Activation of the phosphatidylinositol-3' kinase pathway and DNA synthesis by a mutant insulin-like growth factor I receptor lacking the NPXY motif

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Abstract

We have investigated the role of the NPXY motif in the insulin-like growth factor I receptor (IGF-IR) by focusing on the activation of the phosphatidylinositol-3' kinase (PI3-K) pathway and DNA synthesis following IGF-I stimulation. For this purpose, we established stable R – cell lines, which are deficient in endogenous IGF-IR, and express human IGF-IR lacking the whole NPEY⁹⁵⁰ sequence (Δ NPEY). The Δ NPEY cells showed an apparent autophosphorylation of IGF-IR, albeit with reduced sensitivity to stimulation compared with cells expressing similar levels of wild-type IGF-IR. Activation of insulin receptor substrate (IRS)-1 and IRS-2 was severely impaired in Δ NPEY cells even at high concentrations of IGF-I. However, recruitment of p85, a regulatory subunit of PI3-K, to activated IRS-2 was similar between the cell

lines, but recruitment of p85 to IRS-1 was reduced in Δ NPEY cells. Essentially similar levels of p85- or phosphotyrosine-associated PI3-K and Akt activities were observed between the cell lines, although the sensitivity to stimulation was reduced in Δ NPEY cells. Activation of extracellular signal-regulated kinase and DNA synthesis were virtually unaffected by the mutation, in terms of both sensitivity to stimulation and responsiveness. DNA synthesis was completely inhibited by the PI3-K inhibitor, LY294002. These results indicate that the IGF-IR is able to activate the PI3-K pathway and induce DNA synthesis in a normal fashion without the NPXY motif when the receptor is fully activated.

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Introduction

Insulin-like growth factor I receptor (IGF-IR) is a trans-membrane tyrosine kinase which is highly homologous to insulin receptor (IR) (Ullrich *et al.* 1986) and plays pivotal roles in a variety of phenotypes including development, cell growth, transformation, protection from apoptosis and differentiation (Liu *et al.* 1993, Beck *et al.* 1995, Baserga 1997, O'Connor *et al.* 1997). The NPEY sequence in the juxtamembrane region of IGF-IR and IR is a binding site for insulin receptor substrate (IRS)-1 and Shc which, in turn, leads to a strong activation of phosphatidylinositol-3' kinase (PI3-K) and mitogen-activated protein kinase (MAPK) respectively (Kaburagi *et al.* 1993, 1995, Peruzzi *et al.* 1999). These two major downstream pathways are responsible for the above described functions in IGF-IR (Peruzzi *et al.* 1999). The sequence is generally called the NPXY motif and also exists in other growth factor receptors such as IR-related protein, interleukin-4 (IL-4) receptor, and nerve growth factor (NGF) receptor (Kaburagi *et al.* 1995). In addition to these major pathways, IRS-2 also binds the NPXY motif, leading to the

activation of the PI3-K pathway (Van Obberghen *et al.* 2001). Furthermore, 14–3–3 proteins bind the SSSS¹²⁸³ sequence in the C-terminus of IGF-IR, which is missing in IR, and stimulate the MAPK pathway via c-Raf (Peruzzi *et al.* 1999, 2001).

Many studies have reported the effects of mutations of the NPXY motif in the IR on the activation of IRS-1 and Shc, or other biological responses. Mutation of tyrosine residue 960 in the NPEY⁹⁶⁰, deletion of the whole motif, or even deletions of larger portions of amino acids in proximity to the motif in the juxtamembrane region severely impairs activation of IRS-1, Shc or DNA synthesis, but not autophosphorylation of IR (Murakami & Rosen 1991, Kaburagi *et al.* 1993, 1995, Chaika *et al.* 1997, Goalstone *et al.* 2001). On the other hand, similar studies on the IGF-IR are limited and have been controversial; some reports showed that mutation at tyrosine residue 950 in the NPEY⁹⁵⁰ impaired activation of autophosphorylation, IRS-1 or DNA synthesis (Craparo *et al.* 1995, Miura *et al.* 1995a), while other reports found that neither IRS-1 activation nor cell proliferation were affected by the mutation (Romano *et al.* 1999, Navarro &

Baserga 2001). The only report, to our knowledge, concerning the effects of deleting the whole NPEY sequence of the IGF-IR, showed deficient activation of autophosphorylation, IRS-1 and DNA synthesis using CHO cell lines (Hsu *et al.* 1994).

