Baculovirus-directed Expression of the Human Insulin Receptor and an Insulin-binding Ectodomain*

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In this report we describe the use of the baculovirus expression system to overproduce the human insulin holoreceptor (HIR) and a truncated, secretory version of the HIR cDNA (HIRsec) consisting of the α subunit and the extracellular portion of the β subunit (β'). SF9 cells infected with the full-length HIR viruses synthesize a typical size recombinant HIR (rHIR) with an insulin-binding α subunit of apparent M, = 110,000 and a β subunit of apparent M, = 80,000. Un cleaved αβ proreceptor accumulates in infected cells. Both of these forms assemble into higher order disulfide-linked dimers or heterotetramers of apparent M, > 350,000. Insulin-binding activity in cells infected with rHIR viruses is present predominantly on the extracellular aspect of the plasma membrane (>80%). Insulin binding to the full-length rHIR occurs with typical complex kinetics with Kd = 0.5–1 × 10−9 M and receptors are present in large amounts in infected cells (1 × 10^6 receptors/cell; 1–2 mg HIR/10^6 cells). The full-length rHIR undergoes insulin-dependent autophosphorylation; half-maximal activation of α subunit autophosphorylation occurs at 1–2 × 10−8 M. The αβ proreceptor also becomes phosphorylated in vitro. Analysis of tryptic phosphopeptides derived from in vitro autophosphorylated β subunit and αβ proreceptor reveals a pattern of phosphorylation that is indistinguishable from that of authentic placental HIR. SF9 cells infected with rHIRsec viruses synthesize and secrete an (αβ)2 heterotetrameric complex having an insulin-binding α subunit of apparent M, = 110,000 and a truncated β' subunit of apparent M, = 45,000 that lacks kinase activity. The rHIRsec complex purified from the conditioned medium of infected cells binds insulin with high affinity (Kd = 10−16 M).

The human insulin receptor (HIR)1 is an integral mem-

brane glycoprotein composed of two α subunits (apparent M, = 130,000–140,000) and two β subunits (apparent M, = 95,000) linked through disulfide bonds to form an (αβ)2 heterotetrameric complex. The receptor α and β subunits are also becomes phosphorylated in vitro. Analysis of tryptic phosphopeptides derived from in vitro autophosphorylated β subunit and αβ proreceptor reveals a pattern of phosphorylation that is indistinguishable from that of authentic placental HIR. SF9 cells infected with rHIR viruses synthesize and secrete an (αβ)2 heterotetrameric complex having an insulin-binding α subunit of apparent M, = 110,000 and a truncated β' subunit of apparent M, = 45,000 that lacks kinase activity. The rHIRsec complex purified from the conditioned medium of infected cells binds insulin with high affinity (Kd = 10−16 M).

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1 The abbreviations used are: HIR, human insulin holoreceptor; HIRsec, secretory HIR; rHIR, recombinant HIR; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; [125I]Tyrosine, [125I]-labeled tyrosine; [35S]-ATP, [35S]-labeled ATP; [125I]-labeled insulin; [tyrosine-125I]monoiodoinsulin; [35S]-ATP, [35S]-labeled ATP; [125I]-labeled insulin; [tyrosine-125I]monoiodoinsulin.

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a recombinant baculovirus (19) encoding the entire HIR homologous region of the insulin receptor/10 infected cells) of insulin receptor complexes that are fully active with respect to both insulin binding and insulin-dependent tyrosine protein kinase activities. Sf9 cells infected with a virus encoding a secreted soluble insulin-binding protein complex that binds insulin with high affinity and linear binding isotherms.

EXPERIMENTAL PROCEDURES

Materials

Crystalline porcine insulin was from Novo (Copenhagen) and was stored at −20 °C as a stock solution in 0.01 N HCl at 6 mg/ml (10−3 M). Enzymes for molecular biology were purchased from Boehringer Mannheim, New England Biolabs, or Pharmacia LKB Biotechnology Inc. [tyrosine-3H]Monodiodoinsulin was a gift from Dr. Bruce Frank, Lilly Co., and [tyrosine-3H]monodiodoinsulin was purchased from Amersham Corp. All other biochemicals were purchased from Du Pont-New England Nuclear. Agarose-bound lectins were purchased from Vector Laboratories, and N-acetylglucosamine was purchased from Sigma. Triton X-100 (SurfaceAmpe) and disucinamidobenzene were purchased from Pierce Chemical. Protease inhibitors were purchased from Boehringer Mannheim and Sigma. Grace’s insect medium and fetal bovine serum were purchased from GIBCO and Grace’s powder concentrate was purchased from KC Biologics.

Cells

The insect cell line Sf9 (S. frugiperda) was obtained from Dr. Max Summers (Texas A & M University). The transfer vector pAc401 contains the wild-type polyhedron ATG sequence. Plasmids pSP64-HIR-4 and pSP64-HIR-11 (see Fig. 1) contain the full-length insulin receptor cDNA cloned from the B- untranslated region up to and including nucleotide 2998 from the B-untranslated region up to and including nucleotide 2998 of the HIR cDNA insert. A stop codon has been introduced at this point and is followed by a Sall site.

Recombinant plasmids were cotransfected into Sf9 cells along with wild-type AcNPV viral DNA by calcium phosphate transfection procedures as previously described (20). In vivo homologous recombination between the polyhedron sequences in the wild type viral DNA and the recombinant plasmids resulted in the generation of recombinant viruses coding for a fused gene product at a frequency of less than 0.1% (results not shown). The recombinant viruses were then plaque purified by growing the colonies (polyhedron negative) phenotype or by colony hybridization using 32P-RNA probes covering the 5′-1.1 kilobase of the HIR-coding region. The method used for the colony hybridization procedure was essentially identical to that described originally by Summers and Smith (20) with the exception that the 32P-labeled RNA probes instead of DNA probes were used. 32P-RNA probes were synthesized using SP6 RNA polymerase (Promega Biotech) according to the manufacturer’s specifications.

