The Insulin-like Growth Factor Type 1 and Insulin-like Growth Factor Type 2/Mannose-6-phosphate Receptors Independently Regulate ERK1/2 Activity in HEK293 Cells*

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Insulin-like growth factor types 1 and 2 (IGF-1; IGF-2) and insulin-like peptides are all members of the insulin superfamily of peptide hormones but bind to several distinct classes of membrane receptor. Like the insulin receptor, the IGF-1 receptor is a heterotetrameric receptor tyrosine kinase, whereas the IGF-2/mannose-6-phosphate receptor is a single transmembrane domain protein that is thought to function primarily as a clearance receptor. We recently reported that IGF-1 and IGF-2 stimulate the ERK1/2 cascade by triggering sphingosine kinase-dependent "transactivation" of G protein-coupled sphingosine-1-phosphate receptors. To determine which IGF receptors mediate this effect, we tested seven insulin family peptides, IGF-1, IGF-2, insulin, and insulin-like peptides 3, 4, 6, and 7, for the ability to activate ERK1/2 in HEK293 cells. Only IGF-1 and IGF-2 potently activated ERK1/2. Although IGF-2 was predictably less potent than IGF-1 in activating the IGF-1 receptor, they were equipotent stimulators of ERK1/2. Knockdown of IGF-1 receptor expression by RNA interference reduced the IGF-1 response to a greater extent than the IGF-2 response, suggesting that IGF-2 did not signal exclusively via the IGF-1 receptor. In contrast, IGF-2 receptor knockdown markedly reduced IGF-2-stimulated ERK1/2 phosphorylation, with no effect on the IGF-1 response. As observed previously, both the IGF-1 and the IGF-2 responses were sensitive to pertussis toxin and the sphingosine kinase inhibitor, dimethylsphingosine. These data indicate that endogenous IGF-1 and IGF-2 receptors can independently initiate ERK1/2 signaling and point to a potential physiologic role for IGF-2 receptors in the cellular response to IGF-2.

The insulin superfamily family of peptide hormones, consisting of insulin, insulin-like growth factor type 1 (IGF-1),2 insulin-like growth factor type 2 (IGF-2), and the heterodimeric peptide relaxin/insulin-like factors INSL3, INSL4, INSL5, INSL6, and INSL7, regulates diverse biological functions including cell growth, differentiation, and energy balance. Because of its critical role in metabolic homeostasis, insulin signaling has long been the subject of intense investigation. By comparison, the mechanisms of action of IGF-1, IGF-2, and the more recently characterized INSLs are less thoroughly understood.

Although all members of the insulin superfamily share structural homology, their signals are transmitted through binding to a number of structurally dissimilar receptors. IGF-1 and IGF-2 are single chain polypeptides, with sequences 62% identical to that of proinsulin. Both contain a short D domain that is not present in insulin, and unlike insulin, they do not undergo post-translational proteolytic cleavage. Both of the A and B domains remain linked in the mature peptide by a C domain analogous to the C peptide of insulin (1, 2). IGF-1 and IGF-2 bind two structurally distinct sites on the receptor, referred to as the IGF-1 and IGF-2/mannose-6-phosphate (M6P) receptors. The IGF-1 receptor is a heterotetrameric transmembrane receptor tyrosine kinase that is structurally and functionally related to the insulin receptor. It is composed of two extracellular α-subunits that contain the ligand-binding domain and two transmembrane β-subunits that possess intrinsic ligand-stimulated tyrosine kinase activity (3, 4). Ligand binding to the β-subunit activates the β-subunit tyrosine kinase, resulting in tyrosine phosphorylation of intracellular adapter proteins, such as insulin receptor substrate (IRS)-1 and IRS-2, Shc, and Gab1 (5–7). These adapters, in turn, provide docking sites for catalytically active proteins such as Grb2/mSos, which catalyzes Ras activation, and the p85 regulatory subunit of phosphatidylinositol 3-kinase, which activates Akt and promotes cell survival via suppression of the Bcl2-antagonist of cell death (BAD)/Bcl-X apoptotic pathway (8, 9). Both IGF-1 and IGF-2 bind the IGF-1 receptor, albeit with different affinity, and most of their biological activities have been ascribed to activation of the IGF-1 receptor.

