Expression of a functional human insulin receptor from a cloned cDNA in Chinese hamster ovary cells
(heterologous cell system/glucose uptake/autophosphorylation)

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ABSTRACT We have placed human insulin receptor cDNA into a vector under the control of the simian virus 40 (SV40) early promoter and tested its function by transient expression in microinjected Xenopus oocytes and by expression in stably transformed CHO cells. The precursor and the α and β subunits of the receptor were detected by immunoprecipitation from extracts of these cells. The human insulin receptor expressed in CHO cells specifically binds 125I-labeled insulin but not insulin-like growth factor I, displays insulin-stimulated autophosphorylation of the β subunit, and mediates insulin-stimulated 2-deoxyglucose uptake. We conclude that the human insulin receptor is synthesized, processed normally, and functional in this heterologous cell system.

Diabetes mellitus is caused either by a deficiency of insulin or by insensitivity of the target cells to insulin. This latter defect causes the majority of diabetes, and its incidence appears to be increasing.1 An understanding of the defect in these patients requires an understanding of the molecular mechanisms of insulin action. The manifold insulin responses in target cells are initiated by the binding of insulin to its receptor, an integral membrane glycoprotein composed of two α (Mr 135,000) and two β subunits (Mr 95,000) (2, 3). Insulin binding to the α subunit (4) of the receptor results in stimulation of intrinsic phosphokinase activity of the β subunit and autophosphorylation predominantly on tyrosine residues (5, 6). How this activity is related to the unique aspects of the insulin response is unknown.

Recently the human insulin receptor cDNA has been cloned by Ebina et al. (7) and by Ullrich et al. (8). The nucleotide sequence of the cDNA has revealed the primary structure of the single-chain insulin receptor precursor of 1382 amino acids (154 kDa). The α subunit (735 amino acids, 84 kDa) contains a cysteine-rich crosslinking domain; the β-subunit (620 amino acids, 70 kDa) contains both the single transmembrane domain of the receptor and the tyrosine phosphokinase domain with the presumed ATP binding site and potential tyrosine phosphorylation sites (7, 8). These features of the human insulin receptor and of the related epidermal growth factor receptor (9) provide the structural basis for understanding receptor function; they also intensify the mystery surrounding the specific biological effects associated with these molecules.

To elucidate the mechanism(s) of insulin action, the elements of the insulin receptor must somehow be functionally isolated and reconstituted. A heterologous cell system in which the receptor is expressed and then assayed for functional reconstitution would provide an experimental system in which the biological response of the cell to insulin could be explored ultimately in molecular detail. In this paper we demonstrate that the human insulin receptor cDNA can be expressed in heterologous cell systems. By the use of a monoclonal antibody specific for the human insulin receptor (10), it is possible to discriminate between endogenous receptors and those produced via the transfected gene. The human insulin receptor expressed in this system is functional by several criteria.

MATERIALS AND METHODS

Construction of the Expression Vector. The pcDL1 expression vector (kindly provided through the courtesy of T. Yokota and K. Arai, DNAX, Palo Alto, CA) was constructed from Okayama and Berg vectors (11) as follows. The EcoRI–HindIII fragment (2.9 kilobases (kb)) of pcDV1 was ligated with the EcoRI–HindIII fragment (0.54 kb) of pL1, resulting in an EcoRI site downstream from the simian virus 40 (SV40) early promoter and late region introns. The EcoRI–EcoRI cDNA inserts (7) of λIR-10 (nucleotides 62–1101) and λIR-15 (nucleotides 1102–4443) were inserted into the EcoRI site of pcDL1. This expression vector is designated pcDL1-HIR7. The cDNA insert encodes the initiation methionine (amino acid −27), the signal peptide (−27 to −1), and the single-chain precursor (1–1382) of the human insulin receptor.