Southern Blot Analysis of Recombinant Virus

Recombinant viral DNA was purified from the conditioned medium of infected Sf9 cells as described by Smith and Summers (20). Agarose gel electrophoresis, Southern blot transfer of restriction endonuclease cut DNA to nitrocellulose (B&K, Schleicher & Schuell), and hybridization of 32P-labeled DNA probes to nitrocellulose filters were carried out as described by Maniatis et al. (21) and by Promega Biotech Technical Notes (22).

Detection of Expression of Recombinant Insulin Receptor

Sf9 cells were plated in 24-well dishes (Costar) at 3 × 105 cells/well and allowed to attach for 2 h in complete Grace’s medium. Cells were infected with wild-type AcNPV isolate E2 or recombinant viruses encoding the truncated (HIRsec) or full-length (HIR) versions of the human insulin receptor. Two days post-infection, the cell layer and the conditioned medium were assayed for the presence of insulin binding activity by incubating the cells or the conditioned medium with 125I-A1-human insulin for 2 h at 4 °C in binding buffer (Hank’s balanced salts supplemented with 50 mM Hepes, pH 7.6, and bovine serum albumin at 1 mg/ml) and then precipitating the bound insulin with ice-cold 3% trichloroacetic acid. The cell layers were washed twice with ice-cold Hank’s balanced salts and then lysed with 0.1 N NaOH for 30 min at 37 °C. The solubilized cell extracts were then collected and counted in a gamma counter. The insulin binding activity in the conditioned medium was assayed using a polyethylene glycol precipitation assay (23) using 125I-A1-Insulin as tracer.

For the determination of binding affinities of recombinant insulin receptors in whole infected Sf9 cells, insulin binding to attached cells was carried out in a reaction volume of 200 μl with 125I-A1-insulin (30 pmol) and increasing concentrations of unlabeled insulin (10−8 to 10−4 M) for 2 h at 4 °C. The cells were then washed twice in ice-cold binding buffer, lysed with 200 μl of 0.1 N NaOH at 37 °C for 30 min and were then counted in a gamma counter. The data were corrected for nonspecific binding (less than 5% total counts) and analyzed by the method of Scatchard (24) using the IGAND program of Rodbard and Munson (25).

Insulin Binding to Solubilized Insect Cells

Partial purification of the baculovirus-encoded HIR and HIRsec proteins was achieved using wheat germ agglutinin-agarose (Vector Laboratories) in a modification of the affinity procedure of Fujita-Yamaguchi et al. (26). Baculovirus-infected Sf9 cells were harvested and washed once in ice-cold Grace’s medium. Cells were then lysed in buffer A containing 1% Triton X-100 (Pierce Chemical Co.), 50 mM Hepes, pH 7.6, 150 mM NaCl, 2 mM of phenylmethylsulfonyl fluoride, 5 mM K-64, 5 mg/ml Leupeptin, 5 mg/ml pepstatin A, 5 mg/ml aprotinin, and 5 mM EDTA for 30 min at 4 °C. The lysates were centrifuged for 5 min at 10,000 × g at 4 °C and then diluted 2-fold prior to absorption to a wheat germ agglutinin-agarose column. Binding was allowed to proceed for 1 h at 4 °C batch-wise, after which the bound proteins were eluted from the column (Económ gel, Bio-Rad) with 10 volumes of buffer B (buffer A with 0.1% Triton X-100 instead of 1%, and 500 mM NaCl instead of 150 mM), and the bound proteins were subsequently eluted with buffer C (buffer B plus 500 mM N-acetylglucosamine). The eluate was assayed for insulin binding activity as described below and pooled fractions were stored at −80 °C.

Soluble insulin binding assays were performed in a modification of the procedure of Harrison and Itin (27) and the data analyzed by the method of Scatchard (24).

Chemical cross-linking of partially purified recombinant HIR and
HIRsec complexes with 125I-At or 125I-B26-insulin was achieved with disuccinimidyl suberate (Pierce Chemical Co.) essentially as described by Pilch and Czech (28). Insulin receptor preparations were incubated with 125I-At or 125I-B26-insulin (1–2 nM) for 16–20 h at 4°C. Disuccini-
idyl suberate was then added to a final concentration of 200 μM and incubated for 15 min on ice. The reaction was then terminated by the addition of a 10-fold molar excess of Tris-HCl, pH 7.5, and the affinity labeled insulin receptors were immunoprecipitated as described below with monoclonal antibody 83–14. The immune complexes were then resuspended in SDS-sample buffer and were analyzed by SDS-PAGE (29).

**Receptor Kinase Assays**

Insulin receptor autophosphorylation was carried out by a modification of the procedure of Tavare and Denton (30). In some experiments, autophosphorylated insulin receptors were immunoprecipitated with a monoclonal antibody (MAb 83–14, kindly provided by Dr. Kennethiddle, University of Cambridge) directed against the α subunit of the insulin receptor (31). In these experiments, the autophosphorylation reactions were terminated by the addition of EDTA to 10 mM, and the labeled receptor subunits were immunoprecipitated in the presence of 0.1% SDS, 0.5% Triton X-100, 150 mM NaCl, and 50 mM Hepes, pH 8.0, overnight at 4°C. The immune complexes were then bound to protein A-Sepharose beads for 2–4 h at 4°C. The complexes were centrifuged briefly and were washed in the presence of 0.1% SDS, 0.5% Triton X-100, 500 mM NaCl, and 50 mM Hepes, pH 8.0. The immune complexes were then suspended in SDS-sample buffer and the immunoprecipitated proteins were analyzed by SDS-PAGE (29).