Unlike the IGF-1 receptor, the IGF-2/M6P receptor is a type 1 single transmembrane glycoprotein composed of a large extracellular domain made up of 15 homologous cysteine-rich peptide; IRS, insulin receptor substrate; M6P, mannose-6-phosphate; PMA, phorbol myristate acetate; PTX, Bordetella pertussis toxin; RXFP, relaxin-family peptide; siRNA, small interfering RNA; SK, sphingosine kinase; S1P, sphingosine-1-phosphate; SCR, control scrambled siRNA.

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2 The abbreviations used are: IGF, insulin-like growth factor; EGF, epidermal growth factor; DMS, dimethylsphingosine; ERK1/2, extracellular signal-regulated kinases 1 and 2; GPCR, G protein-coupled receptor; INSL, insulin-like growth factor type 2 (IGF-2), and the heterodimeric peptide relaxin/insulin-like factors INSL3, INSL4, INSL5, INSL6, and INSL7, regulate diverse biological functions including cell growth, differentiation, and energy balance. Because of its critical role in metabolic homeostasis, insulin signaling has long been the subject of intense investigation. By comparison, the mechanisms of action of IGF-1, IGF-2, and the more recently characterized INSLs are less thoroughly understood.

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repeats, a single transmembrane region, and a short carboxy-
terminal cytoplasmic tail (10–12). It localizes primarily to the
trans-Golgi network and endosomal compartments, and to a
lesser extent, on the cell surface. Binding of IGF-2- and M6P-
containing ligands occurs at two distinct sites, with repeats 3
and 9 involved in binding proteins bearing M6P moieties and
repeat 11 containing the core IGF-2-binding sites (3, 13). Lack-
ing intrinsic catalytic activity, the IGF-2/M6P receptor is
thought to function primarily as a “clearance receptor,” with
known roles in the internalization, lysosomal trafficking, degra-
dation of IGF-2, prolierin, and glycosylated leukemia inhibi-
tory factor (10, 14, 15), and proteolytic activation of latent
transforming growth factor-β (16, 17). It is less clear whether
IGF-2/M6P receptors initiate transmembrane signals. Endo-
thelial cell migration and angiogenesis in response to the pla-
cental angiogenic hormone, prolierin, is reportedly IGF-2/
M6P receptor-dependent (18). Although some reports suggest
that it plays a role in IGF-2 signaling (19, 20), the prevailing
opinion is that the biological effects of IGF-2 are predominantly
mediated through the IGF-1 receptor or insulin receptor iso-
form A (21–23).

At least some of the recently characterized relaxin/insulin-
like factors bind yet another class of membrane receptor, seven
membrane-spanning G protein-coupled receptors (GPCRs).
INSLs are composed of two peptide chains connected by three
disulfide bonds and share a high degree of structural homology
with insulin (24–26). Based on this structural similarity, it
was initially thought that INSLs would signal by binding to
receptor tyrosine kinases, similar to the insulin and IGF
receptors. Recently, however, it has been shown that relaxin
activates a family of leucine-rich repeat-containing GPCRs,
the relaxin family peptide (RXFP) receptors, previously
known as LGRs, and mediates heterotrimeric G protein-de-
pendent activation of a number of effectors including aden-
yl cyclase (26–30). Although insulin and IGF-1/2 do not
appear to activate RXFP receptors to any significant extent,
these results clearly broaden the signaling repertoire of pep-
tides with homology to insulin.

