Heterologous transmembrane signaling by a human insulin receptor–v-ros hybrid in Chinese hamster ovary cells

(chimeric receptors/protein-tyrosine kinase)

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ABSTRACT A hybrid receptor molecule composed of the extracellular ligand-binding domain of the human insulin receptor and the transmembrane and cytoplasmic (protein-tyrosine kinase) domains of the chicken sarcoma virus US2 transforming protein p68^gr^-ros has been constructed and expressed in Chinese hamster ovary (CHO) cells. The hybrid is processed normally into α and hybrid β subunits, is expressed on the cell surface at high levels, and binds insulin with near-wild-type affinity. Furthermore, insulin stimulates the phosphorylation on tyrosine residues of the hybrid β subunit in vivo and the phosphorylation of an exogenous substrate (poly(Glu,Tyr)) in vitro. Thus the hybrid is capable of heterologous transmembrane signaling. However, the hybrid mediates neither the insulin-activated uptake of 2-deoxyglucose nor the incorporation of [3H]thymidine into DNA, suggesting that the physiological responses mediated by ligand-activated protein-tyrosine kinases may utilize distinct intracellular mechanisms for postreceptor signaling.

The insulin receptor (IR) is a disulfide-linked heterotetramer composed of two α and two β subunits. Insulin binds to cells with high affinity and stimulates the autophosphorylation of the β subunit of the IR predominantly on tyrosine residues (residues 1,022-1,027). The extracellular sequence of the human IR (hIR) protein, deduced from the nucleotide sequence of the human placental IR cDNA (2, 3), indicates a single polypeptide chain precursor (aβ), which is proteolytically processed during biosynthesis to generate α subunit (735 amino acids; Mr = 84,214) and β subunit (620 amino acids; Mr = 69,703). The presumptive membrane-spanning domain bisects the β subunit. Thus the IR is composed of an extracellular domain (composed of the α subunit and about one-third of the β subunit), which binds insulin, and a cytoplasmic domain (about two-thirds of the β subunit), which contains the protein-tyrosine kinase (PTK; EC 2.7.1.112) domain of the IR.

A wide variety of studies implicate the IR kinase in mediating insulin-activated functions such as glucose uptake, glycogen synthesis, and DNA synthesis (4–6). However, it is not known whether the IR kinase domain itself conveys specificity for the initiation of insulin responses by cells. In the present study we have employed molecular genetic methods to test this requirement by constructing a hybrid receptor composed of the extracellular domain of the hIR protein fused to the transmembrane and cytoplasmic PTK domain of the chicken sarcoma virus US2 transforming protein p68^gr^-ros (7–12), with which the hIR is ≈50% identical (2). We find that the heterologous IR-ros hybrid when expressed in Chinese hamster ovary (CHO) cells exhibits insulin-activated PTK activity in vivo and in vitro. However, it is nonfunctional as an IR: it mediates neither the insulin-activated uptake of 2-deoxyglucose nor the incorporation of [3H]thymidine into DNA.

MATERIALS AND METHODS

Construction of an Expression Plasmid Encoding the hIR–ros Hybrid Protein. All manipulations of DNA were according to standard procedures (13). Enzymes were from New England Biolabs or Boehringer Mannheim. Plasmids were propagated in the DH1 strain of Escherichia coli as described (14).