Phosphopeptide mapping of the in vitro autophosphorylated HIR subunit was performed according to the procedure of Tavare and Denton (30). Autophosphorylated subunits were immunoprecipitated with monoclonal antibody 83–14 and separated by SDS-PAGE in the presence of reducing agent. The β subunit and uncleaved α2 proreceptors were located by autoradiography of the untreated gel and the gel chips containing the isolated 70-kDa labeled HIR subunits were treated as described previously (30).

**Metabolic Labeling of Injected Cells**

S2B cells were grown to a density of 1.5–2 × 10^7 cells/ml in Complete Grace's medium plus 0.1% Phorionic Polycl F-64 and then infected at a multiplicity of infection of 10. Twenty-four h post-infection, cells were centrifuged at 2500 rpm for 5 min, washed twice in TNM-FH lacking methionine, and were then resuspended at the original density in TNM-FH (methionine) + 10% dialyzed fetal bovine serum (GIBCO) for 1 h. Cells were then washed once in TNM-FH (methionine) + 10% dialyzed fetal bovine serum + 0.1% Phorionic Polycl F-64 + 100 μCi/μl [35S]methionine (Amersham Corp.) and were cultured for 4 h at 27°C in a New Brunswick Gyrotary shaker at 140 rpm. At the end of this pulse labeling period, cells were centrifuged at 2500 rpm for 5 min, washed twice in Complete Grace's medium, and were then cultured in the original volume of Complete Grace's medium containing 0.1% Phorionic Polycl F-64 for the indicated chase times. Cell harvesting, lysis, and immunoprecipitation of metabolically labeled HIR subunits was described elsewhere.

**Analysis of Oligosaccharide by Endoglycolytic Cleavage**

Partially purified recombinant HIR and HIRsec complexes were incubated with 1 μCi of 125I-tyrosine-B26-insulin overnight at 4°C prior to chemical cross-linking with 200 mM disuccinimidyl suberate as described previously (16, 28, 30). Affinity labeled insulin receptors were then immunoprecipitated (as described above) with monoclonal antibody 83–14 and were then subjected to cleavage with either N-glycanase (Genzyme, Boston) or Endoglycosidase H (Genzyme, Boston) according to the manufacturer's specifications. The reaction products were then separated in SDS-sample buffer and analyzed by SDS-PAGE in the presence of reducing agent (29).

**Electrophoresis and Autoradiography**

Characterization of recombinant proteins was carried out using denaturing SDS-polyacrylamide gel electrophoresis in a discontinuous buffer system (29). Autoradiography was performed at −70°C using Kodak XAR-5 film and a Du Pont Cronex Lightning plus enhancing screen. The molecular weight markers (apparent M) used were myosin (200,000), β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), and ovalbumin (45,000) (Bio-Rad).

**RESULTS**

The two recombinant baculoviruses we constructed are based on a human insulin receptor cDNA derived from human kidney mRNA, and therefore include exon 11 (5, 6, 32). The strategy used for the construction of the HIR recombinant baculovirus encoding the full-length human insulin receptor (HIR virus) is outlined in Fig. 1. The resulting recombinant plasmid (pAc401-HIR) codes for a fusion protein in which the first 2 amino acid residues are contributed by the AcNPV polyhedron gene (Met-Arg), the third amino acid (Pro) is generated as a result of the ligation, and the remaining 1834 amino acids are HIR coding.

The strategy for constructing the truncated HIRsec baculovirus is essentially the same as that used for constructing the HIR virus except that a 3.3-kilobase NcoI-SalI restriction fragment of the mutant HIRsec gene was blunt end ligated into Smal cut pAc401 after treatment of the HIRsec insert.
with the Klenow fragment of DNA polymerase I. The resulting 
transfer plasmid (pAc401-HIRasec) has the same structure 
surrounding the initiation codon as does pAc401-HIR but 
terminates at amino acid residue 917 of the HIR sequence 
and thus lacks the transmembrane and cytoplasmic portions 
of the β subunit (5, 6, 33).

Recombinant baculoviruses were generated by introducing 
pAc401-HIR or pAc401-HIRsec transfer plasmids into the 
Sf9 cell line together with wild-type AcNPV viral DNA by 
calcium phosphate transfection (20). Recombinant viruses 
were identified and subsequently isolated by a combination 
of visual screening and colony hybridization using RNA 
probes directed against HIR-coding region sequences (data 
not shown). The structure of these viruses was confirmed by 
Southern blot hybridization of viral DNA (data not shown).

The viruses were screened for their ability to encode func-
tional insulin receptors in a whole cell insulin binding assay. 
Sf9 cells were infected with recombinant viruses and both the 
cells and infected cell culture supernatants were assayed 
directly for the presence of insulin binding activity. Fig. 2 
shows this analysis for three HIR viral isolates and three 
HIRsec viral isolates. Sf9 cells infected with several inde-
pendently isolated viral clones of both types of viruses had 
significant levels of specific insulin binding activity, while 
neither uninfected cells nor cells infected with wild-type 
AcNPV had significant specific insulin binding activity. 
Insulin binding activity as measured by chemical cross-linking 
of 125I-B26-insulin in cells infected with HIR viruses is present 
on the surface (greater than 80%) or in the cytoplasm (less 
than 20%) of infected cells (data not shown). The insulin 
binding activity of cells infected with the HIRsec viruses is 
partitioned between the medium and the cells as indicated in 
Fig. 2 (see below).