We recently reported that in HEK293 cells, exposure to
IGF-1 or IGF-2 leads to activation of the ERK1/2 mitogen-acti-
vated protein (MAP) kinase cascade through a mechanism
involving sphingosine kinase (SK)-dependent “transactivation”
of G protein-coupled sphingosine-1-phosphate (S1P) receptors
(31). Here, we sought to definitively identify the endogenous
receptor(s) that mediate G protein-dependent ERK1/2 activa-
tion in response to IGF-1 and IGF-2 in HEK293 cells. Through
selective silencing of IGF-1 and IGF-2/M6P receptor expres-
sion, we find that although activation of the IGF-1 receptor is
sufficient to account for the response to IGF-1, activation of
ERK1/2 by IGF-2 occurs in large part through the IGF-2/M6P,
independent of IGF-1 receptor expression. These data suggest
that both IGF-1 and IGF-2/M6P receptors play a physiologic
role in the cellular response to IGFs and suggest that SK-de-
pendent S1P receptor transactivation might serve as a general
mechanism of heterotrimeric G protein-dependent signaling
by multiple classes of non-GPCR.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium, fetal bovine serum, and
penicillin/streptomycin were from Invitrogen. Insulin-like pep-
tides INSL3, INSL4, INSL6, and INSL7 were from Phoenix
Pharmaceuticals (Belmont, CA). IGF-1, IGF-2, and phorbol
12-myristate 13-acetate (PMA) were from Sigma. Double-
stranded small interfering (si)RNAs were purchased from
Ambion (Austin, TX). GeneSilencer™ transfection reagent
was from Gene Therapy Systems (San Diego, CA). Primers for
real-time PCR were from Integrated DNA Technologies (Cor-
alville, IA). RNAesy kits were from Qiagen Corp. (Valencia, CA),
and iScript cDNA synthesis kits and iQ™ SYBR® Green Super-
mix kits were from Bio-Rad Laboratories. Pertussis toxin was
from List Biological Laboratories (Campbell, CA). S1P and di-
methylphosphoglycerone (DMS) were provided by the Medical Un-
iversity of South Carolina (MUSC) Lipidomics Core (Depart-
ment of Biochemistry, MUSC, Charleston, SC). Goat
polycyclonal anti-IGF-1 receptor (Ab-2) and anti-IGF-1 receptor
phospho-Tyr1158 were from Calbiochem. Mouse monoclonal
anti-human CD222 M6P/IGF2R was from Novus Biologicals
(Littleton, CO). Rabbit polyclonal anti-ERK1/2 and phospho-
ERK1/2 IgG were from Cell Signaling Technology (Beverly,
MA). Horseradish peroxidase-conjugated donkey anti-rabbit
IgG was from Amersham Biosciences.

Cell Culture—HEK293 cells were obtained from the Ameri-
can Type Culture Collection and maintained in minimum
essential medium supplemented with 10% fetal bovine serum
and penicillin/streptomycin.

siRNA Down-regulation of IGF-1 and IGF-2 Receptor
Expression—IGF-1 receptor expression was down-regulated
using human IGF-1 receptor sequence-specific validated
siRNA (vendor ID number 74). Down-regulation of IGF-2
receptor was performed by targeting two different sites located
657 and 2050 nucleotides downstream of the start codon using
sequence-specific siRNAs (5′-GGAGGUCAUGCUAGAU-
GUUtt-3′ and 5′-CACAUGUGCAUGCCACUCUtt-3′) for the
first site and sequence-specific siRNAs (5′-GGGUUUUCG-
UUUGACUUUt-3′ and 5′-UAAGUCAAAAAACCCGt-3′)
for the second site. Scrambled siRNA sequences (5′-
AGGUCACUGUCGAGAAdTdT-3′ and 5′-UCUCCGGAACUGUCAcGUdTdT-3′) were used
as negative controls. HEK293 cells were seeded in collagen-coated
10-cm dishes at a density of 2 × 10⁵ cells/dish 24 h before
transfection. Cells were transfected using Gene Silencer®
siRNA transfection reagent according to the manufacturer’s
protocol. 24 h after transfection, cells were split into multwell
plates, as appropriate, and serum-deprived overnight in growth
medium supplemented with 0.5% bovine serum albumin and 10
mm HEPES, pH 7.4, prior to stimulation. The efficiency of
the knockdown was determined by quantitative real-time PCR for
receptor mRNA and immunoblotting for IGF-1 and IGF-2
receptor protein 48 h after transfection.

Quantitative Real-time PCR—Total cellular RNA was iso-
lated using the RNasy kit according to the 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 specific primers. For IGF-1 receptor, the forward primer was 5’-GCACCATCCTCAAGGGCAATTG-3’, and the reverse primer 5’-AGGAAGGCAAGGAACCAAAG-3’. For IGF-2R receptor, the forward primer 5’-GAGGCGAGGCAAAG-3’, and the reverse primer was 5’-TGTGCCAGCATCTCAG-3’. For β-actin, the forward primer was 5’-ATTGGCAATGACGGTTCC-3’, and the reverse primer was 5’-GGTAGTTTCGTCGATGCCACA-3’. Real-time PCR results were analyzed using Softmax® Pro software (Molecular Devices Corp.). IGF-1 and IGF-2 receptor mRNA abundance was normalized to β-actin as an endogenous control.