The hybrid hIR-ros cDNA was assembled in plasmid pECE, a 2.9-kilobase (kb) expression vector (4), as follows. Plasmid pETAp, which contains the full-length (~4.5-kb) hIR cDNA inserted into the EcoRI/Xba I sites of pECE (4), was (i) digested to completion with Xba I, (ii) digested partially with Aar II [which leaves a 3' overhang at base pair (bp) 2983 of the hIR cDNA], and (iii) rendered blunt-ended by treatment with the large (Klenow) fragment of E. coli DNA polymerase for 30 min at room temperature, followed by an additional 30 min at room temperature in the presence of all four deoxynucleoside triphosphates (P-L Biochemicals; 100 µM). Then the ~5.8-kb fragment containing the PECE vector and 2983 bp of the hIR cDNA, with 3' and 3' blunt ends, was purified by electrophoresis in low-gelling-temperature agarose (SeaPlaque, FMC, Rockland, ME); the product is designated pETAp. The transmembrane domain and the entire cytoplasmic domain (including the PTK domain) of the transforming protein p68^gr^-ros were provided by a Hae II (bp 800) to Pvu II (bp 3011) fragment of UR2 DNA (11). After removal of the 3' Hae II overhang (see above), this ~2.2-kb fragment was ligated with pETAp and used to transform DH1. A plasmid with the appropriate orientation of the insert was designated pEHR-ros. As a result of the blunt-end ligation of hIR and p68^gr^-ros sequences, the expected junction of the cDNA is

\[
\begin{align*}
\text{IR} & \quad \text{p68}^{\text{gr}^-\text{ros}} \\
\text{921} & \quad 142 \\
\text{Tyr-Lys-Ala-Glu-Glu-Thr} & \\
5'-\text{TAT TTA GCG GAG AAG} & \\
3'-\text{ATA AAT CGC CTC TGG} &
\end{align*}
\]

The first G of the Ala codon is contributed by the Asp-922 codon of the hIR cDNA [which is interrupted by the Aat II site at bp 2983 (2)], while the second (C) and third (G)

Abbreviations: IR, insulin receptor; hIR, human IR; PTK, protein-tyrosine kinase; mAb, monoclonal antibody.

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nucleotides of the Ala codon are derived from the Pro-141 codon of p68\textsuperscript{gag-ros} which is interrupted by the \textit{Hae} II site at bp 800 (11). This DNA sequence was confirmed by the dideoxy chain terminator method (15). The hIR-ros protein thus contains 921 residues derived from the hIR [all of the \(\alpha\) subunit and all of the extracellular portion of the \(\beta\) subunit, except for 8 amino acids prior to the transmembrane domain (2)], the Ala residue at the hybrid junction, 10 residues encoded by viral gag sequences, 6 extracellular residues, and the transmembrane domain (29 residues) and cytoplasmic domain (367 residues) encoded by \(\text{ros}\) sequences (11). The cDNA contains 72 bp of 3' untranslated sequence after the stop codon (bp 2036) at the end of the p68\textsuperscript{gag-ros} sequence (11).

**Expression and Characterization of the Hybrid Receptor in CHO Cells.** The expression plasmid pehIR.ros was transfected into CHO cells and transformants expressing high levels of the hIR-ros protein were selected as described (16). One resulting cell line that exhibits a level of fluorescence about 200 times control (equivalent to \(\approx 2 \times 10^6\) cell surface molecules per cell; see ref. 4) was selected for further study (data not shown).

The binding of \(^{125}\text{I}\)-labeled insulin (\(^{125}\text{I}\)-insulin) to cultured cells, purification of IR proteins, metabolic labeling of cells, immunoprecipitations, kinase assays, covalent crosslinking of the IR with \(^{125}\text{I}\)-insulin, and uptake of 2-deoxy[\(^3\text{H}\)]glucose were performed as described (4).

Thymidine uptake was measured in semiconfluent cells in 24-well plates after 32 hr at 37°C in serum-free Ham's F-12 medium. Cells were incubated with insulin for 1 hr at 37°C, 0.75 \(\mu\)Ci of \(^{3}\text{H}\)thymidine (New England Nuclear, 20 Ci/ mmol; 1 Ci = 37 GBq) was added, and after 45 min cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline and solubilized with 0.4 ml of 1% NaDodSO\(_4\). Tri- chloroacetic acid was added to the lysate to a final concentration of 10% and the resulting precipitate was pelleted by centrifugation (2000 \(x\) g, 20 min, 4°C). After aspiration of the supernatant, the pellet was dissolved in 0.5 ml of 0.4 M NaOH, followed by 0.25 ml of 1 M Tris-HCl, pH 7.4, and 0.25 ml of 1 M HCl and the radioactivity of the solution was then measured.