Fig. 3 shows a time course of expression of insulin binding 
activity for cells infected with the HIR virus. Insulin binding 
activity increased from 36 h post-infection until peak levels 
of cell-associated insulin binding activity were found between 
72 and 96 h post-infection. The maximum increase in specific 
insulin binding was approximately 10-fold. The appearance 
of insulin binding activity in cells infected with the HIRsec 
viruses paralleled that of cells infected with HIR viruses; 
insulin binding activity in the cells increased from 36 h 
post-infection to a maximum at 72-96 h post-infection. At 
early times post-infection (24-48 h) most of the insulin bind-
ing (up to 70%) was associated with the cell layer in HIRsec-
infected cells and only 20-30% was present in the conditioned 
medium as a soluble secretory protein complex. At late times 
post-infection (72-96 h) up to 45% of the insulin binding 
avtivity was present in the conditioned medium of HIRsec 
cells.

Immunofluorescent staining of live and formaldehyde-fixed 
infected cells with a monoclonal antibody (83 14) (31) di-
rected against the α subunit of HIR confirmed that the 
recombinant HIR is expressed on the surface of infected cells 
(data not shown). Control experiments using the same anti-
body failed to reveal any specific staining of either uninfected 
or wild-type AcNPV infected Sf9 cells. These data confirm 
the results of insulin binding in indicating that the orientation 
of the recombinant HIR in the plasma membrane is correct

**Fig. 2. Insulin-binding screen of recombinant viruses. Sf9** 
cells were plated in 24-well dishes (3 × 10^5 cells/well), infected 
with wild-type AcNPV virus isolate E2 (WT), recombinant viruses 
encoding either HIR or HIRsec. Two days post-infection, the cell layer 
(upper panel) and the conditioned medium (lower panel) were assayed 
for the presence of insulin binding activity by a direct insulin binding 
assay (28). Black bars indicate the specific binding of 125I-A26-insulin 
precipitated from cell extracts or from conditioned medium (cpm/ 
well) when the binding was done in the absence of unlabeled porcine 
insulin. Open bars indicate the nonspecific binding of 125I-A26-insulin 
precipitated from cell extracts or from conditioned medium when the 
binding reaction was done in the presence of 10^-6 M porcine insulin. 
Each data point represents the average of three experiments. The 
letters below each pair of bars designate the individual clonal isolates 
of each virus.

**Fig. 3. Time course of HIR expression. Sf9** cells were plated 
in 24-well dishes (3 × 10^5 cells/well) and were infected with wild-type 
AcNPV virus isolate E2 (WT), recombinant HIR virus (HIR), or with 
Complete medium (Mock). At 24 h intervals post infection, cells were 
washed twice with ice-cold phosphate-buffered saline and then lysed 
with buffer containing 1% Triton X-100. Cell lysates were then 
analyzed for insulin binding activity with a soluble insulin receptor 
assay. Specific binding was determined in the absence of unlabeled 
porcine insulin, while nonspecific binding of 125I-A26-insulin was 
determined in the presence of 10^-6 M unlabeled porcine insulin. 
Counts were normalized with respect to cell number.
at least with respect to the α subunit.

Correct expression of the recombinant HIR sequences in infected S/9 cells should result in the accumulation of insulin receptor subunits. Fig. 4 shows the results of experiments in which the α and β subunits of various recombinant HIRs were characterized by SDS-PAGE. Panel A of Fig. 4 shows the results of an experiment in which detergent-solubilized infected cell proteins were incubated with 125I-B26-insulin, treated with the chemical cross-linker disuccinimidyl suberate, immunoprecipitated with an anti-HIR monoclonal antibody, and then analyzed by SDS-PAGE in the presence of reductant. Lanes 1 and 2 contain in them 125I-B26-insulin-labeled proteins immunoprecipitated from the NIH 3T3 3.5 cell line that expresses approximately 5 × 10⁶ receptor complexes/cell (15). In these cells, the insulin binding α subunit has an apparent Mᵦ = 140,000, and the uncleaved proreceptor has an apparent Mᵦ = 190,000–200,000 (see lanes 1 and 2, panel A). When 125I-B26-insulin-labeled HIR subunits synthesized by these cells are analyzed by SDS-PAGE in the absence of reductant, a single labeled species of apparent Mᵦ >350,000 is observed. This corresponds to the (αβ)₂ heterotetrameric HIR complex.

In HIR-infected insect cell lysates (lanes 3 and 4), two anti-HIR reactive proteins are cross-linked specifically to 125I-B26-insulin. The larger has an apparent Mᵦ = 185,000, while the smaller has an apparent Mᵦ = 110,000. In the absence of reductant, a single immunoreactive species of apparent Mᵦ >350,000 is detected (panel B, Fig. 4).

In HIRsec-infected cells (lanes 5 and 6), two anti-HIR reactive proteins are detected. The larger of these two proteins has an apparent Mᵦ = 140,000–145,000, while the smaller has an apparent Mᵦ = 110,000. This smaller cross-linked protein comigrates with the Mᵦ = 110,000 125I-B26-insulin-labeled protein synthesized in HIR-infected insect cells and corresponds to the mature processed α subunit. The larger insulin-binding species corresponds in size to the predicted molecular mass for the uncleaved HIRsec proreceptor from which approximately 45 kDa of β subunit have been deleted.

