Characterization of a Hybrid Receptor Formed by Dimerization of the Insulin Receptor-Related Receptor (IRR) with the Insulin Receptor (IR): Coexpression of cDNAs Encoding Human IRR and Human IR in NIH-3T3 Cells†

Hsiang-Yiang Jui, Domenico Accili, and Simeon I. Taylor*

Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Bethesda, Maryland 20892-1770

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ABSTRACT: In many tissues, the insulin receptor-related receptor (IRR) is colocalized with the homologous receptors for insulin and insulin-like growth factor-I (IGF-I). Since a ligand for the IRR has not yet been identified, it has been proposed previously that IRR may be activated and transduce its signal via formation of hybrids with the insulin and IGF-I receptors. To test this hypothesis, we have coexpressed the human IRR and the human insulin receptor (IR) in NIH-3T3 cells. Although IRR/IR hybrid receptors were detected in these cells by using immunoprecipitation techniques, only a small proportion of each receptor was assembled into hybrids. While insulin was capable of stimulating insulin receptor autophosphorylation in these cells, there was no detectable increase in the total phosphotyrosine content of IRR. We conclude that the IRR/IR hybrid receptor does not play a major role in IRR signal transduction in response to insulin in NIH-3T3-hIRR/hIR cells.

The members of the insulin receptor family [insulin receptor, IGF-I receptor, and insulin receptor-related receptor (IRR)] are characterized by a heterotetrameric structure, composed of two $\alpha\beta$ heterodimers linked by disulfide bonds (Pilch & Czech, 1980; Van Obberghen et al., 1980; Czech et al., 1980; Ullrich et al., 1985, 1986; Ebina et al., 1985; Shier & Watt, 1989; Jui et al., 1994). Because of their structural similarities, insulin receptor $\alpha\beta$ heterodimers have been demonstrated to dimerize with IGF-I receptor $\alpha\beta$ heterodimers during receptor biosynthesis. By using coimmunoprecipitation techniques, IR/IGF-IR hybrid receptors have been demonstrated to exist in human placenta and a variety of cultured cell lines including NIH-3T3 cells, HepG2 hepatoma cells, IM-9 lymphocytes, and KB cells (derived from human epidermoid carcinoma) (Moxham et al., 1989; Soos & Siddle, 1989; Kasuya et al., 1993). Hybrid IR/IGF-IR receptors have also been detected in transfected cell lines coexpressing both receptors (Soos et al., 1990; Frattali & Pessin, 1993; Seely et al., 1995).

The physiological significance of these IR/IGF-IR hybrid receptors is not clear. Purified hybrid receptors bound IGF-I with high affinity similar to IGF-I heterotetramers. However, they bound insulin with much less affinity than insulin receptor heterotetramers (Frattali & Pessin, 1993; Soos et al., 1993; Seely et al., 1995). Therefore, it appears that IR/IGF-IR hybrid receptors would be responsive primarily to IGF-I rather than insulin at physiological concentrations of the ligands.

In situ hybridization analysis revealed that the mRNAs encoding IRR, insulin receptors, and IGF-I receptors were colocalized in several tissues, including dorsal root and trigeminal ganglia, as well as kidney distal tubules (Reinhardt et al., 1993). This observation raised the possibility that IRR molecules may form hybrids with insulin and IGF-I receptors in these tissues. Indeed, endogenous IRR has been detected in several human neuroblastoma tissues. In at least one neuroblastoma cell line, some IRR molecules were assembled in hybrids with insulin and IGF-I receptors (Kovacina & Roth, 1995). In this report, we describe experiments in which we have coexpressed IRR and insulin receptors in NIH-3T3 cells to study the role of the IRR/IR hybrid receptors in IRR signaling.

EXPERIMENTAL PROCEDURES

Materials. The NIH-3T3-hIR and NIH-3T3-hIRR cells are NIH-3T3 cells expressing human insulin receptors and human IRR [($\sim$1−2 $\times$ 10^6 receptors per cell], respectively, as described previously (Jui et al., 1994; Kadowaki et al., 1988). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The antipeptide antibodies used in this study include Ab713, an antibody directed against the carboxyl terminus of the human IRR (Jui et al., 1994), as well as Ab53 and Ab50, antibodies directed against the tyrosine kinase domain and the carboxyl terminus of the human insulin receptor, respectively (Cama et al., 1988). The antibody B10 is a polyclonal antibody specific to the α-subunit of the human insulin receptor (Zhang & Roth, 1991).

