Secretion of the extracellular domain of the human insulin receptor from insect cells by use of a baculovirus vector

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To explore the utility of the baculovirus/insect-cell system for the expression of a soluble secreted human insulin-receptor (hIR) extracellular ligand-binding domain, we have engineered a recombinant virus encoding an hIR deletion mutant which is truncated eight residues from the beginning of the predicted transmembrane domain (i.e. 921 residues). Within 24 h after infection of Sf9 cells with virus, insulin-binding activity begins to accumulate in the culture medium, and reaches a maximum between 48 and 72 h. The intracellular transit and processing of this secreted receptor, designated 'AchIR01', is quite slow. After 24 h in pulse-chase experiments ~50 % of the metabolically labelled protein is still inside the cell. This protein accumulates as a non-cleaved hIR precursor which is glycosylated, but the carbohydrate is entirely endoglycosidase H (endoH)-sensitive (i.e. high mannose). Approximately one-half of the receptor in the culture medium (i.e. ~25 % of the total) is in the form of non-cleaved precursor, and about one half of its carbohydrate chains are now endoH-resistant. The remainder of the protein is proteolytically processed hIR (α -plus truncated β -subunits). None of these hIR species exhibit O-linked carbohydrate. Only the processed form of the receptor in the medium binds insulin. This insulin-binding protein is secreted as a dimer ($\alpha\beta$)₂, and binds insulin with an affinity which is comparable with that of both the wild-type hIR as well as the secreted form of the hIR expressed in mammalian cells. Despite the rather inefficient processing and altered glycosylation of the AchIR01 protein in insect cells, this high-affinity insulin-binding protein accumulates in the medium at levels (mg/litre) of about 100 times that achieved in a mammalian-cell system.

INTRODUCTION

The *Spodoptera frugiperda* (fall armyworm) (Sf9) insect-cell system has proved to be an effective method for the production of significant (milligram) quantities of a variety of proteins by the use of recombinant baculovirus (*Autographa californica*) expression vectors [for a review, see Luckow & Summers, 1988]. There are as yet only a few examples of the use of this system for the synthesis of mammalian transmembrane glycoproteins (e.g. Greenfield *et al.*, 1988; reviewed in Luckow & Summers, 1988), and significant differences in the glycosylation patterns of insect and mammalian cells have been reported (Butters & Hughes, 1981; Butters *et al.*, 1981; Hsieh & Robbins, 1984; reviewed in Kornfeld & Kornfeld, 1985). Whereas insect cells transfer a high-mannose oligosaccharide [Glc₃Man₉(GlcNAc)₂] from a lipid donor to an asparagine residue of an elongating polypeptide chain, this carbohydrate chain may or may not be subsequently trimmed to the trimannosyl core oligosaccharide [Man₃(GlcNAc)₂Asn...], and terminal 'capping' sugars (galactose, fucose, sialic acid) are not subsequently added. The addition of N-acetylgalactosamine to O-linked glycans at serine or threonine residues has been reported for insect cells (Butters & Hughes, 1981; Butters *et al.*, 1981). Thus a prerequisite for the use of the baculovirus/Sf9 system for the expression of a complex mammalian glycoprotein is the demonstration

that such potential alterations in carbohydrate composition do not adversely effect the biological properties and/or physiological function of the protein of interest.

The insulin receptor (IR) is a disulphide-linked heterotetramer ($\alpha\beta$)₂, whose biosynthesis is rather complex (for a recent discussion, see Olson *et al.*, 1988). The IR is synthesized as a single-polypeptide-chain precursor (1355 amino acids; *M_r* 153917) that is proteolytically cleaved after dimerization into α -subunits (735 amino acids; *M_r* 84214) and β -subunits (620 amino acids; *M_r* 69703). Both subunits of the IR are glycosylated at asparagine residues (Olson & Lane, 1987). There are 13 potential N-linked glycosylation sites in the α -subunit, and four in the β -subunit (Ebina *et al.*, 1985). Glycosylation of the IR precursor is a prerequisite for both the acquisition of insulin binding and for cleavage into α - and β -subunits, as the IR expressed in cells treated with tunicamycin does not bind insulin and is not processed; however, the carbohydrate can subsequently be enzymically removed from the glycosylated precursor without compromising the ability of the protein to bind insulin (Olson & Lane, 1987). Thus glycosylation, dimerization, post-translational proteolytic cleavage of subunits and maturation of carbohydrate chains transpire during the intracellular transit of the IR.