**RESULTS AND DISCUSSION**

**IR-ros Hybrid Protein Is Expressed on the Surface of Transfected CHO Cells.** The fact that CHO.1R.ros cells bind the hIR monoclonal antibody (mAb), which recognizes only the native hIR (17), indicates that the extracellular domain of the IR-ros molecule approximates the native conformation of the hIR external domain. Furthermore, binding studies with \(^{125}\text{I}\)-insulin demonstrate that the CHO.1R.ros cells bind insulin with near wild-type affinity (data not shown). Half-maximal displacement of labeled insulin occurred with \(\approx 0.9\) nM unlabeled insulin for the CHO.1R.ros cells, as compared to \(\approx 0.3\) nM insulin for a wild-type receptor. Thus IR-ros receptor displays only a modest loss of affinity for insulin (decrease by a factor of \(\approx 3\)).

**IR-ros Protein Is Processed Normally Into \(\alpha\) and Hybrid \(\beta\) Subunits.** In CHO cells that express the wild-type hIR (CHO.T cells), the hIR mAb specifically immunoprecipitates labeled bands of \(\approx 200, 135,\) and 95 kDa, consistent with the molecular masses of the IR precursor and \(\alpha\) and \(\beta\) subunits, respectively (4, 17) (Fig. 1A, lane 2 from left). These bands are not present when a control antibody (normal mouse IgG) is employed (Fig. 1A, lane 1). The hIR mAb recognizes in CHO.1R.ros cells the same three bands, which are not present in the precipitate formed by the control serum (Fig. 1A, lanes 4 and 5). Note that the labeling of the hybrid \(\beta\) subunit (relative to that of the \(\alpha\) subunit) is significantly less than that of the wild-type hIR; the cytoplasmic domain of p68\textsuperscript{gag-ros} has 6 (labeled) methionine residues, versus 17 for the hIR. The hybrid IR-ros is also recognized by a rabbit polyclonal antibody specific for the ros sequences of p68\textsuperscript{gag-ros} (Fig. 1A, lane 6; L.-H.W., unpublished results). Since this antibody does not recognize the hIR (Fig. 1A, lane 3), these results indicate that the epitopes of p68\textsuperscript{gag-ros} recognized by this antibody are present in the hybrid IR-ros molecule.

To further assess the immunological cross-reactivity between the hIR and IR-ros proteins, we employed a radioimmunoassay with a panel of mAbs directed against extracellular (the \(\alpha\) class mAbs) or cytoplasmic (the \(\beta\) class mAbs) determinants of the hIR (5, 18). As illustrated in Table 1, the three mAbs that recognize extracellular determinants of the hIR precipitate the hybrid IR-ros with the same affinity as the wild-type hIR (from term human placenta). In contrast, none of the mAbs bound to IR-ros.

**Table 1. Binding of the IR-ros hybrid receptor by mAbs to the IR**

<table>
<thead>
<tr>
<th>mAb</th>
<th>Antibody class*</th>
<th>Placenta bound, normalized cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>hIR</td>
<td>(\alpha_1)</td>
<td>140</td>
</tr>
<tr>
<td>5D9</td>
<td>(\alpha_1)</td>
<td>910</td>
</tr>
<tr>
<td>3D7</td>
<td>(\alpha_2)</td>
<td>260</td>
</tr>
<tr>
<td>1G2</td>
<td>(\beta_1)</td>
<td>2100</td>
</tr>
<tr>
<td>7D5</td>
<td>(\beta_1)</td>
<td>1200</td>
</tr>
<tr>
<td>1B5</td>
<td>(\beta_1)</td>
<td>3370</td>
</tr>
<tr>
<td>17A3</td>
<td>(\beta_2)</td>
<td>2490</td>
</tr>
<tr>
<td>2A4</td>
<td>(\beta_2)</td>
<td>2480</td>
</tr>
<tr>
<td>2G7</td>
<td>(\beta_2)</td>
<td>3230</td>
</tr>
<tr>
<td>1H5</td>
<td>(\beta_3)</td>
<td>1320</td>
</tr>
<tr>
<td>2D4</td>
<td>(\beta_4)</td>
<td>3060</td>
</tr>
</tbody>
</table>

*Antibody class was determined as described (18).