Expression of Human Insulin Receptors in NIH-3T3-hIRR Cells. To establish cell lines stably expressing both human IRR and insulin receptors, NIH-3T3-hIRR cells were transfected with pBPV-hIR, an expression plasmid encoding the human insulin receptor (Kadowaki et al., 1988). Prior to transfection, cells were grown to 50−80% confluence in a T-75 flask. To prepare the plasmid−liposome mixture for transfection, 10 μg of pBPV-hIR plus 1.5 μg of pY3-Hyg^r [a plasmid encoding hygromycin resistance (Blochlinger &

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* Address correspondence to this author at the National Institutes of Health, Building 10, Room 9S-213, Bethesda, MD 20892. Telephone: (301) 496-4658. FAX: (301) 402-0573. e-mail: Simeon_Taylor@nih.gov.

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Diggelmann, 1984)] was mixed with 0.4 mg/mL Lipofectamine reagent (Gibco BRL, Gaithersburg, MD) in a total of 3.2 mL of Opti-MEM (Gibco BRL), incubated for 45 min at room temperature, and then diluted with 6.4 mL of Opti-MEM. The plasmid−liposome mixture was overlaid on cells that had been prewashed 2 times with Opti-MEM, and cells were transfected for 12 h at 37 °C. Thereafter, 8 mL of fresh DMEM containing 20% FBS was added to the plasmid−liposome mixture, and transfection was continued for another 12 h. To isolate stably transfected clonal cell lines, cells were grown in DMEM containing 10% FBS and selected for resistance to the antibiotic hygromycin (200 μg/mL; Pharmacia Biotech, Piscataway, NJ). Multiple clones (≈50) were screened by PCR for the presence of expression plasmids for both IRR and insulin receptors. Among these, 10 clones were selected for further analysis. In the final experiments, we studied four clones that represent a range of levels of expression of insulin receptors, ranging from H48 (with the lowest level of insulin receptor expression) to H24 and H37 (with the most insulin receptors).

Biotinylation and Streptavidin Detection of Cell Surface Receptors. For biotinylation of cell surface receptors, confluent monolayers of cells in 10-cm tissue culture dishes were washed 3 times with ice-cold DPBS (Dulbecco's phosphate-buffered saline with calcium and magnisium) and then incubated on ice with 0.5 mg/mL NHS-LC Biotin (5 mL per dish; Pierce, Rockford, IL) for 30 min (Levy-Toledano et al., 1993). The biotinylation reaction was stopped by washing cells 3 times with ice-cold DPBS containing 15 mM glycine. Thereafter, cells were solubilized on ice in 1 mL of Kahane buffer (KB: 0.5% Triton X-100, 0.3 M NaCl, 0.025 M NaH_2PO_4, pH 7.4, and 0.02% sodium azide) containing 20 mM *n*-octyl glucoside and the following protease inhibitors (Boehringer Mannheim, Indianapolis, IN): leupeptin (10 mg/mL), antipain dihydrochloride (10 mg/mL), and phenylmethanesulfonyl fluoride (1 mM). This mixture of protease inhibitors has been chosen because it has been successful in experiments with solubilized insulin receptors from a variety of cells and tissues; it has not been specifically evaluated for its ability to inhibit proteolysis of IRR. Biotinylated receptors were immunoprecipitated with several anti-receptor antibodies (see above) (1:200 dilution) overnight at 4 °C. Antibody 713 immunoprecipitates approximately 70% of IRR under our conditions; this was determined by sequential immunoprecipitations with the antibody. In similar sequential immunoprecipitation experiments, we did not detect a difference in the efficiency of immunoprecipitation; however, because of the relatively low level of hybrid molecules, it was not possible to quantitate this as precisely as with IRR itself. Immune complexes were precipitated with protein A−agarose (Gibco-BRL) and washed 3 times with KB−0.1% BSA, 3 times with KB, and once with TBS (0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, and 0.02% NaN_3). The final pellet was eluted from beads by boiling the samples in Laemmli's sample buffer containing 80 mM DTT for 5 min, and fractionated by $NaDodSO_4$−PAGE (8.5%). Proteins were transferred from the gels to nitrocellulose membranes by electroblotting. The nitrocellulose membranes were soaked in PBS-T (phosphate-buffered saline, pH 7.4, containing 0.1% Tween-20) containing 10% nonfat milk for 2 h at room temperature to block sites for nonspecific protein binding. After washing, blots were incubated for 1 h at room temperature in PBS-T containing horseradish peroxidase-conjugated streptavidin (1:500 dilution; Amersham, Arlington Heights, IL). Thereafter, blots were washed extensively in PBS-T, followed by detection with Enhanced Chemiluminescence (ECL, Amersham) using the manufacturer's protocol.