The deduced sequence of the human IR (hIR; Ebina *et al.*, 1985; Ullrich *et al.*, 1985) predicts two large domains on either side of a single membrane-spanning

Abbreviations used: hIR, human insulin receptor; AchIR01, the extracellular domain of the hIR as engineered and secreted from insect cells; hIR01, the extracellular domain of the hIR as engineered and secreted from mammalian cells; SV40, simian virus 40; endoH, endoF, endoglycosidase (endo- β -N-acetylglucosaminidase) H, F; mAbs, monoclonal antibodies; PAGE, polyacrylamide-gel electrophoresis; WGA, wheat-germ agglutinin.

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domain, with 929 residues extracellularly (all of the α -subunit and about one-third of the β -subunit) and 403 residues in the cytoplasm (the C-terminal two-thirds of the β -subunit). Both of these large domains have now been engineered (truncated) and expressed as discrete, soluble molecules in mammalian cells: i.e. a secreted functional dimer of the extracellular domain which binds insulin with high affinity (Ellis *et al.*, 1988b; see also Whittaker & Okamoto, 1988), and a monomeric cytoplasmic domain with the expected tyrosine-specific protein kinase activity (Ellis *et al.*, 1987). These findings suggest a new experimental strategy to explore the structure of the functional domains of such large complex transmembrane glycoproteins, provided that each domain can be produced in sufficient quantity for biochemical, and ultimately structural, analysis (Ellis, 1988). The baculovirus system has now been successfully employed for the expression of the soluble human IR protein-tyrosine kinase domain in Sf9 cells (Ellis *et al.*, 1988a; Herrera *et al.*, 1988), and ~10 mg of this enzyme have been routinely purified from ~800 ml of cultured cells (B. Clack & L. Ellis, unpublished work). In the present study we explored the feasibility of using this expression system for the synthesis of a secreted soluble hIR extracellular ligand-binding domain.

MATERIAL AND METHODS

All manipulations of DNA were carried out by the use of standard procedures (Maniatis *et al.*, 1982). Enzymes were from New England Biolabs or Boehringer Mannheim. Plasmids were propagated in the DH1 strain of *Escherichia coli* (Hanahan, 1983).

A ~3 kb cDNA fragment encoding 921 of the extracellular amino acids of the hIR was derived from plasmid pehIR01, a ~5.8 kb simian-virus-40 (SV40) early promoter expression vector designed to express the extracellular domain of the hIR as a soluble secreted protein in mammalian cells (Ellis *et al.*, 1988b). The truncated hIR protein encoded by this plasmid, designated 'hIR01', contains 921 residues derived from the hIR (all of the α -subunit and all of the extracellular portion of the β -subunit, except for eight amino acids prior to the transmembrane domain) and two residues (Ala-Arg) derived from polylinker sequences of the pECE expression vector (Ellis *et al.*, 1986). The C-terminal sequence of hIR01 (beginning with residue 920) is therefore...YLAR as against...YLDVPSNIAK...for the C-terminal sequence of the extracellular domain of the wild-type hIR.

pehIR01 was (i) digested to completion with restriction endonuclease *Nco*I, which cleaves at a single site within the codon for the initiation methionine of the signal peptide at position -27 of the hIR protein, (ii) rendered 'blunt-ended' by incubation with the large fragment of *E. coli* DNA polymerase (Klenow) in the presence of all four deoxynucleotide triphosphates (dG, dA, dT and dC), (iii) digested partially with *Bam*HI to cleave at a vector site 245 bp from the 3' end of the hIR cDNA (this 245-bp 3'-untranslated vector sequence includes SV40 polyadenylation signals), and the ~3.2-kb hIR fragment was purified by electrophoresis in low-gelling-temperature agarose (SeaPlaque; FMC, Rockland, ME, U.S.A.). This 5'-blunt-ended 3'-*Bam*HI fragment was then cloned into the baculovirus expression plasmid pAc373.2 (Ellis *et al.*, 1988a), by the use of unique *Sma*I and *Bam*HI sites

in the vector, and thus placed downstream from the polyhedrin promoter. The resulting plasmid is designated 'pAchIR01'. In this configuration, the complete 5'-untranslated leader sequence of the polyhedrin gene is employed, and protein synthesis initiates at the Met +1 codon of polyhedrin. The expected nucleotide and deduced protein sequence at the N-terminus of the protein is therefore:

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Met-Arg-Pro-Met...
5'...ATAAAT ATG CGC CCC ATG...3'
3'...TATTTA TAC GCG GGG TAC...5'