**Fig. 1.** (A) Identification of metabolically labeled \(^{125}\text{I}\)methionine, 16 hr wild-type hIR or hybrid hIR-ros proteins in CHO.T or CHO.1R.ros cells, respectively. After extraction with nonionic detergent, immunoprecipitation was carried out with the following antibodies: NM, normal mouse IgG control antibody; IR, hIR-specific mAb (17); ros, rabbit polyclonal antibody specific for the ros sequences of p68\textsuperscript{gag-ros} transforming protein (L.-H.W., unpublished results). Labeled proteins were visualized by NaDodSO\(_4\)/PAGE and fluorography. (B) Covalent crosslinking with \(^{125}\text{I}\)-insulin of human placental IR or the hIR-ros hybrid in vitro (see text).
of the mAbs directed against four distinct antigenic sites of the cytoplasmic portion of the hIR β subunit recognize the IR-ros hybrid (Table 1), nor do they recognize the chicken p68gag-ros transforming protein (5). Thus we conclude that the PTK domains of the hIR and P68gag-ros and thus IR-ros are immunologically divergent.

A prominent feature of the deduced amino acid sequence of the α subunit of the hIR is the cysteine-rich region: this domain may be involved in the formation of the intermolecular disulfide bridges required for the oligomeric structure of the receptor. How does the presence of heterologous p68gag-ros transmembrane and cytoplasmic domains influence this intermolecular behavior of the hybrid IR-ros molecule? Electrophoretic analysis under nonreducing conditions of total cell lysates of CHO.IR.ros cells covalently cross-linked with 125I-insulin reveals several high molecular weight species (Mr >> 200,000) after immunoprecipitation with either the hIR mAb (Fig. 1B, lane 5) or ros-specific antibody (Fig. 1B, lane 6) but not control (normal mouse IgG, Fig. 1B, lane 4) antibodies. The mobility of these oligomers is indistinguishable from that observed for cross-linked hIR purified from human term placenta (Fig. 1B, lane 2). Thus the IR-ros hybrid also displays the propensity for oligomerization characteristic of the wild-type hIR.

The IR-ros Hybrid Exhibits Ligand-Activated Transmembrane Signaling. Is the hybrid IR-ros molecule capable of transmembrane signaling? CHO.T cells metabolically labeled with 32P; exhibit a basal level of phosphorylation of the hIR β subunit (Fig. 2A), which is known to occur predominantly at serine residues (20). Incubation of such cells with insulin stimulates the incorporation of 32P; into the β subunit, both total and on tyrosine (~8-fold and ~14-fold, respectively). In contrast, CHO.IR.ros cells have very little detectable basal phosphorylation. Insulin stimulates the incorporation of 32P; into the hybrid IR-ros β subunit by ~15-fold (total), and ~10-fold on tyrosine. Thus the hybrid molecule expressed in CHO cells exhibits insulin-dependent phosphorylation in vivo.

Is the observed phosphorylation of the IR-ros hybrid a consequence of autophosphorylation (i.e., intramolecular)? Since (i) CHO cells have endogenous rodent IRs [2000–3000 cell surface IRs per cell (21)], and (ii) insulin-responsive cells contain a non-IR, insulin-dependent serine kinase (20), it is possible that the insulin-dependent phosphorylation that we observe is a result of phosphorylation in trans by one of these alternative mechanisms. To distinguish between the various possibilities we have examined the kinase activity of IR-ros hybrid in vitro with the use of a specific mAb (17A3) that can distinguish endogenous CHO IR from the IR-ros hybrid (see Table 1) and completely blocks IR autophosphorylation on tyrosine residues.