Protein Immunoblotting— Appropriately transfected HEK293 cells were split to collagen-coated 12-well plates, serum-deprived overnight, and preincubated in the presence or absence of inhibitors as described in the figure legends. 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. Samples containing 20 μg of cell protein were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane for immunoblotting. Proteins were detected with polyclonal IgG against IGF-1 receptor, phospho-ERK1/2, IGF-1 receptor, or IGF-1 receptor pY1158 as appropriate, with horseradish peroxidase-conjugated polyclonal donkey anti-rabbit IgG as secondary antibody. For immunoblotting, the IGF-2/M6P receptor, mouse monoclonal anti-human M6P/IGF2R IgG, was used, with horseradish peroxidase-conjugated anti-mouse IgG as secondary antibody. Total ERK1/2 was measured to confirm equal loading of the whole cell lysate samples. Immune complexes were visualized by enzyme-linked chemiluminescence and quantified using a Fluor-S Multimeter.

RESULTS

IGF-1 and IGF-2, but Not Insulin or INSLs, Potentially Activate ERK1/2 in HEK293 Cells—Insulin superfamily peptides interact with several classes of membrane receptor, and in some cases, a single peptide can bind to more than one receptor type. This is particularly the case for insulin, IGF-1 and IGF-2, each of which can bind to the IGF-1, IGF-2, and insulin receptors (1, 2, 32, 33). Therefore, we initially tested a panel of insulin-like peptides for the ability to activate ERK1/2 in HEK293 cells. As shown in Fig. 1, IGF-1 and IGF-2 both potently activated endogenous ERK1/2 in HEK293 cells. As shown in Fig. 1, IGF-1 and IGF-2 both potently activated endogenous ERK1/2, although IGF-1 was somewhat more efficacious at maximal concentrations. Insulin elicited a response only at high concentrations, consistent with effects mediated by low affinity binding to IGF-1 receptors (34–36). The INSLs tested, INSL3, INSL4, INSL6, and INSL7, were inactive even at high concentrations, indicating that the G protein-coupled RXFP receptors are either not coupled to ERK1/2 activation in this cell line.

Since both IGF-1 and IGF-2 can activate the IGF-1 receptor tyrosine kinase, we next compared the EC₅₀ for IGF-1- and IGF-2-stimulated IGF-1 receptor activation with the EC₅₀ of each for ERK1/2 activation. As shown in Fig. 2, IGF-2 was as efficacious but 2.3-fold less potent than IGF-1 at stimulating tyrosine autophosphorylation of the IGF-1 receptor β-subunit. This is consistent with the reported lower affinity of IGF-2 binding to the IGF-1 receptor (32, 33). In contrast, IGF-1 and IGF-2 were equipotent activators of ERK1/2, with EC₅₀ values of 11.0 and 11.8 nm, respectively. This apparent discrepancy prompted us to consider the possibility that IGF-2 might mediate ERK1/2 activation through another receptor, particularly when present at concentrations that produced submaximal IGF-1 receptor activation.

Down-regulating IGF-1 and IGF-2/M6P Receptors Has Differential Effects on IGF-1- and IGF-2-stimulated ERK1/2 Activation—To test the hypothesis that both IGF-1 and IGF-2/M6P receptors played a role in IGF-mediated ERK1/2 activation, we employed RNA interference to down-regulate endogenous receptor expression singly and in combination and determined the effect on the ability of IGF-1 and IGF-2 to activate ERK1/2. These assays employed concentrations of IGF-1 and IGF-2 (10 nm) that approximated the EC₅₀ for ERK1/2 activation, at which concentration IGF-2 was less effective than IGF-1 at activating the IGF-1 receptor.