The subunit molecular weight of the catalytically active β subunit was determined by labeling it with 32P₀₄ in an in vitro autophosphorylation reaction, immunoprecipitating the autophosphorylated HIR complexes with a monoclonal anti-HIR antibody, and then analyzing the reaction products by SDS-PAGE. The results of this analysis are shown in Fig. 5. In panel A, immunoprecipitated proteins were treated with reductant prior to separation by SDS-PAGE, while in panel B the immunoprecipitated proteins remain unreduced.

In the absence of excess unlabeled porcine insulin, a 4–10-fold increase in 32P₀₄ incorporation into the β subunit is obtained. In the absence of reductant, all of the HIR-specific 32P₀₄ label is present in a complex of apparent Mᵦ >350,000, which corresponds to the (αβ)₂ heterotetrameric complex.

From the data presented in Figs. 4 and 5, we conclude that the insulin-binding α subunit of recombinant HIR expressed in insect cells. The insulin binding properties of recombinant HIR subunits produced in the overproducing murine cell line (HIR 3T3 3.5) (16) and in insect cells (S/9 cells) were analyzed by chemically cross-linking human 125I-tyrosine-B26-insulin to solubilized HIR with disuccinimidyl suberate. Specifically cross-linked HIR subunits were detected following immunoprecipitation with a monoclonal anti-HIR antibody and protein A-Sepharose beads by electrophoresis on 4–10% gradient gels in the presence (panel A) or absence (panel B) of reductant. In both panels, lanes 1 and 2 indicate HIR cell 3T3 3.5 extract; lanes 3 and 4, S/9 cells infected with HIR virus extract; lanes 5 and 6, S/9 cells infected with HIRsec virus extract. Lanes 1, 3, and 5 show cross-linking in the absence of excess unlabeled porcine insulin. Lanes 2, 4, and 6 show cross-linking in the presence of excess (10⁻⁴ M) porcine insulin. Molecular mass standards (in daltons) are indicated with arrows.
in Sf9 cells has a relative $M_t = 110,000$, that the $\beta$ subunit has a relative $M_t = 80,000$ and is autophosphorylated in an insulin-dependent fashion, and that the $\alpha$ and $\beta$ subunits assemble normally into a heterotetrameric ($\alpha\beta_2$) complex with a relative $M_t > 350,000$. Since the $M_t = 185,000$ species can be cross-linked to insulin, it is phosphorylated in response to insulin, is immunoprecipitable with a species-specific anti-HIR monoclonal antibody, and is assembled into a higher molecular mass disulfide-bonded oligomeric species seen on non-reducing gels, we conclude that this species is the HIR proreceptor polypeptide that has not been proteolytically cleaved at the RKRR tetrabasic sequence at the $\alpha\beta$ subunit junction. Densitometric analysis of the experiments shown in Fig. 4 indicates that the uncleaved proreceptor accounts for 30–60% of the HIR subunits in an infected cell culture as probed by these chemical cross-linking experiments (data not shown).

HIRsec-infected insect cells also synthesize insulin-binding $\alpha$ subunits of apparent $M_t = 110,000$ and 145,000. The $M_t = 145,000$ species is presumably the uncleaved HIRsec proreceptor polypeptide and the $M_t = 110,000$ polypeptide is the mature, fully processed $\alpha$ subunit.

To determine more precisely the ratio of cleaved to uncleaved rHIR subunits synthesized in infected cells, HIR-infected Sf9 cells virus were pulsed for 4 h with $[^{35}S]$methionine (24-h post-infection) and then chased with medium containing an excess of unlabeled methionine for varying lengths of time (Fig. 6). Panel A of Fig. 6 shows the results of such a pulse-chase experiment, while panel B shows the results of steady-state labeling of proreceptor and $\alpha$ subunits of rHIR.

Uncleaved rHIR proreceptor is converted to mature, cleaved $\alpha$ subunit very slowly relative to the rate at which conversion occurs in mammalian cells (3, 4). Following a 4-h pulse labeling, mature $\alpha$ subunit accounts for 15–20% of the total radiolabel incorporated specifically into rHIR subunit. Following a 24-h chase period, mature $\alpha$ accounts for approximately 35–40% of total label in immunoprecipitated rHIR subunits. Under steady-state labeling conditions (Fig. 6, panel B), there are equal amounts of label incorporated into uncleaved proreceptor and cleaved $\alpha$ subunits (proreceptor = 50–60%, $\alpha$ subunit = 40–50%). We therefore conclude that maximally 50% of rHIR proreceptor is converted to mature $\alpha$ and $\beta$ subunits and that both rHIR proreceptor and cleaved $\alpha$ and $\beta$ rHIR subunits are equally stable in Sf9 insect cells.

We estimate the approximate $t_\alpha$ of rHIR proreceptor to be approximately 24 h, while $t_{\alpha\beta}$ for appearance of mature $\alpha$ subunit is similar. In these experiments we have been unable to accurately measure the rate of appearance of the $\beta$ subunit due to comigrating $[^{35}S]$methionine-labeled bands that were coprecipitated with it in the gel system used for these studies.

