The Tyrosine Kinase Activity of the Chicken Insulin Receptor Is Similar to That of the Human Insulin Receptor

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Received October 28, 1999; Accepted December 27, 1999

The tyrosine kinase activity of a chimeric insulin receptor composed of the extracellular domain of the human insulin receptor (IR) and the intracellular domain of the chicken IR was compared with wild-type human IR. The degrees of autophosphorylation, phosphorylation of IRS-1, and in vitro phosphorylation of an exogenous substrate after stimulation by human insulin were similar to that seen with the human IR. We conclude that the insulin resistance of chickens is not attributable to a lower level of intrinsic tyrosine kinase activity of IR.

Key words: insulin receptor; chicken; tyrosine kinase; IRS-1

Avian species, including chickens, are hyperglycemic, with the fasting blood glucose level of chickens being ~200 mg/100 mL, approximately twice as high as that of humans and many other mammals including rodents. The infusion of insulin into birds cause an only slight decrease in blood glucose levels and even high doses of insulin do not usually result in hypoglycemia. Collectively, the avian species are resistant to insulin. The basis for this insulin resistance has long been a matter of debate. The existence of the insulin receptor and of a substrate of the insulin receptor (IRS-1) in the tissues of chickens has been reported. The phenomena of ligand-stimulated phosphorylation of the insulin receptor and IRS-1 have also been observed. One of the possible explanations for the reduced sensitivity to insulin of the bird is the difference in the signaling activity of the insulin receptor itself. However, the structure of the chicken insulin receptor has not been characterized, and its signaling capabilities have not yet been studied. The mammalian insulin receptor is a heterotetrameric transmembrane protein, with the tyrosine kinase activity of this protein being essential for the signaling pathway. A comparison of the tyrosine kinase activity of the chicken insulin receptor with that of the human receptor is indispensable for determining if the difference in insulin sensitivity is attributable to a difference in the structure of the intracellular domain of the respective receptor. In this study, we attempted to directly compare the tyrosine kinase activity of the human and chicken insulin receptors. To this goal, a chimeric insulin receptor cDNA the extracellular domain of which was derived from the human receptor and the transmembrane and intracellular domains of which were from the chicken receptor was created and stably expressed. In vivo and in vitro tyrosine kinase assays were done in these cell lines.

A brain cDNA library from a newly hatched male White Leghorn was constructed using RiboClone cDNA synthesis system (Promega) and a Lambda gt10 system (Promega). The library was screened for chicken insulin receptor cDNA using a C-terminal portion of a human insulin receptor cDNA. The obtained cDNA was subcloned into the pGEM-4Z vector (Promega), and its nucleotide sequence was analyzed by an automated DNA sequencer (Shimadzu, Japan) (data not shown: GenBank accession number: AF111857). The 3692-bp cDNA clone included regions corresponding to the entire β-subunit and part of the α-subunit. Overall, the intracellular domain of the chicken insulin receptor was highly similar to that of the human insulin receptor. The amino acid sequence of the tyrosine kinase domain was 93% identical in accordance with the report by

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Abbreviations: IR, insulin receptor; IRS-1, insulin receptor substrate-1; WGA, wheat germ agglutinin
Savo et al.5) The similarities of the juxtamembrane and C-terminal domains between the human and chicken insulin receptor were 79% and 69%, respectively. The C-terminal extension reported in the Drosophila insulin receptor11) was not contained in the chicken insulin receptor.

An artificial cDNA that encoded a chimeric insulin receptor composed of the extracellular domain of the human insulin receptor and of the intracellular domain of the chicken receptor was created by making use of Ssp I sites contained in both human and chicken insulin receptor cDNAs (Fig. 1). Human insulin receptor cDNA constructed in the pGEM-4Z vector was digested with Ssp I, and a fragment containing part of the vector and the N-terminal region of insulin receptor cDNA was purified. The chicken insulin receptor construct was also digested with Ssp I, and a fragment containing part of the vector sequence and the C-terminal region of the receptor was obtained. Ligation of these fragments resulted in a reconstruction of the vector sequence and in the generation of a human-chicken chimeric insulin receptor cDNA. The cDNA was subcloned into the pBPV mammalian expression vector (Pharmacia) as previously described.10) The chimeric and wild-type insulin receptors were stably expressed in NIH-3T3 mouse fibroblast, and the receptor number and \(K_d\) were calculated by Scatchard analysis using \(^{125}\)I-insulin. Among the cell lines obtained, two lines with the wild-type receptor and two lines with the chimeric receptor expressing similar numbers of respective receptors were chosen and used for further study. The numbers of expressed receptors in these cell lines are shown in Table 1. We and others have already shown that the activities of chimeric proteins of this sort, such as those of human insulin and IGF-I receptors, reflect the structural context of intracellular domains.12,13)

Intact cell tyrosine kinase assays of the expressed receptors were done essentially as described previously.14)