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where the first methionine residue is Met +1 of polyhedrin, the arginine and proline residues are derived from the vector sequence (including the *Sma*I site) and the second methionine is Met -27 of the signal peptide of the hIR (Ebina *et al.*, 1985). Thus this plasmid configuration results in the addition of three amino acids to the signal peptide of the hIR. The above DNA sequence was confirmed by subcloning an ~300-bp *Eco*RV [which cuts 5' at bp -98 of the polyhedrin gene (Summers & Smith, 1987) and 3' at a unique site at bp 253 of the hIR (Ebina *et al.*, 1985)] fragment which includes this junction into the unique *Sma*I site of M13mp19 (Yanish-Perron *et al.*, 1985) and determining the nucleotide sequence of both strands of the fragment by the dideoxy-chain-terminator method (Sanger *et al.*, 1977). Plasmid pAchIR01 also includes the 245-bp 3'-untranslated sequences with polyadenylation signals of SV40 origin (derived from the pehIR01 plasmid; see above), followed (3' to the *Bam*HI site, which is the unique *Bam*HI site located at bp +171 of the polyhedrin gene) by interrupted (and now non-coding) polyhedrin gene sequences [see Fig. 1 of Ellis *et al.* (1988a) and Figs. 3 and 5 of Summers & Smith (1987)]. Plasmid pAchIR01 was used to transfect Sf9 cells and recover recombinant virus encoding the AchIR01 protein by the methods described by Summers & Smith (1987). All of the experiments described herein utilized a single recombinant virus designated 'AchIR01 D8.2'. Sf9 cells were seeded into flasks at a density of 3×10^6 cells/25 cm² flask. After attachment of the cells the medium was removed and virus inoculum was added (in a total volume of 1 ml) at a multiplicity of infection (MOI) of 10. After 1 h of incubation at 27 °C, 4 ml of complete culture medium was added.

Metabolic labelling of cells, immunoprecipitation of labelled proteins and gel-filtration chromatography were as described by Ellis *et al.* (1986, 1988a,b). The binding of insulin [mono[¹²⁵I]iodinated porcine insulin (90 μ Ci/ μ g) from New England Nuclear and unlabelled porcine insulin from Lilly] to AchIR01 protein immobilized by anti-IR monoclonal antibodies on microtitre plates was done as described by Morgan & Roth (1986) and Ellis *et al.* (1988b). EndoH, neuraminidase and O-glycanase were purchased from Genzyme (Boston, MA, U.S.A.), and endo F was from du Pont/New England Nuclear (Wilmington, DE, U.S.A.); all of these enzymes were used according to the manufacturer's instructions. The panel of anti-IR monoclonal antibodies employed were generated in the laboratories of Dr. Richard Roth (Stanford University) (Roth *et al.*, 1982; Morgan & Roth, 1986; Morgan *et al.*, 1986) and Dr. Kenneth Sidle (University of Cambridge, U.K.) (Soos *et al.*, 1986).

Fig. 1. Accumulation of insulin-binding activity and in the culture medium of Sf9 cells infected with recombinant AchIR01 virus

(a) Aliquots of medium were assayed for ${}^{125}\text{I}$ -insulin binding by a solid-phase assay (Morgan & Roth, 1986; Ellis *et al.*, 1988b), by successively binding, on a microtitre plate, rabbit anti-mouse IgG, an anti-IR monoclonal antibody (mAb 18.26 of Soos *et al.*, 1986), and culture medium, followed by incubation with ${}^{125}\text{I}$ -insulin for 4 h at 4°C . (b) ${}^{125}\text{I}$ -insulin binding in non-ionic-detergent (1% Triton X-100) cell extracts or culture medium of Sf9 cells infected with AchIR01 virus: ▨, binding activity of cell extract; ■, binding activity of medium.

RESULTS AND DISCUSSION

The hIR extracellular domain is secreted from Sf9 cells infected with recombinant AchIR01 virus

Within 24 h of infection of Sf9 cells with recombinant AchIR01 virus, insulin-binding activity is detectable in the culture medium (Fig. 1a). This activity continues to accumulate in the medium over the next 24–48 h and is followed by a decline which is coincident with the beginning of cell lysis. When insulin binding was assayed in cell lysates as compared with the medium of infected cells, little increase in binding was observed in the extracts over a 98 h time course, during which time the activity in the medium dramatically increases, peaks and begins to decline (Fig. 1b). As assayed by a solid-phase method

Fig. 2. Recognition by a panel of anti-IR mAbs (Roth *et al.*, 1982; Morgan & Roth, 1986; Morgan *et al.*, 1986; Soos *et al.*, 1986) of the insulin-binding protein secreted into the medium of Sf9 cells infected with AchIR01 virus

The solid-phase assay was as in Fig. 1.