The contribution of N-linked oligosaccharides to the apparent size of the baculovirus-encoded recombinant HIR subunits was determined by cleavage of $[^{125}I]$-insulin-labeled HIR subunits with Endoglycosidase H and N-glycanase. Cleavage of $[^{125}I]$-insulin-labeled recombinant HIR with N-glycanase at the linkage between asparagine and the proximal N-acetylgalactosamine results in a reduction in apparent $M_t$ of the $\alpha$ subunit from 110,000 daltons to 103,000–105,000 daltons, a decrease of 5,000–7,000 daltons (Fig. 7). The $[^{125}I]$-insulin-labeled $\alpha\beta$ proreceptor undergoes an apparent shift of approximately 30 kDa from 185 to 155 kDa. Cleavage with Endoglycosidase H results in a similar decrease in size for both the proreceptor and $\alpha$ subunit. The fact that recombinant HIR is sensitive to Endoglycosidase H suggests that the N-linked sugars are not of the complex type and therefore do not have terminal branching structures. This conclusion is supported by the results of studies in which baculovirus-encoded HIR was assessed for its ability to bind to a variety of lectin affinity supports (data not shown). While recombinant HIR bound to lectins specific for the constituent sugar residues of the N-linked core (wheat germ agglutinin, concanavalin A, lentil, and pea lectins), it did not bind to lectins specific for terminal N-acetylgalactosamine residues (i.e. *dolichos biflorus* agglutinin) nor was it sensitive to treatment with neuraminidase (data not shown).

Fig. 8 shows the results of comparative binding experiments in which the insulin binding properties of recombinant HIRs...
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Binding experiments reveals that ligand binding occurs with typical complex kinetics (as reflected in curvilinear Scatchard plots with $K_d = 2-4 \times 10^{-9} \text{ M}$ and $K_{u2} = 1 \times 10^{-7} \text{ M}$). Binding affinity studies with detergent solubilized, partially purified recombinant HIR indicate that both binding affinities increase such that $K_{d1} = 0.3-0.6 \times 10^{-9} \text{ M}$ and $K_{u2} = 0.8 - 1 \times 10^{-7} \text{ M}$ (see Fig. 8, middle panel). Scatchard analysis of insulin binding to intact cells showed that infected insect cells have $5-8 \times 10^{5}$ low affinity sites and $1-2 \times 10^{6}$ high affinity sites, with a total of $7-10 \times 10^{6}$ insulin binding sites/cell. This corresponds to approximately $1-2 \text{ mg HIR/liter}$ of cells at cell densities of $1-2 \times 10^{9} \text{ cells/liter}$.

Competitive binding experiments with HIRsec polypeptides purified from the culture supernatant of infected Sf9 cells are shown in Fig. 8, lower panel. Upon Scatchard transformation of the binding data, a linear plot is obtained from which a $K_d = 0.5-1 \times 10^{-6} \text{ M}$ is derived.

Characterization of the insulin-dependent autophosphorylation activity of the recombinant HIR is shown in Figs. 9 and 10. Fig. 9, panel A, is an autoradiogram of autophosphorylated HIR subunits that have been activated in the presence of increasing amounts of porcine insulin at $22^\circ \text{ C}$. The incorporation of $^{32}\text{P}0_4$ into both the mature $\beta$ subunit (apparent $M_r = 80,000$) and the uncleaved proreceptor (apparent $M_r = 185,000$) increases with increasing insulin concentration. This result is shown graphically in Fig. 9, panel B, in which the percent maximal stimulation of autophosphorylation activity has been measured by densitometric scanning of the autoradiogram shown in panel A. From these data, half-maximal stimulation of insulin-dependent autophosphorylation of the recombinant HIR $\beta$ subunit occurs at $1-2 \times 10^{-9} \text{ M}$ insulin, while half-maximal stimulation of insulin-dependent autophosphorylation of the recombinant HIR proreceptor occurs at $2-2.5 \times 10^{-9} \text{ M}$ insulin. These values for the mature receptor

![Fig. 8. Scatchard analysis of insulin binding to recombinant HIR. Top, Scatchard transformation of binding isotherm for solubilized recombinant HIR partially purified from an overproducing murine cell line NIH 3T3 3.5. $K_{d1} = 1-2 \times 10^{-9} \text{ M}; K_{u2} = 1 \times 10^{-7} \text{ M}$. Inset, binding isotherm of solubilized HIR purified from NIH 3T3 3.5 cells. Middle, Scatchard transformation of binding isotherm for recombinant full-length HIR holorecceptor expressed in Sf9 insect cells. $K_{d1} = 2-4 \times 10^{-9} \text{ M}; K_{u2} = 1 \times 10^{-7} \text{ M}$. Inset, binding isotherm of solubilized HIR holoreceptor from Sf9 cells infected with HIR-encoding baculovirus. Bottom, Scatchard transformation of binding isotherm for recombinant secretory HIRsec-truncated receptor expressed in the medium of Sf9 insect cells. $K_{d} = 0.5-1 \times 10^{-6} \text{ M}$. Inset, binding isotherm of secreted HIRsec-truncated receptor purified from the conditioned medium of Sf9 insect cells infected with HIRsec-encoding baculovirus.](image-url)

![Fig. 9. Dose response curve of insulin-dependent autophosphorylation. Partially purified insulin receptor was autophosphorylated and immunoprecipitated as detailed under "Experimental Procedures." After SDS-PAGE in the presence of reductant on a 4-10% gradient gel and following autoradiography, the films were scanned using an LKB soft laser densitometer and the data were plotted as % maximal stimulation versus log [insulin]. The data were normalized by setting the basal level of autophosphorylation at 0 and the maximal level of autophosphorylation at 1. Closed circles, $\beta$ subunit autophosphorylation. Open circles, proreceptor autophosphorylation. Half-maximal stimulation for $\beta$ subunit = $1-2 \times 10^{-9} \text{ M}$. Half-maximal stimulation for proreceptor = $2-2.5 \times 10^{-9} \text{ M}$. Inset, autoradiogram of the autophosphorylated HIR subunits. $\beta$ subunit, 80 kDa; proreceptor, 185 kDa.](image-url)
are in close agreement with the values obtained with partially purified placental HIR.