In this study, we attempted to clarify the role of the NPXY motif in the IGF-IR by focusing on the activation of the PI3-K pathway and DNA synthesis upon insulin-like growth factor-I (IGF-I) stimulation, which thus far has been controversial. For this purpose, we established stable R- cell lines, which are deficient in endogenous IGF-IR, and express human IGF-IR lacking the whole NPEY sequence (Δ NPEY). Activities were compared with those in cells expressing similar levels of wild-type IGF-IR after stimulation with varying concentrations of IGF-I.

Materials and Methods

Materials

Antibodies against IGF-IR β -subunits, mouse IgG conjugated with horseradish peroxidase (HRP), rabbit IgG-HRP and Protein A/G PLUS-Agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine antibody (PY20) was purchased from Transduction Laboratory (Lexington, KY, USA). Anti-PKB/AKT pSer(473) antibody was purchased from Biosource (Camarillo, CA, USA). Anti-ACTIVE MAPK (ERK2 pThr(183)pTyr(185)) antibody and pfu polymerase were purchased from Promega (Madison, WI, USA). Anti p85 antibody was purchased from Upstate (Lake Placid, NY, USA). The ECL Western blotting analysis system and [γ -³²P]ATP were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). Ready Cap was purchased from Beckmann (Fullerton, CA, USA). Recombinant human IGF-I and pCR-Blunt vector were purchased from Invitrogen (Carlsbad, CA, USA). LY294002 and PD98059 were purchased from Sigma Aldrich (St Louis, MO, USA). Phosphatidylinositol was purchased from Avanti (Alabaster, AL, USA).

Plasmid construction

The Δ NPEY mutant was derived from the human IGF-IR cDNA (Ullrich *et al.* 1986). For deletion of the corresponding nucleotides, PCR-assisted *in vitro* mutagenesis was utilized. The first PCR was performed using the primers 5'-GTACCGGATCGATATCCACA GCTGC and 5'-CATCAGCAGCGCTGAACACAGA GGCATAC. The latter primer was designed to lack 12 nucleotides corresponding to the NPEY⁹⁵⁰ in the IGF-IR cDNA. The second PCR was performed using the first PCR product as a 5'-primer and 5'-AAATCTTCGGCT ATGCAATTCC as a 3'-primer. pfu polymerase was used

for both PCRs. The second PCR product was ligated to pCR-Blunt vector and the correct deletion was confirmed by a dideoxy sequencing method. The SmaI-HindIII fragment of the plasmid, including the deleted region, was used to replace the same region of the IGF-IR in pBluescript (Miura *et al.* 1995a). The XhoI-NotI fragment of pBPV IGF-IR (Miura *et al.* 1995a) was replaced by the same region of the pBluescript IGF-IR, including the deletion. The final expression plasmid was designated pBPV Δ NPEY.

Cell lines and culture conditions

R- cells are fibroblast cell lines derived from mouse embryos, with a targeted disruption of the IGF-IR genes (Sell *et al.* 1994). R- cells were co-transfected with pBPV IGF-IR or pBPV Δ NPEY and pPDV6+ encoding the puromycin resistance gene (Miura *et al.* 1995b), by a standard calcium-phosphate precipitation method. Cells were selected in 4 μ g/ml puromycin and the resultant clones expressing similar levels of IGF-IR were used in this study. All cell lines were maintained in growth medium as described previously (Tezuka *et al.* 2001). For serum-starvation, growth medium was replaced by serum-free medium containing 1 mg/ml bovine serum albumin and incubated overnight.