Transfection of Mammalian Cells. pcDL1-HIR7 plasmid DNA (10 μg for 2 × 10^6 cells) and pSV2 neo DNA (2 μg) (12) were introduced into cells by the calcium phosphate precipitation technique, with the addition of a glycerol shock after 4 hr (12). After a 24-hr exposure to the DNA, the cells were trypsinized and replated at a 1:5 dilution. Within 24 hr, the antibiotic G418 (GIBCO) was added to the medium to a concentration of 400 μg/ml. After 2 weeks, independent colonies were picked using trypsin/EDTA-saturated 3MM paper discs and transferred to a 24-well plate. When confluent, the cells were incubated with a monoclonal antibody (10 nM) specific for the human insulin receptor (10) for 1 hr at 4°C. After washing, the cells were incubated with affinity-purified 125I-labeled rabbit anti-mouse IgG (10^5 cpm per well) (Cappel Laboratories, Cochranville, PA) for 1 hr at 4°C. The cells were washed three times with Dulbecco's phosphate-buffered saline (PBS), pH 7.3/1% bovine serum albumin and then were solubilized with 0.03% NaDodSO4.

Immunoprecipitation of Metabolically Labeled Proteins. Oocytes. Individual Xenopus oocytes, manually stripped of fragments of ovarian tissue, were oriented on plastic mesh grids and microinjected with about 10 nl of supercoiled pcDL1-HIR7 DNA (1 mg/ml), aiming for the oocyte germinal

Abbreviations: IGF, insulin-like growth factor; NP-40, Nonidet P-40; SV40, simian virus 40; kb, kilobase(s).

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vesicle. After about 4 hr of incubation in modified Barth's medium (MBSH; ref. 25), 30 undamaged injected oocytes and 30 un.injected controls were selected and incubated in groups of 5 oocytes per 30 μl of MBSH containing 30 μCi each of [35S]methionine and [35S]cysteine (Amersham, about 1300 Ci/mmol; Ci = 37 GBq) in Tris-buffered saline, potassium, streptomycin, and gentamycin. After 44 hr at 21°C the oocytes were harvested, rinsed with MBSH, and homogenized in 1 ml of 20 mM Tris Cl, pH 8.1/1.50 mM NaCl/1 mM EDTA/1% (vol/vol) Nonidet P-40 (NP-40)/1% sodium deoxycholate/0.1% NaDodSO4/1% aprotinin/1 mM phenylmethylsulfonyl fluoride. Pigment and yolk were removed by centrifugation (20,000 × g, 20 min). The supernatant was divided into two portions and subjected to immunoprecipitation overnight at 4°C with either a control monoclonal antibody or a pool of monoclonal antibodies that recognize both the α and β subunits of the insulin receptor (13). In both cases, the antibodies were used in a preformed complex with formalin-fixed Staphylococcus aureus (Pansorbin, Calbiochem), with rabbit anti-mouse IgG (Cappell Laboratories) as a bridge. After immunoprecipitation the complex was washed extensively. Bound proteins were released by heating with NaDodSO4/PAGE sample buffer and half the sample was electrophoresed in a 10% gel (14).

Mammalian cells. CHO and CHO-HIR3 cells (5 × 107) were metabolically labeled with [35S]methionine as follows: After a 2.5-hr incubation with methionine-free medium, cells were incubated for 16 hr with [35S]methionine (Amersham, 1255 Ci/mmol, 50 μCi/ml) and then for 4 hr with medium containing unlabeled methionine. An NP-40 (1%) extract was made from each culture, and labeled protein was immunoprecipitated using either normal mouse IgG or human insulin receptor-specific monoclonal antibody as described (10). [35S]-labeled proteins were visualized by autoradiography (without fluorography) of the gel with Kodak X-Omat film for 52 hr at −70°C. Molecular weights were calibrated using 14C-labeled protein standards (Amersham).