Insulin Binding to Immunoprecipitated Receptors. Three T-75 flasks of confluent NIH-3T3-hIRR and NIH-3T3-hIRR/hIR cells were solubilized in 1.5 mL of KB, and receptors were immunoprecipitated with anti-receptor antibodies immobilized on protein A−agarose (Gibco BRL). The immobilized receptors were washed 3 times with KB−0.1% BSA, 2 times with KB, and 2 times with binding buffer (120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 15 mM sodium acetate, 10 mM glucose, 1 mM EDTA, 50 mM HEPES, pH 7.8, and 10 mg/mL BSA), and then resuspended in binding buffer to a total volume of 500 μL. For insulin binding, 80 μL of immobilized receptors was incubated overnight at 4 °C with ∼0.1 nM [^{125}I]insulin (360 Ci/g; Dupont-NEN, Wilmington, DE) in the presence or absence of unlabeled insulin (1 μM) in a total volume of 100 μL. Thereafter, the immobilized receptors were washed 3 times with ice-cold binding buffer, and radioactivity was quantified in a gamma counter.

Phosphorylation of Receptors in Intact Cells. Confluent monolayers of cells in 10-cm tissue culture dishes were incubated overnight in serum-free DMEM containing 0.1% of insulin-free BSA. The serum-starved cells were incubated with increasing concentrations of insulin (10^{-9}−10^{-6} M) for 2 min at 37 °C. The phosphorylation reaction was stopped by washing cells with 3 mL of ice-cold PBS and then freezing cells on a liquid nitrogen bath. Thereafter, cells were solubilized as described above, except that phosphatase inhibitors (1 mM sodium pyrophosphate, 2 mM sodium vanadate, and 200 mM NaF) were added to the lysis buffer. After immunoprecipitation, phosphorylated receptors were detected by Western blotting with a monoclonal anti-phosphotyrosine antibody (UBI; 1:4000 dilution) followed by ECL.

RESULTS

Detection of IRR/IR Hybrid Receptors in NIH-3T3 Cells Coexpressing IRR and Insulin Receptors. To see whether IRR and insulin receptors can form hybrid receptors, NIH-3T3-hIRR cells were stably cotransfected with cDNA encoding the human insulin receptor. Cell surface proteins were biotinylated, immunoprecipitated with anti-receptor antibodies, and detected by Western blotting with streptavidin. In control experiments (Figure 1A), insulin receptors in the NIH-3T3-hIR cell lysate were immunoprecipitated with either an antibody specific for the hIR β-subunit (Ab50; lane 4) or an antibody specific for the hIR α-subunit (AbB10; lane 5). Neither antibody immunoprecipitated IRR in extracts prepared from the NIH-3T3-hIRR cells (lanes 9 and 10). In contrast, an anti-hIRR antibody (Ab713) reacted with IRR, but not insulin receptors (compare lane 8 to lane 3). Furthermore, when both cell lysates were mixed together, insulin receptors were immunoprecipitated by both Ab50 and AbB10, but not Ab713; similarly, IRR was immunoprecipitated by Ab713, but not by Ab50 or by AbB10. Both receptors were immunoprecipitated by an antipeptide antibody direct against a conserved sequence present in both