In these experiments, insulin stimulates the phosphorylation of poly(Glu,Tyr) by about 2-fold (Fig. 2B). Neither the basal nor the insulin-dependent level of substrate phosphorylation is affected by the addition of mAb 17A3. Thus we conclude that the observed phosphorylation of an exogenous substrate in vitro is a consequence of the stimulation by insulin of the IR-ros hybrid molecule. It is therefore likely that the observed tyrosine phosphorylation of the IR-ros hybrid β subunit in vivo (see Fig. 2A) is a consequence of insulin activation of this hybrid kinase. Thus this molecule is apparently capable of this initial aspect of transmembrane signaling—i.e., transduction of the insulin signal across a

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** (A) Phosphorylation in vivo of the wild-type hIR or hIR-ros hybrid in metabolically labeled (32P; CHO.T or CHO.IR.ros cells, respectively. CHO.IR.ros or CHO.T [which express ~5 x 10^6 wild-type cell surface IRs per cell (4)] cells were metabolically labeled for 4 hr at 37°C with [32P], and subsequently incubated with or without 1 μM porcine insulin for 5 min at 37°C. Immunoprecipitates with the appropriate antibodies were then prepared from nonionic detergent extracts of such cells and examined by NaDodSO4/PAGE and autoradiography. To directly compare total phosphorylation with phosphorylation on tyrosine residues, a companion gel for each experiment was treated at alkaline pH, which preferentially hydrolyzes serine phosphate versus tyrosine phosphate (19). The amount of radioactivity incorporated into the IR β subunit was quantitated in each case by measuring the radioactivity in the excised band of ~95-kDa protein. To account for differences in receptor expression between the two cell types, values have been normalized by using amounts of immunoprecipitated receptor from parallel cultures of [35S]methionine-labeled cells. (B) Phosphorylation in vitro of an exogenous substrate [poly(Glu,Tyr)] by the IR-ros hybrid protein. The IR-ros hybrid was partially purified (by the use of wheat germ agglutinin affinity chromatography) from nonionic detergent extracts of CHO.IR.ros cells. The endogenous rodent IR copurifies with the IR-ros hybrid under these conditions. Such extracts were incubated with the exogenous substrate poly(Glu,Tyr) (whose only phosphorylation sites are tyrosine residues), [γ-32P]ATP, 1 μM porcine insulin when indicated, and mAb 17A3 when indicated. Amounts of poly(Glu,Tyr) phosphorylation were determined by NaDodSO4/PAGE and autoradiography, excision of the labeled species, and liquid scintillation counting.
heterologous transmembrane domain (derived from p68gag-rs) and stimulation of a heterologous PTK domain.

It is of interest to note that the IR-ros molecule exhibits a high level of basal autophosphorylation of the hybrid β subunit in vivo. This phosphorylation is not stimulated by 1 μM porcine insulin (data not shown). This contrasts with the in vivo phosphorylation results of Fig. 2A, in which the IR-ros phosphorylation is very low basally and is stimulated by insulin. p68gag-rs is also phosphorylated basally both in vivo (predominantly on serine but also on tyrosine) and in vitro [exclusively (8)]. Thus the extracellular domain of the h1r is able to regulate the activity of the IR-ros hybrid in vivo (presumably in concert with cellular tyrosine phosphatases), while the PTK domain of the hybrid is autonomously active in vitro.

CHO.IR.ros cells grow to contact inhibition and do not form foci or grow in soft agar. Furthermore, the incubation of such cells with porcine insulin at a range of concentrations (10⁻⁶ to 10⁻¹² M) has no influence on their growth in plastic culture dishes or in soft agar (data not shown), even though the IR-ros molecule is phosphorylated on tyrosine residues in vivo in the presence of insulin (see Fig. 2A; see also below). The inability of IR-ros to transform CHO cells could be due to resistance of this cell line to transformation by the UR2.ros oncogene or to the structural modification of the p68gag-rs molecule produced by construction of the IR-ros hybrid.