RNA Blot-Hybridization Analysis. Cells were grown in a 6-cm diameter dish and, before confluent, were washed twice with PBS and once with Tris-buffered saline (TBS: 20 mM Tris Cl, pH 7.5/150 mM NaCl). Cells were scraped off the plate with 0.4 ml of TBS/0.5% NP-40 and were mixed briefly, on a Vortex, in a 1.5-ml Eppendorf tube. Nuclei were pelleted in a microcentrifuge (30 sec at 4°C), and NaDodSO4 was added to the supernatant fluid to a final concentration of 0.5%. Following phenol/chloroform (3 times) and chloroform (1 time) extraction, the RNA was precipitated by addition of 1 ml of cold ethanol. Twelve micrograms of total RNA was electrophoresed in a formaldehyde/agarose gel, transferred to nitrocellulose (15) and hybridized with a nick-translated Pst I–EcoRI (1.5 kb) fragment of the human insulin receptor cDNA (7). 28S (=4.7 kb) and 18S (=1.9 kb) ribosomal RNAs were used as electrophoresis standards.

Binding of 125I-Labeled Insulin to Mammalian Cells. CHO-HIR3 or control CHO cells were plated in 24-well plates in Ham's F-12 medium with 10% fetal bovine serum. When confluent, the cells were washed twice with PBS plus 1% bovine serum albumin and then incubated with the same buffer containing 80 μM 125I-labeled insulin (120 μCi/μg) plus the indicated concentration of nonradioactive ligand. After 4 hr at 4°C, the cells were washed three times and solubilized with 0.1% NaDodSO4. The results are expressed as the percentage of total cpm bound per 50 μg of protein. The unlabeled ligands were either insulin or the monoclonal anti-human insulin receptor.

Autophosphorylation of the Insulin Receptor. CHO-HIR3 or control CHO cells at confluence in four 75-cm2 culture flasks were lysed and passed over a wheat germ agglutinin-agarose column to partially purify the insulin receptor, as described (10). The peak fraction of the insulin-binding activity for each cell type was concentrated 10-fold on a Centricon (Amicon). After a 1-hr incubation with 100 μM insulin for 1 hr at 24°C, [γ-32P]ATP (10 μM, 5 Ci/μl, 23 Ci/mmol) and MnCl2 (2 mM) were added, and the reaction continued for 60 min at 24°C. The reaction was stopped by the addition of 1% NaDodSO4, and samples were treated with 2-mercaptoethanol and analyzed by NaDodSO4/PAGE (14). Following autoradiography, the labeled β-subunit bands (M, 95,000) were cut out for liquid scintillation counting.

Insulin Stimulation of 2-Deoxy[3H]glucose Uptake. CHO-HIR3 or control CHO cells were plated as for the binding studies. After reaching confluence, the cells were washed and incubated in buffer containing 140 mM NaCl, 1.7 mM KCl, 0.9 mM CaCl2, 1.47 mM potassium phosphate, and 0.1% bovine serum albumin at pH 7.4. After 30 min at 37°C with the indicated concentration of insulin, the cells were incubated for 10 min with 0.1 mM 2-deoxy-o-[1,2-3H(N)]glucose (0.4 mCi/ml, New England Nuclear). Cells then were washed three times with ice-cold buffer containing 100 mM phloretin and were solubilized with 0.03% NaDodSO4/α-lactalbumin were assayed for protein and radioactivity. The results are expressed as cpm incorporated per 50 μg (CHO-HIR3 cells) or 70 μg (CHO cells) of protein (the average quantities of protein per well). The experiments were also performed in the presence of human insulin receptor-specific monoclonal antibody (30 nM). When the results are normalized with respect to protein, the values for CHO-HIR3 cells are increased by 40%, resulting in a higher basal level uptake in CHO-HIR3 cells. This higher basal activity was not observed in all experiments.

RESULTS AND DISCUSSION

An expression plasmid, pcDL1-HIR7, containing the entire coding sequence of the human insulin receptor cDNA (7), was constructed (Fig. 1). In this vector the human insulin receptor cDNA is placed under the transcriptional control of the early promoter of SV40. The transcriptional unit is completed with the SV40 late-region introns and polyadenylation signal.