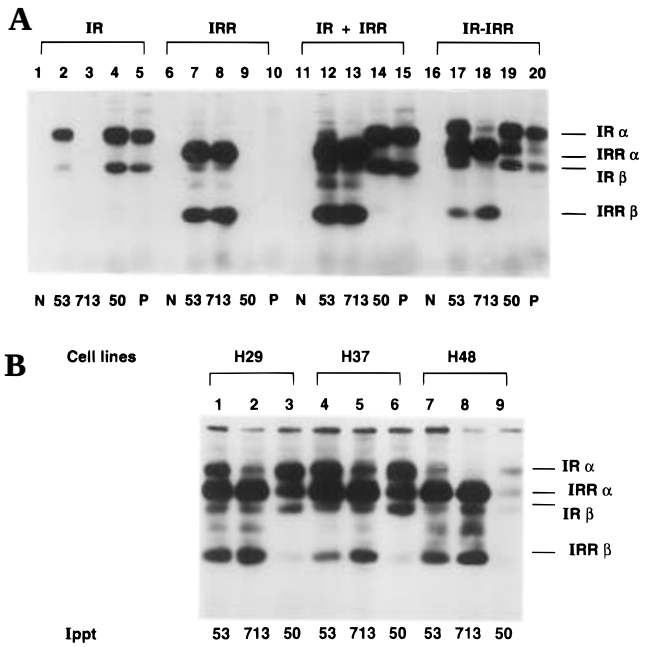

FIGURE 1: Detection of IRR/IR hybrid receptors in NIH-3T3-hIRR/hIR cells. (Panel A) NIH-3T3 cells expressing human insulin receptors (lanes 1−5), human IRR (lanes 6−10), and the mixture of these two cell lines (11−15), as well as cells coexpressing both receptors (clone H24, lanes 16−20), were biotinylated, and receptors were immunoprecipitated as indicated: N, normal serum; 53, Ab53 directed against the tyrosine kinase domain of the hIR; 713, Ab713 directed against the carboxyl terminus of the hIRR; 50, Ab50 directed against the carboxyl terminus of the hIR; and P, polyclonal AbB10 directed the α-subunit of the human insulin receptor. (Panel B) Similar studies were carried out in three different clonal lines of NIH-3T3-hIRR/hIR cells.

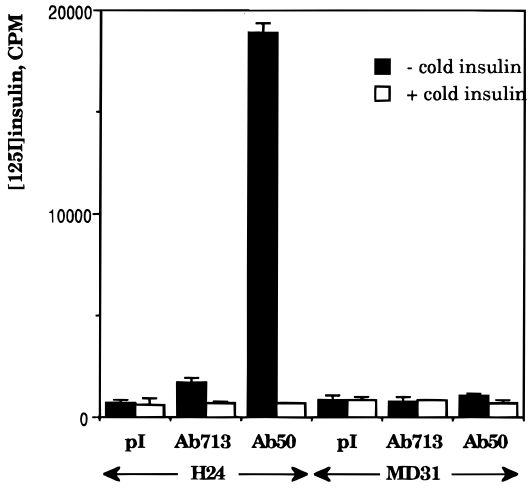

FIGURE 2: [^{125}I]Insulin binding to immobilized receptors. NIH-3T3 cells coexpressing IRR and insulin receptors (clone H24) or expressing IRR alone (clone MD31) were solubilized and immunoprecipitated with preimmune serum (pI), Ab 713, and Ab50. [^{125}I]-Insulin binding to the receptors in each immunoprecipitate was measured in the absence (filled bar) or presence (open bar) of excess unlabeled insulin (1 μM). The data (means of triplicate determinations) are representative of the results of two independent experiments.

tyrosine kinase domains (Ab53; lanes 2, 7, and 12). In four NIH-3T3-hIRR/hIR cell clones coexpressing IRR and insulin receptors (clone H24, Figure 1A, lanes 16−20; and clones H29, H37, H48, Figure 1B), insulin receptors were immunoprecipitated with both Ab53 and Ab50, whereas IRR was immunoprecipitated with both Ab53 and Ab713. In addition, a small portion of insulin receptors was detected in the Ab713 immunoprecipitates (Figure 1A, lanes 18; and Figure 1B, lanes 2, 5, 8). Similarly, a fraction of IRR molecules was coimmunoprecipitated with insulin receptors by Ab50 (Figure 1A, lane 19; and Figure 1B, lanes 3, 6, 9). Because of the small number of biotinylation sites on the extracellular domains of the β-subunits and the low level of hybrid formation, the bands corresponding to the IRR β-subunit in the Ab50 immunoprecipitates are less intense than the bands corresponding to the α-subunit. The NIH-3T3-hIR cells express approximately (1−2) $\times$ 10^6 receptors per cell estimated by insulin binding (Kadowaki et al., 1988). Our biotinylation experiments indicated that the parental NIH-3T3-hIRR cells and their derived NIH-3T3-hIRR/hIR cells express IRR at a similar level. (This conclusion is based upon an assumption that both receptors are biotinylated with comparable efficiency under our conditions.) The quantity of hybrid receptors in doubly transfected cells was correlated with the level of expression of the individual recombinant receptors. For example, clone H48 expressed markedly fewer insulin receptors than clones H29 and H37 (Figure 1B). Accordingly, the level of hybrid receptors detected in clone H48 was also much lower than in clones H29 and H37. These results confirmed the previous observation that IRR and insulin receptors could form hybrids (Kovacina & Roth, 1995). However, even in NIH-3T3-hIRR/hIR cells with high-level overexpression of both receptors (e.g., clone H37 in Figure 1B), <20% of IRR β-subunits were present in the form of IR/IRR hybrids. A similarly small percentage of insulin receptor molecules assembled into hybrids with IRR.

