeric G protein-dependent and required SK1 activity, we tested the hypothesis that the S1P generated in response to IGF was transactivating endogenous S1P receptors. To determine whether IGF treatment generated sufficient S1P to activate the receptor, we transfected cells with a GFP-tagged S1P1 receptor (39) and employed it as a biological sensor for physiologically relevant quantities of S1P. A characteristic feature of most GPCRs is ligand-dependent internalization (51). As shown in Fig. 7, GFP-S1P1 receptors expressed in HEK293 cells undergo a striking rearrangement from a uniform distribution on the plasma membrane to a punctate distribution within endosomal vesicles upon exposure to S1P, consistent with activation-dependent receptor internalization. Treatment with either IGF-1 or IGF-2 produced a similar pattern, suggesting that IGF stimulation activates S1P receptors. To test whether this activation was SK-dependent, the experiments were repeated in the presence of DMS. As shown, pretreatment with DMS completely blocked IGF-1- and IGF-2-stimulated GFP-S1P1 receptor internalization, suggesting that SK activation is required for IGF-stimulated S1P receptor activation. Predictably, S1P-stimulated GFP-S1P1 receptor internalization was insensitive to DMS, because inhibiting SK would not be expected to affect receptor activation by exogenously supplied ligand.

As noted previously, HEK293 cells express mRNA for the S1P1, S1P2, and S1P3 receptors, which couple to both G_{i/o} and G_{q/11} family G proteins (44–48). Unfortunately, nonselective S1P receptor antagonists that potently inhibit all five S1P receptor subtypes are not currently available. We therefore tested whether VPC23019, a selective S1P1/S1P3 receptor antagonist (52), would inhibit S1P- and IGF-stimulated ERK1/2 activation. As shown in Fig. 8, preincubation with a maximally effective concentration of VPC23019 inhibited ERK1/2 activation by a half-maximal concentration of IGF-1 and IGF-2-stimulated ERK1/2 activation was observed in VPC23019-treated cells. In contrast, PMA-stimulated ERK1/2 activation was insensitive to VPC23019 (data not shown). Although these results do not definitively exclude an S1P receptor-independent role for S1P in IGF-stimulated ERK1/2 activation, the combined findings that IGF treatment leads to DMS-sensitive S1P1 receptor activation, that an S1P1/S1P3 receptor antagonist blocks IGF-stimulated ERK1/2 activation as effectively as it blocks the action of exogenous S1P, and that simultaneous inhibition of G_{i/o} and G_{q/11} signaling virtually eliminates the IGF response strongly suggest that transactivation of endogenous S1P receptors accounts for most, if not all, of the IGF-dependent ERK1/2 activation in HEK293 cells.

**DISCUSSION**

Although to date only seven membrane-spanning receptors have been shown to possess ligand-activated guanine nucleo-
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by these receptors remain poorly understood. Some evidence suggests that the heptahelial GPCR architecture is not required for a receptor to directly catalyze heterotrimeric G protein activation (32). For example, Gαo and Gβ subunits have been shown to co-precipitate with the IGF-1 receptor, and IGF-1 stimulation reportedly increases the amount of receptor-associated Gαo, while decreasing the amount of bound Gβ (22, 23). Similar co-precipitation of the insulin receptor with Gαo, Gαq, and Gβγ has been observed, along with insulin-dependent GTP-loading of Gαq in adipocyte membranes (25). Three purified peptides derived from sequences in the insulin receptor β subunit have been shown to accelerate GTP exchange on purified heterotrimeric G proteins in vitro (33), although this result has not been reproduced with the intact receptor. Two of these peptides contain sites of tyrosine autophosphorylation, and the one corresponding to residues 1325–1345 of the receptor activates Gαq only when tyrosine is phosphorylated. However, the role of IGF-1 receptor tyrosine kinase activity in initiating G protein-dependent signals in intact cells is uncertain, as some studies have reported responses that are insensitive to pharmacologic inhibition of the kinase (28, 34), whereas others suggest that it is required (21). Similar approaches suggest a direct role for the structurally distinct IGF-2/M6P receptor in G protein activation. IGF-2 treatment elicits IGF-2/M6P receptor-dependent GTP loading of Gαi2, but not Gαs, or Gαo, in Balb/c 3T3 cell membranes, and a putative G protein activator sequence was identified within residues 2410–2423 of the IGF-2/M6P receptor based on the ability of the peptide to selectively activate Gαi2 in vitro (31, 35).

An alternative to the model of direct G protein coupling by nonheptahelial receptors is the hypothesis that they activate G proteins indirectly by transactivating heptahelial GPCRs. Such a model is inherently attractive in that it does not require one to postulate the existence of conformationally regulated G protein activator sequences within the structure of many disparate types of membrane receptor. A conceptually similar form of signal convergence accounts for transactivation of EGF receptors. It is now clear that diverse stimuli originating from GPCRs, cytokine receptors, and even receptor-independent signaling molecules like reactive oxygen species share the ability to transactivate EGF receptors by catalyzing matrix metalloprotease-dependent release of EGF family peptide growth factors (53). EGF receptor transactivation accounts for the ability of many receptors that lack intrinsic tyrosine kinase activity to initiate Ras-dependent signals affecting cell proliferation, differentiation, apoptosis, or migration. Even other receptor tyrosine kinases, such as the IGF-1 receptor, share the ability to induce EGF receptor ligand shedding (7, 8). Given the number of non-GPCRs that have been reported to signal via pertussis toxin-sensitive G proteins, it is possible that S1P receptor transactivation represents an analogous form of signal convergence, in this case one that permits diverse stimuli to access heterotrimeric G protein signaling pathways. Indeed, our data are consistent with results obtained for the PDGF receptor, which has been shown to activate SK and cause recruitment of β-arrestins to S1P1 receptors, whereas inhibition of SK activity blocks PDGF-stimulated Rac activation and chemotaxis (36).