Fig. 3. Synthesis of the truncated hIR extracellular domain in Sf9 cells

At 48 h after infection of Sf9 cells with AchIR01 virus, cells were metabolically labelled with [${}^{35}\text{S}$]methionine/[${}^{35}\text{S}$]cysteine for 15 min and 'chased' for either 6 or 24 h. Immunoprecipitates were prepared with anti-IR mAb 83.14 (Soos *et al.*, 1986) from either non-ionic-detergent (Triton X-100) extracts of cells (lanes 3 and 4) or from culture medium (lanes 1 and 2). Analysis was by SDS/PAGE on 10% (w/v) gels and fluorography. Molecular masses (*M*) of standard proteins are indicated at the left of this, and subsequent, Figures.

(Morgan & Roth, 1986; Ellis *et al.*, 1988b) employing a panel of anti-IR monoclonal antibodies (mAbs; Roth *et al.*, 1982; Morgan & Roth, 1986; Morgan *et al.*, 1986; Soos *et al.*, 1986) which recognize 12 distinct epitopes of the extracellular domain of the IR, the AchIR01 protein secreted from Sf9 cells is recognized by each of these mAbs with a relative affinity comparable with that of the wild-type hIR or the truncated hIR01 protein secreted

from mammalian cells; those mAbs which block insulin binding to intact cells (51, 25.49, 47.9, 83.14) or have low affinity for the IR (3D7, 18.146) exhibit reduced levels of ^{125}I -insulin binding (Fig. 2; cf. Fig. 6 of Ellis *et al.*, 1988b).

To examine the biosynthesis of the extracellular domain of the hIR 48 h after infection with virus, cells were metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine for 15 min, 'chased' for either 6 or 24 h, and both the medium and a non-ionic-detergent extract of the cells were treated with control (normal mouse IgG; results not shown) or anti-IR monoclonal antibody (IR mAb; mAb 83.14 of Soos *et al.*, 1986; Fig. 3). After 6 h of chase, most of the labelled protein specifically recognized by IR mAb is present in the cell extract, and migrates with an M_r of $\sim 138\,000$ [as estimated by SDS/polyacrylamide-gel electrophoresis (PAGE) on 10% (w/v) gels], as expected for the glycosylated precursor (lane 3; the deduced M_r of the precursor is 105 119). A polypeptide of the same size is also present in the medium (lanes 1 and 2). After 24 h of chase, $\sim 50\%$ of the labelled protein is still present as precursor in cell extracts (lane 4). Of the remaining $\sim 50\%$ now in the medium, one labelled band has the same M_r as that of the intracellular precursor, whereas the second has an M_r of $\sim 108\,000$, as expected for the glycosylated α -subunit after cleavage of the precursor (lane 2; the deduced M_r of the α -subunit is 84 214). The difference in size of these two bands, namely $\sim 29\,000$, is about that expected for the glycosylated extracellular portion of the truncated β -subunit, whose deduced M_r is 20 905. To reveal this smaller band requires a longer exposure of the film than that in Fig. 3 (see Fig. 4 below). Note that the processed α -subunit is also detectable intracellularly in cell extracts, although in quantities substantially less than that of the precursor (Fig. 3, lanes 3 and 4); this intracellular processed receptor is likely to account for the small increase in insulin binding observed in cell extracts during the time course of the infection (cf. Fig. 1b). Thus these pulse-chase experiments demonstrate that the biosynthesis of the extracellular domain of the hIR in this cell system is somewhat inefficient, as a substantial fraction of the polypeptide accumulates inside the cell as a non-processed precursor which does not bind insulin (cf. Fig. 1b).

The AchIR01 protein is glycosylated

Treatment of the intracellular precursor after 24 h of chase (see above) with endoH, which cleaves between the di-GlcNAc residues of high-mannose (non-complex) oligosaccharide chains (Tarentino & Maley, 1974; Tarentino *et al.*, 1974), results in the increase in the mobility of the protein ($M_r \sim 135\,000$; as estimated by SDS/PAGE on 5–15% gradient gels) to an M_r ($\sim 110\,000$) close to that deduced for the non-glycosylated precursor (105 119), which suggests that all of the N -linked carbohydrate attached to the precursor is of the high-mannose form (Fig. 4; cf. lanes 2 and 4). This change in mobility ($\sim 25\,000$) is consistent with the presence of about eight asparagine-linked high-mannose carbohydrate chains. This glycosylation of the nascent precursor polypeptide chain is quite rapid, as periods of label as short as 2.5 min fail to catch the transition in M_r due to addition of carbohydrate (results not shown).