We first tested the ability of this plasmid (pcDL1-HIR7) to express the human insulin receptor in Xenopus oocytes. After injection of the plasmid, oocytes were metabolically labeled with [35S]cysteine and [35S]methionine, and nonionic

![Diagram](image-url)
detergent (NP-40) extracts were prepared from control and injected oocytes and incubated with insulin receptor-specific monoclonal antibodies. These antibodies specifically immunoprecipitated three labeled polypeptides from the extract of injected oocytes; the apparent Mr's (200,000, 135,000, and 95,000; Fig. 2, lane 2) are consistent with those of the insulin receptor precursor and α and β subunits, respectively (16). These proteins were not precipitated from control oocytes (Fig. 2, lane 4), by the control antibody (lanes 1 and 3), or from mock-injected oocytes (data not shown). Thus the injection of the expression vector into oocytes not only results in the synthesis of a protein of the size expected for the human insulin receptor precursor, but also in the processing of the precursor into the α and β subunits.

For further studies, we established stable rodent [CHO, rat hepatoma (HTC), and mouse L (LTA)] cell lines by cotransfection of the human insulin receptor expression plasmid, pcDL1-HIR7, with a plasmid conferring resistance to the neomycin analog G418 (see legend to Fig. 1). Twenty-five, 12, and 19 G418-resistant colonies were picked randomly from about 100 colonies of CHO, HTC, and LTA cell lines, respectively. These permanent cell lines were assayed for the presence of the human insulin receptor on the cell surface by selective binding of a monoclonal antibody specific for the human insulin receptor. This monoclonal antibody also blocks binding of insulin to human cells (10). Of the CHO and HTC cell lines, 10 and 4, respectively, bound about 10 times control quantities of the monoclonal antibody, whereas the 3 LTA clones that were positive bound only 2 times control quantities of the monoclonal antibody. We chose for further study one of the CHO clones (designated CHO-HIR3) that bound the most antibody (monoclonal antibody binding to the surface of these cells was also detectable by indirect immunofluorescence; data not shown). The morphological character of the CHO-HIR3 and the control CHO cells was indistinguishable. Blot-hybridization analysis of total RNA from CHO-HIR3 cells revealed the presence of a human insulin receptor mRNA of about 4.7 kb, a size expected from the receptor cDNA encoded by this expression vector (Fig. 2).

**Fig. 2.** Immunoprecipitation of 3H-labeled human insulin receptor from *Xenopus* oocytes microinjected with the expression vector pcDL1-HIR7. Each lane of the gel contained protein immunoprecipitated from 7 *Xenopus* oocytes. Lanes 1 and 2: proteins immunoprecipitated from microinjected oocytes by control monoclonal antibody (lane 1) and anti-insulin receptor monoclonal antibodies (lane 2). Lanes 3 and 4: control oocytes plus control antibody (lane 3) and anti-insulin receptor monoclonal antibodies (lane 4). Exposure to x-ray film was for 20 hr at −70°C.

To examine the protein expressed in these cells, we immunoprecipitated metabolically labeled ([35S]methionine) proteins from nonionic detergent extracts of CHO-HIR3 and CHO cells (Fig. 3B). Two polypeptides of Mr = 135,000 and 95,000 (corresponding to the α and β subunits, respectively) were immunoprecipitated from CHO-HIR3 (lane 4) but not CHO (lane 2) cells by the human insulin receptor-specific monoclonal antibody. Normal mouse IgG did not immunoprecipitate these proteins (lanes 1 and 3). We conclude that the transfected rodent cells, like the *Xenopus* oocyte, process the insulin receptor precursor into mature α and β subunits.