At present, little is known about how PDGF receptors, IGF

FIGURE 6. Down-regulation of sphingosine kinase 1 expression by RNA interference inhibits IGF-1- and IGF-2-stimulated ERK1/2 activation. A, HEK293 cells were transfected with control scrambled siRNA (SCR) or siRNA targeted to hSK1 (SK1 siRNA). RNA was isolated 48 h after transfection, and mRNA levels of SK1 and β-actin were determined by quantitative real-time PCR as described under "Experimental Procedures." B, the level of SK1 protein expression in HEK293 cells transfected with control scrambled siRNA or siRNA targeted to hSK1 was determined by immunoblotting whole cell lysates prepared 48 h after transfection. A representative SK1 immunoblot is shown above a bar graph depicting the mean ± S.D. for three independent experiments. C, serum-deprived HEK293 cells transfected with scrambled siRNA or siRNA targeted to hSK1 were stimulated for 10 min with 10 nM IGF-1 or 10 nM IGF-2. Basal (NS, non-stimulated), and IGF-1- and IGF-2-stimulated ERK1/2 phosphorylation in whole cell lysate samples was determined as described. A representative phospho-ERK1/2 immunoblot is shown above a bar graph depicting the mean ± S.D. for three independent experiments. The change in ERK1/2 phosphorylation is expressed as the fold increase above the basal level in non-stimulated control scrambled siRNA-transfected cells (*, less than in control scrambled siRNA-treated cells; p < 0.05).
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**myc-S1P<sub>1</sub>-GFP Receptor**

<table>
<thead>
<tr>
<th></th>
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**FIGURE 7. IGF-1 and IGF-2 stimulate sphingosine kinase inhibitor-sensitive internalization of S1P<sub>1</sub>-GFP receptors.** Serum-deprived HEK293 cells cotransfected with untagged pcDNA3-hSK1 and pEGFP-myc-S1P,R-GFP were pretreated with vehicle (upper panels) or 20 μM DMS (lower panels) for 30 min prior to stimulation with vehicle (NS, non-stimulated), 10 nM IGF-1, 10 nM IGF-2, or 5 nM S1P for 10 min. After stimulation, cells were fixed, and the distribution of S1P,R-GFP was determined by confocal fluorescence microscopy. Shown are representative confocal fields from one of three independent experiments that gave similar results.

**FIGURE 8.** The competitive S1P<sub>1</sub>/S1P<sub>3</sub> receptor antagonist VPC23019 attenuates IGF-1- and IGF-2-stimulated ERK1/2 activation. Serum-deprived HEK293 cells were pretreated with 10 μM VPC23019 (VPC) for 30 min prior to stimulation for 10 min with 10 nM IGF-1, 10 nM IGF-2, or 5 nM S1P. Basal (NS, non-stimulated), IGF-1, IGF-2, and S1P-stimulated ERK1/2 phosphorylation in whole cell lysate samples was determined as described under "Experimental Procedures." A representative phospho-ERK1/2 immunoblot is shown. The change in ERK1/2 phosphorylation is expressed as the -fold increase above the basal level in non-stimulated cells not exposed to VPC23019.* less than in vehicle-treated cells; p < 0.05.

In summary, our data suggest that in HEK293 cells, heterotrimeric G protein-dependent ERK1/2 activation in response to IGF-1 and IGF-2 can be explained on the basis of SK1-dependent transactivation of endogenous S1P receptors. Given the structural diversity of receptors that rely in part on heterotrimeric G subunits or β-arrestins and on endogenous IGF-1 receptor immunoprecipitates from HEK293 cells (data not shown), and thus we have been unable to test this hypothesis. However one recent report suggests that IGF-1 receptors do form heterologous complexes with GPCRs. In MDA-MB-231 tumor cells, IGF-1 induced cell migration reportedly requires CXCL12-independent transactivation of CXCR4 chemokine receptors (56). In these cells, IGF-1 receptor immunoprecipitates contain CXCR4, G<sub>α</sub> and G<sub>β</sub> subunits, and IGF-1 stimulation causes release of G<sub>α</sub> and G<sub>β</sub> from CXCR4.

In summary, our data suggest that in HEK293 cells, heterotrimeric G protein-dependent ERK1/2 activation in response to IGF-1 and IGF-2 can be explained on the basis of SK1-dependent transactivation of endogenous S1P receptors. Given the structural diversity of receptors that rely in part on heterotrimeric G protein activation to mediate their cellular effects, from receptor tyrosine kinases for PDGF, insulin, IGF-1, EGF, and fibroblast growth factor to single transmembrane integrins, T cell receptors, and the IGF-2/M6P receptor (32, 57, 58), it appears increasingly likely that G protein signaling by non-GPCRs reflects GPCR transactivation either because of enzymatic production of a GPCR ligand, as in the case of IGF-stimulated SK1 activation, or in the context of heterologous receptor complexes. With the development of more effective SK inhibitors and S1P receptor antagonists, it should be possible to determine the generality of S1P receptor transactivation as a mediator of non-GPCR signals and establish the physiologic events regulated by this novel form of receptor cross-talk.
Insulin-like Growth Factors Mediate Heterotrimeric G Protein-dependent ERK1/2 Activation by Transactivating Sphingosine 1-Phosphate Receptors
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doi: 10.1074/jbc.M605339200 originally published online August 22, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M605339200

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