The IR-ros Hybrid Does Not Function as an IR in Vivo. One of the rapid responses of insulin-responsive cells is the insulin-dependent increase in the uptake of glucose (reviewed in ref. 1). Wild-type CHO cells or CHO.IR.ros cells were incubated with increasing concentrations of unlabeled porcine insulin for 30 min at 37°C and then for 10 min in the presence of 2-deoxy[³H]glucose (Fig. 3A). CHO cells exhibit a dose-dependent increase (≈2.5-fold maximum) in the uptake of 2-deoxy[³H]glucose, with a half-maximal response observed at about 0.95 nM insulin. While the maximal stimulation (≈3-fold) observed with CHO.IR.ros cells is comparable to that of CHO cells, the response observed with CHO.IR.ros differs in three respects from that of CHO cells: the half-maximal response is observed at 5.5 nM insulin (a decrease in sensitivity by a factor of ≈6), the maximal response is reduced by ≈40%, and the basal uptake is reduced by ≈20%. We have observed comparable results with another CHO.IR.ros cell line, which expresses ≈10⁵ cell surface IR-ros molecules per cell (data not shown). Thus the IR-ros molecule not only does not function as an IR but also interferes in some way with the endogenous rodent IR. These results are reminiscent of our previous observations of the consequences of introducing hIRs with nonfunctional cytoplasmic domains into CHO cells [compare the T-t mutant (4) and the IAR mutant (22)].

As the IR-ros hybrid is ineffective in mediating one aspect of the rapid response of cells to insulin (glucose uptake), it is possible that the hybrid is simply ineffective acutely (30 min in the experiments of Fig. 3A) and requires chronic insulin stimulation to generate an insulin response. We therefore incubated CHO or CHO.IR.ros cells overnight (14 hr) at 37°C with increasing concentrations of unlabeled porcine insulin and then for 45 min with [³H]thymidine (Fig. 3B), as a measure of the insulin-dependent stimulation of DNA synthesis. CHO cells exhibit a dose-dependent increase in the uptake of [³H]thymidine, with a half-maximal response at ≈0.3 nM insulin and a maximal stimulation of ≈2.8-fold.

In contrast, CHO.IR.ros cells exhibit a half-maximal response at 0.75 nM insulin (a reduction by a factor of ≈2.5 in sensitivity), with a maximal stimulation of ≈4.5-fold. Both the maximal and basal levels of uptake are reduced with respect to CHO cells, by ≈10% and ≈20%, respectively. Thus the IR-ros hybrid does not mediate either this long-term aspect of insulin action (thymidine uptake, Fig. 3B) or the short-term uptake of 2-deoxyglucose (Fig. 3A).

The PTK Family—Specificity for the Physiological Response? All of the evidence to date supports the hypothesis that the IR requires ligand-activated PTK functions to initiate the insulin response by cells (1, 4–6). The present study adds further support to this conclusion. By substitution of a homologous PTK for that of the h1r, we find that although such a hybrid is capable of insulin-dependent transmembrane signaling (phosphorylation of the hybrid β subunit on tyrosine residues), the hybrid IR-ros molecule does not function as an IR in such cells: it mediates neither short-term (uptake of 2-deoxyglucose) nor long-term (incorporation of [³H]thymidine) effects of insulin. This suggests (i) that the h1r PTK domain conveys a specificity for initiating the insulin response that the p68gag-rs PTK (though ≈50% identical) cannot and (ii) that a functional h1r extracellular domain alone is not sufficient for generation of the insulin response (e.g., ligand-induced aggregation).

![Fig. 3](image-url) Insulin-dependent uptake of 2-deoxy[³H]glucose (A) or [³H]thymidine (B) by wild-type CHO or CHO.IR.ros cells.
While the IR-ros hybrid is nonfunctional as an IR, the fact that it can mediate one aspect of transmembrane signaling (ligand-dependent tyrosine phosphorylation) encourages us to pursue the experimental strategy of transmembrane receptor domain swaps employed in the present study as an approach to the role of individual PTK domains in the generation of the biological specificity of the transmembrane receptors of which they are an integral part.

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