Fig. 3, A and B, shows the effects of optimal IGF-1 receptor down-regulation (75 nm siRNA; 48 h after transfection). When compared with cells transfected with scrambled control siRNA, IGF-1 receptor mRNA levels measured by quantitative reverse transcription-PCR were reduced by 84%, which corresponded with an 87% reduction in receptor protein measured by immunoblot. As shown in Fig. 3C, down-regulation of IGF-1 receptors reduced IGF-1- and IGF-2-stimulated IGF-1 receptor β-subunit phosphorylation by about 90%, proportional to the reduction in receptor level. As shown in Fig. 3D, IGF-1 receptor down-regulation significantly reduced ERK1/2 activation by both IGF-1 and IGF-2. As a percentage of the agonist-induced increase in ERK1/2 phosphorylation, the IGF-1 response was reduced by 77%, whereas the IGF-2 response decreased by 64%.

FIGURE 1. Effect of insulin superfamily peptides on ERK1/2 activity in HEK293 cells. Serum-starved HEK293 cells were stimulated for 10 min with the indicated concentrations of insulin, IGF-1, IGF-2, INSL3, INSL4, INSL6, and INSL7, and whole cell phospho-ERK1/2 was determined by immunoblotting. Phospho-ERK1/2 is expressed as fold-change after normalization to basal levels. Results shown represent mean ± S.D. of six separate experiments. At ligand concentrations ≥100 nm, IGF-1 increased ERK1/2 phosphorylation 22.4 ± 1.6-fold, whereas IGF-2 increased ERK1/2 phosphorylation 15.2 ± 0.9-fold. The IGF-1 response was more than the IGF-2 response; p < 0.01.

FIGURE 2. IGF-2-stimulated IGF-1 receptor activation with the EC₅₀ for ERK1/2 activation. As shown in Fig. 2, IGF-2 was as efficacious but 2.3-fold less potent than IGF-1 at stimulating tyrosine autophosphorylation of the IGF-1 receptor β-subunit. This is consistent with the reported lower affinity of IGF-2 binding to the IGF-1 receptor (32, 33). In contrast, IGF-1 and IGF-2 were equipotent activators of ERK1/2, with EC₅₀ values of 11.0 and 11.8 nm, respectively. This apparent discrepancy prompted us to consider the possibility that IGF-2 might mediate ERK1/2 activation through another receptor, particularly when present at concentrations that produced submaximal IGF-1 receptor activation.
Although these data clearly indicate that both IGF-1 and IGF-2 can engage the ERK1/2 cascade through the IGF-1 receptor, the finding that the IGF-2 response was less dependent upon IGF-1 receptor expression led us to test whether the IGF-2/M6P receptor also played a role in the response. Fig. 4 shows the effects of an identical set of experiments performed under conditions of IGF-2/M6P receptor down-regulation. As shown in Fig. 4, A and B, optimal IGF-2/M6P receptor down-regulation (100 nM siRNA; 48 h) reduced IGF-2/M6P receptor mRNA and protein levels by 74 and 83%, respectively. Under these conditions, there was no change in the level of IGF-1 receptor expression or, as shown in Fig. 3, IGF-stimulated phosphorylation of the IGF-1 receptor β-subunit. IGF-1-stimulated ERK1/2 activation was unaffected by IGF-2/M6P receptor down-regulation. In striking contrast, IGF-2-stimulated ERK1/2 activation was reduced by 74%. Since the ability of IGF-2 to activate the IGF-1 receptor was unchanged in IGF-2/M6P receptor knockdown cells (Fig. 3), the loss of signal probably represents loss of a direct IGF-2/M6P receptor-mediated signal and not decreased delivery of IGF-2 to the IGF-1 receptor.

Fig. 5 shows the effects of simultaneous down-regulation of both the IGF-1 and the IGF-2/M6P receptors. As shown in Fig. 5, A and B, simultaneous transfection of IGF-1 and IGF-2/M6P receptor siRNAs down-regulated both receptors to a degree...
comparable with that achieved in the individual siRNA transfections. When compared with cells treated with scrambled control siRNA, IGF-1 and IGF-2/M6P mRNA levels were reduced by 88 and 84%, respectively, with a 90% reduction in the expression of each receptor. Fig. 5C confirms that a comparable reduction in IGF-1- and IGF-2-stimulated IGF-1 receptor β-subunit phosphorylation was achieved. As shown in Fig. 5D, when both the IGF-1 and the IGF-2/M6P receptors were down-regulated simultaneously, the reduction in IGF-1-stimulated ERK1/2 activation was 86%, which was not significantly different from the 77% reduction achieved by IGF-1 receptor knockdown alone. In contrast, IGF-2-stimulated ERK1/2 activation was abolished when both receptors were down-regulated.