[^{125}I]Insulin Binding to Ab713 Immunoprecipitates. To determine whether insulin could bind to the IRR/IR hybrid receptor, we measured total [^{125}I]insulin binding to the Ab713 immunoprecipitate. Ab713 has the ability to immunoprecipitate two molecular species: IRR and hybrid IRR/IR receptors. Because insulin does not bind to IRR, any [^{125}I]-insulin binding detected in the Ab713 immunoprecipitates must be attributed by the hybrid IRR/IR receptors. On the other hand, since Ab50 is specific for the insulin receptor, it would immunoprecipitate all molecules that bind [^{125}I]insulin. As we predicted, no [^{125}I]insulin binding was observed in the Ab713 immunoprecipitate from cells that express only IRR (NIH-3T3-hIRR cells; Figure 2). In cells expressing both IR and IRR (NIH-3T3-hIRR/hIR cells), a large amount of [^{125}I]insulin binding was detected in the Ab50 immunoprecipitate; this observation confirmed that insulin interacted normally with the insulin receptor in NIH-3T3-hIRR/hIR cells. In contrast, at most a small quantity of [^{125}I]insulin bound specifically to the Ab713 immunoprecipitate; the small increase of [^{125}I]insulin binding detected in the Ab713 immunoprecipitate was not statistically significant. Of course, these observations do not rule out the possibility that insulin binds with very low affinity (i.e., below our sensitivity to measure insulin binding) to the IRR/IR hybrid receptor (see below). However, such low-affinity binding is unlikely to be quantitatively significant at physiological concentrations of insulin.

Insulin-Stimulated Receptor Phosphorylation in NIH-3T3-hIRR/hIR Cells. We pursued this question by investigating whether insulin stimulated phosphorylation of IR/IRR hybrid receptors. By carrying out these experiments in the presence of high concentrations of insulin, this allows for the

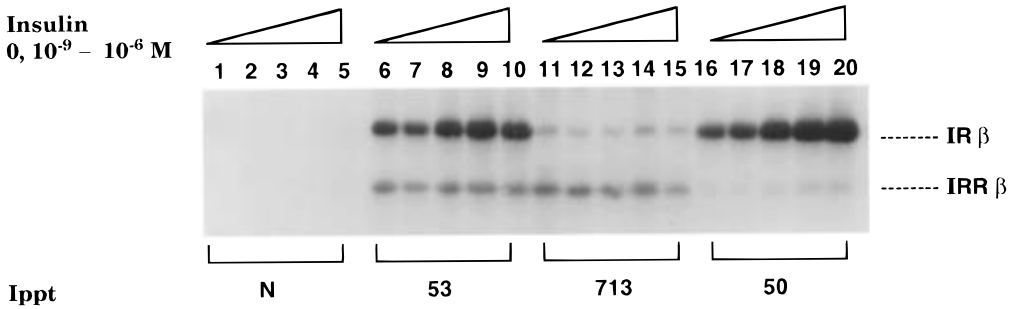

FIGURE 3: Insulin-stimulated receptor tyrosine phosphorylation in NIH-3T3 cells coexpressing IRR and insulin receptors. NIH-3T3-hIRR/hIR cells (clone H24) were incubated with increasing concentrations of insulin, 0, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} M. Receptors were immunoprecipitated with the indicated antibodies (N: normal serum) followed by blotting with an anti-phosphotyrosine antibody.