Western blotting

Western blotting was performed using the ECL system as described previously (Tezuka *et al.* 2001). Briefly, serum-starved cells were stimulated or unstimulated with IGF-I and digested in a lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 0.1% SDS, 1 mM EDTA, 100 mM NaF, 1 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μ g/ml aprotinin). Equal amounts of cell lysates were separated in SDS-polyacrylamide gel and proteins were transferred to a nitrocellulose membrane. Filters were probed with primary antibodies against target proteins and then with secondary antibodies conjugated with HRP. Proteins were visualized with the ECL system. For immunoprecipitation, cells stimulated or unstimulated with IGF-I were digested in a lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 100 μ M NaVO₄, 1 mM NaF, 1 mM PMSF and 1 μ g/ml aprotinin). Cell lysates were immunoprecipitated with the indicated antibodies bound to Protein A/G Agarose. Immunoprecipitates were analyzed by Western blotting as described above. NIH image was used to quantitate intensity of bands. Results were expressed as a percentage of maximal response to IGF-I, after subtraction of values from unstimulated conditions.

Kinase assay for phosphatidylinositol-3' kinase

PI3-K assay was performed as described previously (Watanabe *et al.* 2002) using phosphatidylinositol and

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as substrates. IRS-1-, IRS-2-, p85-, or phosphotyrosine-associated PI3-K activity was determined.

DNA synthesis

Cells (5×10^4) were plated in 6-well plates and medium was changed to serum-free medium including 1 mg/ml bovine serum albumin. After incubation for 48 h, cells were stimulated with various concentrations of IGF-I for 20 h and 37 kBq $[\text{H}^3]\text{thymidine}$ were added. Two hours after incubation, cells were extensively washed with serum-free medium and lysed with 0.5 M NaOH. Cell lysates were transferred to Ready Cap and dried at 70 °C for 1 h. Radioactivity was measured by a scintillation counter. When the PI3-K inhibitor, LY294002, or the MEK inhibitor, PD98059, was used, it was added 1 h prior to the addition of IGF-I.

Statistical analysis

All statistics were analyzed by Student's *t*-test or one-way ANOVA, with $P < 0.05$ considered significant.

Results

Levels of wild-type (WT) and mutant IGF-IR lacking the NPXY motif, expressed in R- cells, are shown in Fig. 1A. Similar levels of proreceptor and IGF-IR β -subunit were expressed in WT and Δ NPEY cells. No expression was detected in R- (puro) cells, which were transfected with a plasmid carrying the puromycin resistance gene alone. Scatchard analysis revealed that both cell lines have approximately 1×10^6 receptors per cell, with a K_d of ~ 0.6 nM. Autophosphorylation of IGF-IR was monitored by Western blotting, using an anti-phosphotyrosine antibody (PY20) after stimulation with varying doses of IGF-I ranging from 1 to 125 ng/ml (Fig. 1B), and its intensity was quantitated by NIH image as shown in Fig. 1C. Compared with WT cells, levels of autophosphorylation of the mutant IGF-IR were significantly lower at low IGF-I concentrations. The intensity, however, increased and reached almost comparable levels to that in WT cells at 125 ng/ml IGF-I. This was unexpected because autophosphorylation was severely impaired in CHO cells expressing the same mutant IGF-IR, as reported by Hsu *et al.* (1994).

Since autophosphorylation was induced in the mutant receptor, we examined its ability to phosphorylate IRS-1 and IRS-2. Consistent with most reports on IR with mutations in the NPXY motif (Murakami & Rosen 1991, Kaburagi *et al.* 1993, 1995, Chaika *et al.* 1997, Goalstone *et al.* 2001), tyrosine phosphorylation of IRS-1 was severely impaired in cells expressing the Δ NPEY mutant (Fig. 2A). Similarly, phosphorylation of IRS-2 was also

impaired in Δ NPEY cells (Fig. 2B). It should be noted that activation of IRS-1 and IRS-2 was not abrogated as shown by high doses of IGF-I. Since activated IRS-1 and IRS-2 associate with p85, a regulatory subunit of PI3-K, and cause PI3-K activation, the levels of p85 recruitment to IRS-1 and IRS-2 were examined. p85 was recruited to IRS-1 in a manner that correlated with IRS-1 activation levels in WT cells. However, p85 recruitment to IRS-1 was barely detectable in Δ NPEY cells (Fig. 2A). Interestingly, however, essentially the same levels of p85 were recruited to IRS-2 in both cell lines even though IRS-2 activation was severely impaired in Δ NPEY cells (Fig. 2B). Quantitative analysis is shown in Fig. 2C and D.