We have also mapped the sites into which $^{32}$PO$_4$ is incorporated in in vitro phosphorylated mature β subunit and in the uncleaved proreceptor using the two-dimensional phosphopeptide mapping system described by Tavaré and Denton (30). Fig. 10, panel A, is an autoradiogram of the phosphopeptides obtained from tryptic digestion of in vitro autophosphorylated recombinant β subunit, while panel B shows an autoradiogram of the phosphopeptides obtained from tryptic digestion of in vitro autophosphorylated recombinant proreceptor. Panel C shows a schematic tracing of the previous characterized phosphopeptides that are obtained from in vitro autophosphorylated placental HIR β subunit analyzed in the same chromatographic system (19). Several tentative conclusions can be drawn from these maps. First, the spectrum of phosphopeptides obtained from both the mature β subunit and the uncleaved proreceptor appear to be highly similar, if not identical (compare panel A with panel B). Second, based on the assignments of Tavaré and Denton (19), there are phosphopeptides from each of the 3 tyrosine phosphate-acceptor domains of the β subunit, i.e., the juxtamembrane region containing tyrosines 953, 960, and 972 (C1', C2, and C3 in Fig. 10C), the “kinase/catalytic” domain containing tyrosines 1316 and 1322 (B1 in Fig. 10C), and the carboxyl-terminal site(s) containing tyrosines 1316 and 1322 (B1 in Fig. 10C). Finally, phosphopeptides from the kinase/catalytic domain of the β subunit include mono- and biphenylphosphorylated peptides, as shown by the presence of phosphopeptides A1, A2, B2, B3, and C1.

**DISCUSSION**

The baculovirus expression system has been used successfully to express a wide variety of active proteins, including nuclear localized, cytoplasmic, membrane-associated, and secreted proteins (19 and references therein). Recently, several groups have used this system to express the epidermal growth factor receptor (36) and a soluble version of its cytoplasmic kinase domain (37) and soluble versions of the insulin receptor family (16, 17) and extracellular ligand binding domains (18). In this paper, we describe the expression of the full-length human insulin holoreceptor and a truncated, secreted version of the extracellular ligand-binding domain in baculovirus-infected Sf9 insect cells.

At the structural level, baculovirus-encoded HIR is highly similar to HIR expressed in mammalian systems. First, the HIR proreceptor polypeptide is proteolytically processed albeit inefficiently, into mature α and β subunits that, respectively, bind insulin with high affinity and catalyze autophosphorylation of HIR in response to insulin. Second, both the proteolytically cleaved α and β subunits and the uncleaved proreceptor assemble into an $\alpha(\beta_2)_2$ heterotetrameric complex. Finally, the assembled heterotetrameric complex is present on the cell surface and is inserted into the plasma membrane with the proper orientation.

The full-length recombinant HIR produced in insect cells is also in most respects functionally identical with the authentic placental or hepatic human insulin receptors. Competitive binding experiments indicate that the kinetics of insulin binding to this recombinant HIR are indistinguishable from authentic placental HIR. The recombinant HIR has complex curvilinear binding with two classes of insulin-binding sites; the high affinity sites have $K_u = 0.5-2 \times 10^{-9}$ M, while the low affinity sites have $K_u = 1.2 \times 10^{-7}$ M. These values are in close agreement with the values obtained with partially purified receptor preparations from human placenta and liver as well as from recombinant HIR expressed in mammalian cell lines (see Fig. 8). The insulin-dependent autophosphorylation activity of the recombinant HIR expressed in baculovirus-infected Sf9 cells is also virtually indistinguishable from that of authentic placental HIR. Like partially purified HIR, half-maximal stimulation of HIR autophosphorylation occurs between 1–2 $\times 10^{-8}$ M insulin, which is in good agreement with previously published values of 6–8 $\times 10^{-9}$ M (34, 35). Moreover, the sites in the β subunit that are phosphorylated in vitro in response to insulin appear to be identical to the sites that are phosphorylated in vitro on partially purified placental HIR (Fig. 8).

Baculovirus-encoded HIR subunits ($\alpha = 110$ kDa, $\beta = 80$ kDa) are smaller than their mammalian counterparts ($\alpha = 135-145$ kDa, $\beta = 95$ kDa) because the type of asparagine-linked oligosaccharides added differ. While both mammalian and insect cells initially transfer a high mannose oligosaccharide dolichol-phosphate precursor (Glc$_3$Man$_n$(GlcNAc)$_2$) to asparagine residues in the nascent polypeptide chain, mammalian cells subsequently trim this high mannose structure to a core oligosaccharide (Man$_n$(GlcNAc)$_2$) and then add a variety of terminal sugar residues (38). Insect cells, on the other hand, do not add terminal sugars after trimming the high mannose structure (39). These differences in N-linked oligosaccharide structure in
baculovirus-encoded HIR do not appear to alter its function from that of the fully glycosylated placental HIR. Furthermore, it would appear that these differences in carbohydrate side chains do not impair the folding or acquisition of tertiary structure of the nascent proreceptor molecule in the endoplasmic reticulum/Golgi complex (3, 40, 41).

In these studies we have assessed the contribution of N-linked oligosaccharides only to baculovirus-encoded HIR subunit size. The presence of O-linked oligosaccharides on the HIR β subunit has been reported (42) although neither the precise structure of this carbohydrate nor its functional significance is understood. Since Sf9 cells are capable of O-linked glycosylation (see Ref. 19 for review) of exogenously expressed proteins, we assume that the putative O-linked site(s) on the HIR β subunit expressed in these cells are occupied.