The specificity of ligand binding was examined by assaying binding of 125I-labeled insulin or insulin-like growth factor I (IGF-I) to CHO-HIR3 cells and CHO cells (Fig. 4A). In the absence of unlabeled insulin, 1.8% of the 125I-labeled insulin was specifically bound by CHO-HIR3 cells, whereas only 0.5% was bound by control CHO cells. The binding of 125I-labeled insulin to both of these cell lines was inhibited in a dose-dependent fashion by unlabeled insulin. The human insulin receptor-specific monoclonal antibody blocked insulin binding, with progressively increasing effectiveness at higher concentrations, to CHO-HIR3 but not CHO cells (Fig. 4A), indicating that the increased binding observed in the transfected cells is via the human insulin receptor. In contrast, both cell types bound 0.4% of 125I-labeled IGF-I (data not shown). This confirms the previous conclusion that the isolated cDNA encodes the human insulin receptor and not the IGF-I receptor (7). It also indicates that the human insulin receptor binds little IGF-I under these conditions. The binding of 125I-labeled insulin is inhibited competitively by unlabeled insulin at lower concentrations (Fig. 4A). The inhibition takes the form of a typical dissociation curve. At higher concentrations, the analysis is complicated by the known binding of insulin to IGF-I receptors (17) and by negative cooperativity (18). It is well known that binding of insulin to its receptor is characterized by curvilinear Scatchard plots and therefore is difficult to analyze (19); however, such plots are useful in making comparisons between data. A Scatchard plot analysis (20) suggests that under these assay conditions CHO-HIR3 cells display approximately 14,000, and CHO cells about 2800, high-affinity sites per cell. The apparent dissociation constants are 5 x 10⁻¹⁰ and 4 x 10⁻¹⁰ M, respectively. The latter estimate is in agreement with the data for CHO cells obtained by Podskalny.
et al. (21) and indicates that the affinities for insulin of the expressed human insulin receptor and the endogenous hamster insulin receptor are very similar.

We next examined whether the insulin receptor from CHO-HIR3 cells displays insulin-activated phosphokinase activity. In partially purified total cellular insulin receptor preparations from both CHO-HIR3 and CHO cells, the $M_r$ 95,000 $\beta$ subunit of the receptor was exclusively phosphorylated (Fig. 4b). The incorporation of $^{32}$P into the $\beta$ subunit was 10 times greater in CHO-HIR3 cells than in CHO cells in both the presence and the absence of insulin. Insulin stimulated the observed phosphorylation 2-fold in both the CHO-HIR3 and the control CHO cells.

The 10-fold increase in phosphokinase activity observed in these preparations is significantly greater than the 5-fold increase in the number of cell surface insulin receptors detected on intact CHO-HIR3 cells. However, the 10-fold increase correlates well with the number of receptors detected in total cellular extracts by immunoassay (data not shown). Thus the difference is accounted for by an increased level of intracellular human insulin receptor in CHO-HIR3 cells.

To assess whether the human insulin receptor expressed in the CHO-HIR3 cells exhibits a physiological response, we assayed the insulin stimulation of glucose uptake. In initial experiments, it was found that 10 pM insulin stimulated 2-deoxyglucose uptake 15% in CHO cells and 75% in CHO-HIR3 cells. This led to a more systematic study of the insulin response, in which we varied the insulin concentration over four orders of magnitude (Fig. 4c). The maximal stimulation by insulin of glucose uptake by both CHO-HIR3 cells and CHO cells was similar ($\approx$100%), but the insulin levels required for this effect were dramatically different for the two cell lines. This increase in sensitivity was confirmed in four separate experiments. For one-half the maximal response, CHO cells required an $\approx$30 times greater concentration of insulin than CHO-HIR3 cells. The increased sensitivity is not due to an increased affinity, since the estimated dissociation constants for the human and the hamster insulin receptors are very similar (Fig. 4a). From the apparent dissociation constant, we calculated that $\approx$17% of the cell surface receptors ($\approx$500 per cell) must be occupied for half-maximal response in CHO cells, whereas only 0.8% of the total receptors ($\approx$100) must be occupied for half-maximal response in CHO-HIR3 cells. Thus, there are "spare" receptors in both cell types (22). The number of occupied receptors required for the biological response appears to be significantly different in the two cell types. This implies either that the human receptors expressed in CHO-HIR3 cells are more effective in transducing the insulin signal than are the endogenous receptors or that the receptors in CHO-HIR3 cells are in a different functional state [e.g., more effective clusters (23, 24) of the receptors might be generated at the higher receptor concentrations present in the membrane of CHO-HIR3 cells].

The ability to express the human insulin receptor in this heterologous cell system permits the further analysis of structure–function relationships of the receptor molecule and the role it plays in eliciting the various physiological responses to insulin.

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