Confluent cell cultures were washed with ice-cold phosphate-buffered saline and frozen in liquid nitrogen. The cells were thawed on ice in the presence of freshly prepared lysis buffer (50 mm Heps, pH 7.6, 150 mm NaCl, 1% (v/v) Triton X-100, 2 mm sodium orthovanadate, 100 mm NaF, 10 mm EDTA, and 1 mm phenylmethylsulfonyl fluoride). The lysate was separated on a 4-20% gradient gel, and phosphorylated proteins were detected using a 4G10 antiphosphotyrosine antibody (Upstate Biotechnology) and ECL detection reagent (Amer sham). As shown in Fig. 2, faint bands of the phosphorylated \(\beta\)-subunit of the endogenous receptor were seen in the mock-transfected Neo cells after insulin stimulation (panel A). In cells expressing wild-type receptors (WT2) and chimeric receptors (CHR6), prominent bands were observed at 1, 5, and 60 min after stimulation (panel B). The experiment using other clones (WT6 and CHR20) gave essentially the same result. Densitometric analysis of the bands from repeated experiments showed no significant difference between the wild-type and chimeric receptors at any time.

An in vitro kinase assay was done as described.14) Confluent cells were washed with ice-cold phosphate-buffered saline and frozen on liquid nitrogen. The cells were thawed on ice in the presence of freshly prepared lysis buffer (50 mm Heps, pH 7.6, 150 mm NaCl, 1% (v/v) Triton X-100, 1 mm phenylmethylsulfonyl fluoride, and 100 units/ml bacitracin). The solubilized receptors were partially purified over a WGA-agarose column as described previously.15) Typically, the third 1-ml fraction eluted from the column was used for ligand binding and the phosphorylation of exogenous substrate. \(^{125}\)I-insulin binding assays of the solubilized insulin receptors were done essentially as described previously15) using 25 \(\mu\)l of freshly prepared WGA-purified receptors (20 \(\mu\)g/\(\mu\)l). The assay was incubated for 16 h at 4°C. Bound/free counts were used to calculate the appropriate dilution factor for receptor preparations in each to achieve equivalent ligand-binding concentrations. Portions of WGA-purified receptor preparations were stimulated at 4°C

| Table 1. Cell Clones Used and the Number of Expressed Receptors |
|------------------|------------------|------------------|
| Cell line | Receptor number (x 10^4/cell) | Expressed receptor |
| Neo | ND | mock transfected |
| WT2 | 2.5 | human insulin receptor |
| WT9 | 2.8 | human insulin receptor |
| CHR6 | 2.2 | chimeric insulin receptor |
| CHR20 | 4.1 | chimeric insulin receptor |
in the presence or absence of human insulin in a total volume of 30 μl of buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 0.04% Triton X-100, and 0.01% BSA). Phosphorylation was started by the addition of the artificial substrate poly(Glu: Tyr) 4:1 (Sigma), 5 μCi of [γ-32P]ATP (final activity 6 μCi/mmol), 50 μM ATP, 1 mM CTP, and 50 mM magnesium chloride to a total volume of 90 μl. The reaction was continued for 30 min at 24°C. The reaction was stopped by spotting 70 μl of reaction mixture on a 4-cm² Whatman No. 3MM filter paper and then immediately immersing the paper in ice-cold 10% trichloroacetic acid and 10 mM sodium pyrophosphate. After extensive washing, the filter paper was dried, and the filter-bound radioactivity was measured by counting in a liquid scintillation counter. The nonspecific filter-bound radioactivity was measured on a sample containing WGA-purified receptors, buffer, and radiolabeled ATP.

Figure 3 shows the results of the in vitro kinase assay. No difference in the activity of the phosphorylating exogenous substrate was observed between wild-type and chimeric receptors.

These data indicate that the activity of the intracellular domain of the chicken insulin receptor is essentially the same as that of the human insulin receptor in regard to both ligand-induced autophosphorylation and the phosphorylation of other substrates.

In addition to the receptor itself, transient insulin-stimulated phosphorylation of a 180-kDa protein was seen in WT and CHR cells (Fig. 2B). We have previously shown that this band, which was observed in a study using NIH-3T3 cells, consists of endogenous IRS-1. These data show that the intracellular domain of the chicken insulin receptor phosphorylates mouse IRS-1 as effectively as the human insulin receptor. Chicken IRS-1 cDNA has been cloned and shown to be highly homologous to its mammalian counterpart. Taking this fact and the results of this study into account, it is conceivable that the activated chicken insulin receptor also effectively phosphorylates chicken IRS-1.

It could be concluded from this study that the insulin resistance of chickens is not due to a decreased intrinsic tyrosine kinase activity of the insulin receptor. Rather, it could be attributable to other steps of the insulin action, which may include the binding kinetics of the ligand, post IRS-1 signaling, non-IRS-1 signaling, the interaction with phosphatases, or characteristics of the glucose transporters. The evolutionary preservation of the activity of the chicken insulin receptor itself may suggest that the avian insulin receptor may be important other than its potential involvement in glucose homeostasis.

Acknowledgments

The authors are grateful to Dr. Axel Ullrich for providing a human insulin receptor cDNA clone. We thank Ms. Bethel Stannard for assistance in creating cell clones. This work was supported in part by a
Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science, and Culture, Japan.

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