possibility of obtaining evidence of low-affinity binding interactions between insulin and IRR/IR hybrids. Thus, NIH-3T3-hIRR/hIR cells were incubated with increasing concentrations of insulin (10^{-9}—10^{-6} M); receptors were immunoprecipitated and analyzed by Western blotting with an anti-phosphotyrosine antibody (Figure 3). As expected, insulin receptor phosphorylation was increased in a dose-dependent manner in Ab50 immunoprecipitates. Furthermore, a small dose-dependent increase of IRR phosphorylation was also observed, suggesting that IRR/IR hybrid receptors may undergo insulin-stimulated tyrosine phosphorylation (lanes 16—20). However, the very low level of phosphorylation of IRR β-subunits contained in hybrid receptors makes it difficult to quantitate the magnitude of the effect. However, there was no increase in total IRR phosphorylation detected in immunoprecipitates with either Ab713 (lanes 11—15) or Ab53 (lanes 6—10) in response to insulin. Because only a small percentage of IRR molecules exist in the form of IR/IRR hybrids, one might not expect to be able to detect a selective increase in phosphorylation of hybrid receptor species. Nevertheless, these observations support the conclusion that activated insulin receptor heterotetramers do not transphosphorylate IRR heterotetramers to any appreciable extent.

DISCUSSION

We have demonstrated the existence of IRR/IR hybrid receptors in NIH-3T3 cells overexpressing both receptors. However, only a small proportion of IRR was assembled into hybrid receptors in these cells. In the case of IR/IGF-IR hybrid receptors, the proportion of hybrids varies in different cell types, ranging from ~40% in the human placenta and HepG2 cells to ~80% in IM-9 cells. The efficiency of IR/IGF-IR hybrid formation has been estimated by transfecting cells with a fixed concentration of insulin receptor expression plasmid and increasing concentrations of IGF-I expression plasmid (Frattali & Pessin, 1993). At low concentrations of IGF-I receptor expression plasmid, fewer than 20% of the expressed insulin receptors were present as IR/IGF-IR hybrids. As the concentrations of IGF-I expression plasmid increased, a greater proportion of the expressed insulin receptors was assembled into hybrid receptors. In our case, we did not systematically estimate the efficiency with which IRR and IR assemble into hybrids. However, it appeared that higher levels of hybrid receptors were observed when both IRR and insulin receptors were overexpressed at the highest levels (Figure 1B). This observation is consistent with the hypothesis that formation of receptor hybrids is driven, at least in part, by the law of mass action. Nevertheless, it is difficult to quantitate the percentage of hybrid molecules precisely because it is possible that there may be subtle difference in the avidities with which the antibodies bind hybrid and nonhybrid receptors.

The low level of hybrid formation in NIH-3T3-hIRR/hIR cells has limited our ability to study the effects of insulin on IRR/IR hybrid receptors. Total [^{125}I]insulin binding to the Ab713 immunoprecipitate from these cells, which contained both IRR and IRR/IR hybrids, was not significantly greater than nonspecific binding. This may have resulted from either the low level of IRR/IR hybrids or a low affinity for insulin binding to hybrid receptors. It has been demonstrated previously that isolated insulin $\alpha\beta$ half-receptors bind insulin with low affinity, and that a combination of two insulin $\alpha\beta$ half-receptors is essential for high-affinity insulin binding (Sweet et al., 1987). Combination of an insulin $\alpha\beta$ half-receptor and an IRR $\alpha\beta$ half-receptor in a hybrid may lead to a conformation with low affinity for insulin. Unlike insulin receptors, high-affinity IGF-I binding requires only one copy of IGF-I $\alpha\beta$-heterodimer (Feltz et al., 1988). Consequently, both IGF-I heterotetramers and IR/IGF-I hybrid receptors bind IGF-I with high affinity. Based upon this analogy, it is possible that hybrid IRR/IGF-I receptors might bind IGF-I with higher affinity, and that IGF-I would be effective in stimulating autophosphorylation of IRR/IGF-IR hybrid receptors. Nevertheless, in human neuroblastoma cells, only a portion of IRR is present in hybrids with IGF-I receptors (Kovacina & Roth, 1995).

Tyrosine phosphorylation of the majority of IRR expressed in NIH-3T3-hIRR/hIR cells did not increase after insulin stimulation. Therefore, formation of IRR/IR hybrid receptors did not seem to be a major IRR signaling mechanism in these cells. However, it remains possible that IRR might transduce signals through IRR/IGF-IR hybrid receptors. Nevertheless, there is likely to be a novel ligand with the function of regulating the tyrosine kinase activity of IRR. Indeed, in preliminary experiments, we have identified an activity in tissue extracts that increases the phosphotyrosine content of IRR in NIH-3T3-hIRR cells (unpublished observations).

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