The effects of down-regulating IGF-1 and IGF-2/M6P receptor expression on IGF-1- and IGF-2-stimulated ERK1/2 activation are summarized in Fig. 6. As shown in Fig. 6A, only IGF-1 receptor down-regulation affected IGF-1-stimulated ERK1/2 activation, and the degree of inhibition correlated closely with the extent of IGF-1 receptor knockdown. This result supports the conclusion that in HEK293 cells, the effects of IGF-1 are mediated almost exclusively through binding to the IGF-1 receptor tyrosine kinase. In contrast, IGF-2-stimulated ERK1/2 activation was partially inhibited when either the IGF-1 or the IGF-2/M6P receptor was down-regulated independently and eliminated only when the two receptors were down-regulated together. These data support the conclusion that IGF-2 signals in part by binding to the IGF-1 receptor but also by binding to the IGF-2/M6P receptor.
IGF-1 and IGF-2/M6P Receptors Employ a Common Sphingosine Kinase-dependent Mechanism to Regulate ERK1/2 Activity—We recently demonstrated that IGFs can activate ERK1/2 through a novel mechanism involving SK-dependent transactivation of heterotrimeric G protein-coupled S1P receptors (31). To determine whether signals transmitted via the IGF-1 and IGF-2/M6P receptors employed the same or different mechanisms, we exposed HEK293 cells to EC50 concentrations of IGF-1 or IGF-2 in the presence or absence of either DMS, an inhibitor of SK activity, or pertussis toxin (PTX), which inactivates Gi/o family heterotrimeric G proteins. As shown in Fig. 4C, clear demonstrates that down-regulating IGF-2/M6P receptors has no effect on the ability of IGF-2 to activate the IGF-1 receptor.

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Moreover, the response mediated by the IGF-2/M6P receptor is independent of IGF-1 receptor since Fig. 4C clearly demonstrates that down-regulating IGF-2/M6P receptors has no effect on the ability of IGF-2 to activate the IGF-1 receptor.
FIGURE 7. IGF-1 and IGF-2 utilize a common sphingosine kinase-dependent and pertussis toxin-sensitive mechanism to activate ERK1/2. A, 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, 5 μM S1P, or 100 nM PMA. Basal (NS), IGF-1-, IGF-2-, S1P-, and PMA-stimulated ERK1/2 phosphorylation was determined as described. p-ERK1/2, phosphorylated ERK1/2. B, cells were serum-starved overnight in the presence or absence of 500 ng/ml PTX prior to stimulation for 10 min with 10 nM IGF-1, 10 nM IGF-2, 5 μM S1P, or 100 nM PMA. Basal, IGF-1-, IGF-2-, S1P-, and PMA-stimulated ERK1/2 phosphorylation was determined as described. In both panels, a representative phospho-ERK1/2 immunoblot is shown above a bar graph depicting mean ± S.D. for three independent experiments. The change in ERK1/2 phosphorylation is expressed as the -fold increase above the basal level in unstimulated cells not exposed to PTX. *p < 0.05.

with different potency, and that IGF-1 receptors contribute to ERK1/2 activation in response to both ligands. Surprisingly, we found that down-regulating the IGF-2/M6P receptor selectively inhibited the response to IGF-2 but not IGF-1. Loss of IGF-2/M6P receptors had no effect on the ability of IGF-2 to activate the IGF-1 receptor, indicating that the IGF-2/M6P receptor was not acting simply to deliver IGF-2 to the cell surface. Furthermore, the effects of IGF-1 and IGF-2/M6P receptor down-regulation on IGF-2-stimulated ERK1/2 activation were approximately additive, strongly suggesting that these two structurally distinct receptors independently contribute to IGF-2 signaling in HEK293 cells.