We then tested PI3-K activity using phosphatidylinositol and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as substrates (Fig. 3). IRS-1-associated PI3-K activity was reduced in Δ NPEY cells, but to a lesser extent than the difference in IRS-1 activation. This relationship is very similar to the findings reported in a study of insulin-stimulated IR mutated at Y960 (Kaburagi *et al.* 1993). On the other hand, no evidence was obtained that IRS-2-associated PI3-K activity was reduced in Δ NPEY cells; in fact, it was somewhat increased at high concentrations of IGF-I. In order to examine total PI3-K activity, phosphotyrosine- and p85-associated PI3-K activities were also examined. Essentially the same activities were observed at high doses of IGF-I in both cell lines.

We next assessed activation of Akt, a downstream kinase which is activated through PI3-K and has key functions in cell survival and proliferation (Kandel & Hay 1999). Activated PI3-K converts phosphatidylinositol 4,5-P₂ to PI3,4,5-P₃ and this lipid activates phosphoinositide-dependent kinase (PDK), which in turn activates Akt (Kandel & Hay 1999). Akt was activated in Δ NPEY cells, especially at high IGF-I concentrations (Fig. 4A). However, activation was significantly lower in Δ NPEY cells than in WT cells at low IGF-I concentrations (Fig. 4B). Using the same blot, activation of extracellular signal-regulated kinase (ERK), a constituent of the MEK/ERK pathway, another major pathway downstream of IGF-IR, was examined. Exactly the same activation kinetics were observed in Δ NPEY and WT cells (Fig. 4B), demonstrating that the MEK/ERK pathway is not affected by the mutation.

Thymidine incorporation into DNA upon IGF-I stimulation was also monitored in the two cell lines (Fig. 5A). DNA synthesis was clearly stimulated with 5 ng/ml IGF-I through the mutant receptor, and there was no significant difference between the cell lines in terms of IGF-I sensitivity and maximum responsiveness. Several experiments under different conditions (such as incubation time and concentration of $[\text{H}^3]\text{thymidine}$) showed similar results (data not shown). To examine their dependence on PI3-K activity, cells were treated with the specific PI3-K inhibitor, LY294002, before IGF-I stimulation (Fig. 5B). The inhibitor almost completely blocked IGF-I-induced

Figure 1 Expression and autophosphorylation of IGF-IR in WT and Δ NPEY cells. (A) Expression levels of IGF-IR in WT and Δ NPEY cells. Equal amounts of cell lysates were subjected to SDS-PAGE and probed as described in Materials and Methods. β -actin was used as a loading control. (B) Autophosphorylation of IGF-IR in WT and Δ NPEY cells in response to IGF-I. Serum-deprived cells were stimulated with a dose-range of IGF-I and cells were lysed 10 min after stimulation. Cell lysates were subjected to SDS-PAGE and probed with PY20 as described in Materials and Methods. Lane 1, 0; lane 2, 1 ng/ml; lane 3, 5 ng/ml; lane 4, 25 ng/ml; lane 5, 125 ng/ml IGF-I. (C) Quantitative analysis of autophosphorylation of IGF-IR in response to IGF-I. Results are expressed as a percentage of the maximal response to IGF-I and are presented as the mean $\pm$ s.e. of three independent determinants. * $P < 0.02$, ** $P < 0.01$ compared with WT.

DNA synthesis, demonstrating that PI3-K activity is essential for IGF-I-induced DNA synthesis in these cell lines. Similarly, the specific MEK inhibitor, PD98059, completely blocked IGF-I-induced DNA synthesis, showing that the MEK/ERK activity is also essential in the process (data not shown).

Discussion

Our major observations using a mutant IGF-IR, which lacked the whole NPXY motif, expressed in R- cells were as follows: (1) autophosphorylation was comparable

to WT cells at high concentrations of IGF-I, but was reduced at low concentrations; (2) tyrosine phosphorylation of IRS-1 and IRS-2 was impaired, even at high concentrations of IGF-I; (3) levels of p85 associated with activated IRS-1 were reduced, but levels of p85 associated with activated IRS-2 were similar when compared with association in WT cells at high concentrations of IGF-I; (4) total PI3-K and Akt activities were essentially the same as in WT cells at high concentrations of IGF-I; and (5) DNA synthesis was induced normally in terms of both sensitivity to stimulation and responsiveness.