Baculovirus-encoded HIR also differs from mammalian HIR in that the extent of conversion of the proreceptor to mature α and β subunits is lower in insect cells than in mammalian cells. While 80–90% of proreceptor is converted to mature subunits in the overproducing NIH 3T3 3.5 cell line, only 40–60% of the baculovirus proreceptor is proteolytically processed to mature subunits in infected insect cells. A similar relative decrease in the efficiency of proteolytic conversion has been seen for the HIV-1 gp160 glycoprotein expressed in Sf9 cells (43). In this case, less than 5% of the gp160 proprotein is converted into gp40 and gp120 by cleavage at the sequence -REKR-, while in mammalian cells it is considerably higher (15%) (44). It is not clear whether these cleavage sequences are poorly recognized cleavage substrates in insect cells or the overproduction of these proteins simply saturates the converting enzyme(s). Recently, Yoshimasa et al. (45) have studied a human mutant/HIR allele that has a single amino acid substitution in the tetra-basic cleavage site (Arg735-Ser735) that prevents complete cleavage into α and β subunits. They have recently expressed this mutant allele in the baculovirus system and found that it is not processed. This suggests that Sf9 cells recognize the tetra-basic-RKRR- sequence normally.

Surprisingly, the proreceptor is autophosphorylated in response to insulin with an ED50 that is very similar to that of the mature receptor (Fig. 9) and at the same sites (Fig. 10). These findings suggest that the proreceptor tyrosine kinase is fully active as an insulin-dependent autophosphorylating enzyme that the proreceptor can serve as a substrate for transphosphorylation (either intras- or interheterotetramer), or both. From the work of Kakehi et al. (46) with the cleavage defective mutant human proreceptor, it seems clear that the pure uncleaved human insulin proreceptor expressed in transformed lymphocytes has a significantly reduced affinity for insulin. Whether in our experiments the proreceptor merely serves as a substrate for phosphorylation by insulin-activated (αβ)2 heterotetramers is currently being tested directly.

It should be emphasized that the cDNA construct we have used in these expression studies corresponds to the larger of the two known alternatively spliced mRNA species that is generated from the HIR gene (5, 6, 32). The 36-base pair exon 11 is alternatively spliced in a number of tissues (32). The 12 amino acids encoded by this exon are just amino terminal to the -RKRR- cleavage site in the proreceptor. Some cell types express HIR mRNA from which this 36-base pair exon is constitutively excluded (brain, Epstein-Barr virus transformed lymphocytes, and spleen), while others express HIR mRNA in which this exon is constitutively included (liver and kidney). Finally, some cell types express a mixture of exon 11 and 11' mRNAs (placenta and adipose tissues) (32). While the significance of this alternative splicing event is not understood, it is possible that the presence or absence of this 12 amino acid segment exerts regulatory effects on proreceptor processing and/or on the affinity of the proreceptor for insulin and its catalytic activity.

In addition to expressing the full-length HIR cDNA in the baculovirus expression system, we have also expressed a truncated, secretory version of the insulin-binding ectodomain. We find that while this form of the insulin-binding region of the HIR is produced in large quantities, it differs somewhat structurally and functionally from the secreted forms of the HIR ectodomain expressed in other cell types (33). In the first instance, only a fraction of the total HIRsec polypeptides synthesized in these cells is secreted into the conditioned medium (approximately 30–50%). The remainder of the HIRsec polypeptide never leaves the cell, presumably because it is either aggregated or otherwise misfolded. Second, only 50% of the material that is secreted from the cells is proteolytically processed to mature IIR subunits; therefore, only 15–25% of the total HIRsec polypeptide produced by baculovirus-infected cells is processed at the α-β cleavage site.

The second observation concerning the HIRsec construct is that its insulin binding activity differs from that of the full-length HIR complex in that the data from binding displacement experiments yield linear instead of curvilinear plots upon Scatchard transformation. Thus, although HIRsec polypeptides form (αβ)2; heterotetrameric complexes in the conditioned medium, competitive binding experiments with partially purified material reveal only one class of high affinity insulin binding sites with an apparent $K_d = 10^{-10}$ M in agreement with the results of Sissom and Ellis (18) for a similar HIR construct expressed with the baculovirus system. Truncated HIRsec polypeptides synthesized in mammalian cells are all proteolytically processed at the junctional -RKRR- to a greater extent than the HIRsec polypeptides expressed in Sf9 cells, and the mammalian HIRsec polypeptides also have complex curvilinear (as opposed to linear) binding isotherms (33). Perhaps the relative preponderance of uncleaved proreceptor present in the insect material affects the occupancy of both insulin-binding sites, assuming a two-site model for insulin binding, either directly or by altering the conformation of the processed HIRsec subunits in the heterotetrameric (αβ)2 complex. Exposure of normal cells to insulin decreases high affinity binding while leading to receptor down-regulation (47) and this cell-controlled mechanism may be lacking in HIRsec.

In conclusion, these results demonstrate the utility of the baculovirus insect expression system for the overproduction of insulin receptors. The levels of full-length HIR holoreceptor produced in this system (1–2 ng/109 cells) compare favorably with reports from other laboratories that have used the baculovirus expression system to overproduce other integral membrane glycoproteins (36, 43, 48). Given the ease with which Sf9 insect cells can be grown and the relatively high levels of functionally active HIR expression that can be achieved, this system affords the possibility of obtaining sufficient quantities of purified HIR holoreceptor and/or the soluble HIR insulin-binding domain for further characterization of the structural details of insulin binding to HIR and of insulin-dependent receptor activation.

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