The most accepted function of the IGF-2/M6P receptor is in the cellular uptake and degradation of peptides such as IGF-2, proliferin, and leukemia inhibitory factor (10, 14, 15). It also plays a role in the uptake and proteolytic activation of latent transforming growth factor-β (16, 17). Nonetheless, some data support a role for the IGF-2/M6P receptor in IGF-2 signaling (19, 20). In rhabdomyosarcoma cells, IGF-2 stimulates cell migration, apparently by binding the IGF-2/M6P receptor (40). Certain in vitro effects of IGF-2, such as stimulating Ca2+ influx and DNA synthesis in 3T3 fibroblasts (41, 42), have been attributed to activation of heterotrimeric G proteins by the IGF-2/M6P receptor (43, 44). More recently, Leu271IGF-2, which binds preferentially to the IGF-2/M6P receptor, has been shown to stimulate pertussis toxin-sensitive, protein kinase C-dependent, protein phosphorylation and modulate central cholinergic receptor function (45). However, previous attempts to ascribe specific signaling functions to the IGF-2/M6P receptor have been hampered by the fact that IGF-2 also binds and activates the IGF-1 receptor tyrosine kinase. Indeed, several authors have suggested that the biological effects of IGF-2 are mediated through the IGF-1 receptor or insulin receptor isoform A (21–23). In our study, we find that down-regulation of IGF-2/M6P receptors leads to a marked reduction in IGF-2-stimulated, but not IGF-1-stimulated, ERK1/2 activation, consistent with signaling directly via the IGF-2/M6P receptor.

Our results also indicate that IGF-1, acting via the IGF-1 receptor, and IGF-2, acting via both the IGF-1 and the IGF-2/M6P receptors, employ a common heterotrimeric G protein-dependent mechanism to activate ERK1/2. We have previously shown that both IGF-1 and IGF-2 promote membrane translocation and activation of SK1, increase intracellular and extracellular S1P levels, and activate G protein-coupled S1P receptors in HEK293 cells (31). What remains unclear is how these two structurally distinct receptors activate SK. In vitro studies have shown that SK1 has high affinity for lipid bilayers rich in phosphatidylinositol and that conserved Thr54 and Asn89 residues in the putative membrane-binding surface of the protein are necessary for membrane binding in vitro and in cells (46).

Furthermore, protein kinase C-dependent phosphorylation of Ser225 increases the membrane affinity of hSK1. If a phosphorylation-dependent mechanism accounts for IGF-2/M6P receptor-mediated SK1 activation, the identity of the kinase and its mechanism of action remain to be determined.

Another finding of our study is that maximum IGF-2-stimulated ERK1/2 activation is about 30% lower than the maximum IGF-1 response, despite the observation that at high concentrations, IGF-1 and IGF-2 promote equivalent phosphorylation of IGF-1 receptor β-subunit Tyr1158. This difference in efficacy may reflect differences in the pattern of receptor or adapter protein phosphorylation generated by two ligands binding to the same receptor. Analogous results have been reported for the epidermal growth factor (EGF) receptor, ErbB1, where binding of EGF and betacellulin generate similar levels of ErbB1 Y1086 phosphorylation, but betacellulin produces greater Akt activation and a more robust anti-apoptotic response than EGF (47, 48). Alternatively, the reduced IGF-2...
response could result from downstream effects on the ERK1/2 pathway due to concomitant IGF-2 receptor activation by IGF-2 but not IGF-1. Given the complexity of the system and the limitations of our current understanding of IGF-2 receptor signaling, the basis of these differences will have to be addressed in future studies.

In purified reconstituted systems, only seven membrane-spanning GPCRs have been shown to possess intrinsic ligand-activated guanine nucleotide exchange factor activity for heterotrimeric G proteins. Nonetheless, it is clear that heterotrimeric G proteins mediate a subset of signals initiated by diverse non-heptahelical receptors, including the insulin, IGF-1, EGF, and platelet-derived growth factor receptor tyrosine kinases and single membrane-spanning receptors for C-type natriuretic peptide and zona pellucida glycoprotein, integrins, and T cell receptors (49). Apart from our results with the IGF-1 and IGF-2/M6P receptors (31), heterotrimeric G protein activation resulting from SK1-dependent transactivation of S1P receptors has been shown to account for pertussis toxin-sensitive signaling by platelet-derived growth factor receptor (50, 51). Based on this small but growing body of evidence, it is tempting to speculate that S1P receptor transactivation represents a common mechanism of signal convergence that permits structurally diverse non-GPCRs to access heterotrimeric G protein signaling pathways.

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