There are redundant pathways leading to activation of PI3-K by IR or IGF-IR, including IRS-1, IRS-2

Figure 2 Activation of the PI3-K pathway in WT and Δ NPXY cells. (A and B) Tyrosine phosphorylation of IRS-1 or IRS-2 and IRS-1- or IRS-2-associated p85 in response to IGF-I. Cells stimulated or unstimulated with IGF-I were digested and cell lysates were immunoprecipitated (IP) with anti-IRS-1 or IRS-2 antibodies. Immunoprecipitates were separated in SDS-PAGE and probed with PY20 (PY) as described in Materials and Methods. The same blots were reprobed with anti-p85 antibody (p85). WB, Western blot, lane 1, 0; lane 2, 1 ng/ml; lane 3, 5 ng/ml; lane 4, 25 ng/ml; lane 5, 125 ng/ml IGF-I. (C and D) Quantitative analysis of tyrosine phosphorylation of IRS-1, IRS-2, and p85 recruitment in response to IGF-I. Results are expressed as the percentage of the maximal response to IGF-I with a value from the maximal response in WT cells normalized to 100%.

(Tobe *et al.* 1995), and the YXXM motif of the IR or the IGF-IR C-terminus, which directly activates PI3-K (Seely *et al.* 1995). Among the pathways, IRS-1 has been thought to be the primary molecule that stimulates PI3-K (Tobe *et al.* 1995). For example, IRS-1-deficient mouse embryo fibroblasts showed significantly decreased PI3-K activation and DNA synthesis upon IGF-I stimulation. Interestingly, overexpression of IRS-2 in IRS-1-deficient cells increased PI3-K activity to levels comparable with normal cells, while DNA synthesis was not increased. These results indicate that IRS-1 is a stronger contributor to PI3-K activation than IRS-2, and is essential for DNA synthesis through an unknown pathway separate from the PI3-K pathway. Furthermore, this unknown pathway cannot be activated by IRS-2 (Bruning *et al.* 1997). The contribution of the YXXM motif of the IGF-IR C-terminus is reported to be very low (Lamothe *et al.* 1995, Boehm *et al.* 1998). Considering these reports, our results are surprising because PI3-K activation and DNA synthesis were quite normal despite the fact that activation

Figure 3 Activation of PI3-K in WT and Δ NPXY cells. Autoradiograms for immunoprecipitated (IP) IRS-1-, IRS-2-, phosphotyrosine (PY)-, and p85-associated PI3-K activities. PI3-K activities 10 min after stimulation with the indicated doses of IGF-I were measured as described in Materials and Methods. PIP, phosphatidylinositol phosphate.

Figure 4 Akt and ERK activation in WT and Δ NPEY cells. (A) Western blots for phosphorylated Akt and ERK. Cells were stimulated with IGF-I and phosphorylated Akt and ERK were visualized as described in Materials and Methods. (B) Quantitative analysis of Akt and ERK phosphorylation in response to IGF-I. Results are expressed as the percentage of the maximal response to IGF-I and presented as the mean $\pm$ s.e. of three independent determinants. * $P < 0.01$ compared with WT.

of IRS-1 and IRS-2 were severely impaired. However, our cells did retain residual IRS-1 and IRS-2 activity. These residual activities may be sufficient to lead to maximal levels of PI3-K activity, beyond which there is no further increase in kinase activity, even in WT cells

where IRS-1 and IRS-2 activity is much higher. In particular, the key process was the recruitment of p85 to activated IRS-2; similar levels of p85 associated with IRS-2 in Δ NPEY cells despite quite low levels of IRS-2 activation. Although IRS-2 is thought to be a less import-

Figure 5 Stimulation of thymidine incorporation into DNA in WT and Δ NPEY cells upon IGF-I stimulation and its dependence on PI3-K activity. (A) Stimulation of DNA synthesis in the two cell lines. The results are expressed as the percentage stimulation of the maximal response to IGF-I in each cell line and presented as the mean $\pm$ s.e. of triplicate determinants. Basal and maximally stimulated thymidine incorporation into DNA were: WT, 6206–66 953 c.p.m.; Δ NPEY, 4757–62 180 c.p.m. (B) Effects of PI3-K inhibition on IGF-I-induced DNA synthesis. Before stimulation with 25 ng/ml IGF-I, LY294002 (LY) was added at concentrations of 10, 20 or 30 μ M and the two cell lines were processed for DNA synthesis.

ant contributor to PI3-K activity after IGF-I stimulation as described above, compensation by IRS-2 may contribute to obtaining normal levels of PI3-K and Akt activities in Δ NPEY cells. Finally, PI3-K-dependent DNA synthesis in the Δ NPEY cells displayed a response indistinguishable from WT cells.

Another question relates to the mechanism by which IRS-1 and IRS-2 are phosphorylated by IGF-IR without the NPEY motif. Binding of IRS-1 to the IGF-IR requires two sites within the IGF-IR, the NPEY and tyrosine kinase domains (Tartare-Deckert *et al.* 1995). IRS-2 also binds the NPEY domain and the tyrosine kinase domain of IR or IGF-IR via the phosphotyrosine binding domain (PTB) and a newly identified kinase regulatory loop binding (KRLB) domain respectively. Binding of the IRS-2 molecule to the IR mutated at Y960 is reduced by approximately half and binding of IRS-2 to the IGF-IR mutated at Y950 is abrogated in a yeast two-hybrid assay (Sawka-Verhelle *et al.* 1996, Van Obberghen *et al.* 2001). Residual levels of IRS-1 and IRS-2 phosphorylation, which presumably arise through the tyrosine kinase domain of IGF-IR alone, may contribute to activation of the PI3-K pathway and DNA synthesis in mammalian cells, even though mutation at Y950 is enough to abrogate binding in a yeast two-hybrid assay.

Activation of the MEK/ERK pathway is also important for DNA synthesis. As a matter of fact, ERK activation was completely retained in cells expressing the mutant receptor and was essential for DNA synthesis. The MEK/ERK pathway is activated through two different regions in the IGF-IR: the NPXY motif and the SSSS¹²⁸³ motif in the C-terminus. The reports that either region is sufficient to induce full activation of the pathway support our results (Dews *et al.* 2000, Morrione *et al.* 2001, Navarro & Basevga 2001).

The characteristics of the IGF-IR lacking the NPXY motif, as described in this study, may account for the discordant reports concerning the NPEY mutant. As long as the mutant receptor retains residual ability to phosphorylate IRS-1 and IRS-2, relative expression levels of the mutant IGF-IR and IRS-1 or IRS-2 may affect the extent of their phosphorylation. In fact, IR with a mutation at Y960, which has a severely impaired ability to phosphorylate IRS-1, is able to phosphorylate IRS-1 when IRS-1 is overexpressed (Chen *et al.* 1995). Therefore, the results of some studies may have been influenced by the IGF-I concentrations used and the levels of IGF-IR, IRS-1 and IRS-2 expression. Two reports demonstrated that IGF-I-induced DNA synthesis and cell proliferation were severely decreased by the mutation. Expression of the mutant IGF-IR was at a level of $\sim 1 \times 10^5$ receptors per cell in the reports. This low level of expression resulted in seemingly impaired autophosphorylation of IGF-IR (Hsu *et al.* 1994, Miura *et al.* 1995a), as predicted from our results where the level

of autophosphorylation showed reduced sensitivity to stimulation in mutant cells (Fig. 1). Therefore, insufficient activation of IRS-1 or IRS-2 because of impaired receptor tyrosine kinase activity may lead to a failure of DNA synthesis.

In conclusion, we have demonstrated that the NPXY motif in the IGF-IR is not essential for activation of the PI3-K pathway or DNA synthesis. These two activities, however, are probably dependent on residual IRS-1 and IRS-2 stimulation presumably brought about through the tyrosine kinase domain of the receptor. IGF-I concentrations and expression levels of mutant IGF-IR and IRS-1 or IRS-2 in cells could therefore influence downstream signaling events.

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