The trimming of high-mannose oligosaccharide chains in insect cells proceeds preferentially to the trimannosyl

Fig. 4. Digestion of the intracellular (Extract) and secreted (Medium) forms of the AchIR01 protein with endoH (see the text)

Analysis was by SDS/PAGE on a 5–15% gradient gel and fluorography.

core oligosaccharide [$\text{Man}_3(\text{GlcNAc})_2\text{Asn}\dots$], with limited accumulation of higher mannosyl forms (i.e. with five or six mannose chains: Hsieh & Robbins, 1984). Glycoproteins or glycopeptides with trimannosyl oligosaccharide chains are very poor substrates for digestion by endoH (Tarentino & Maley, 1974, 1975; Trimble *et al.*, 1978), although the glycosylated amino acid $\text{Man}_3(\text{GlcNAc})_2\text{Asn}$ is a very good substrate (Trimble *et al.*, 1978). After intracellular transit and secretion into the medium, about one-half of the N -linked carbohydrate chains of the non-cleaved precursor (M_r) are now endoH-resistant: with endoH digestion, the secreted precursor migrates with an M_r of $\sim 120\,000$, a change of $\sim 10\,000$, the equivalent of about three N -linked chains (Fig. 4, cf. lanes 1 and 3). Note that a band of this M_r is also present in the endoH-digested extract (Fig. 4, lane 4), which is likely to be the non-processed precursor destined for secretion. The proteolytically processed α -subunit ($M_r \sim 106\,000$) has an estimated three N -linked endoH-sensitive chains, as its M_r after digestion is $\sim 95\,000$ (a change of $\sim 10\,000$; Fig. 4, cf. lanes 1 and 3). The small extracellular portion of the β -subunit, visible in Fig. 4 at an M_r of $\sim 24\,000$, is endoH-resistant (Fig. 4, cf. lanes 1 and 3, lower band). The β -chain is glycosylated, however, as it is sensitive to digestion with endoF, which cleaves between the di-GlcNAc residues of high-mannose, trimmed-mannose or complex oligosaccharide chains (Elder & Alexander, 1982; results not shown). The acquisition of resistance to endoH digestion by the AchIR01 protein during its intracellular transit is consistent with the trimming of some, but not all, of the attached oligosaccharide chains. Endo- α - N -acetylglactosaminidase (O -glycanase), which is specific for

Fig. 5. Gel-filtration chromatography of the AchIR01 protein on Sephacryl S-300 (Superfine grade)

Individual fractions were assayed for ^{125}I -insulin binding by the solid-phase assay. Each point represents the average of three determinations. The M_r of standard proteins are as indicated.

O-linked oligosaccharide chains attached to serine and/or threonine residues (Endo & Kobata, 1976; Umemoto *et al.*, 1977) is without effect on all of these forms of the AchIR01 protein (results not shown). Thus the trimming of the high-mannose oligosaccharide chains initially attached to the intracellular AchIR01 precursor (see above) is heterogeneous, as has been observed for other glycoproteins synthesized in insect cells (see the Introduction).

The secreted AchIR01 protein is a dimer

The wild-type hIR is isolated in non-ionic-detergent extracts of cells and tissues as a disulphide-linked heterotetramer of $(\alpha\beta)_2$ stoichiometry (Kasuga *et al.*, 1982; Fujita-Yamajuchi, 1984; Krämer *et al.*, 1987). The hIR dimerizes in the endoplasmic reticulum before proteolytic cleavage of the precursor (Olson *et al.*, 1988). To assess potential oligomerization of the secreted AchIR01, culture medium harvested 48 h after infection of Sf9 cells with the AchIR01 virus was fractionated by gel-filtration chromatography on a Sephacryl S-300 column (exclu-

Fig. 6. Covalent cross-linking of peaks from the Sephacryl S-300 (fraction 20) and WGA (fraction 7) columns with ^{125}I -insulin

Samples were incubated with labelled insulin, in the presence (+) or absence (-) of $1\ \mu\text{M}$ -unlabelled porcine insulin (Lily), cross-linked and analysed by SDS/PAGE under reducing conditions and autoradiography.

Fig. 7. Chromatography of the AchIR01 protein (the peak of ^{125}I -insulin binding from a Sephacryl S-300 column) on a WGA-agarose column

Elution was accomplished with $0.3\ \text{M}$ -N-acetylglucosamine (GlcNAc). Fractions were assayed for ^{125}I -insulin binding by the solid-phase assay. Each point represents the average of three determinations. Note the difference in the scales of the y-axis in Figs. 5 and 7; the peak material utilized for this Figure is from a preparation larger than that of Fig. 5.

Fig. 8. (a) Binding of ^{125}I -insulin by the soluble hIR ectodomain secreted from Sf9 cells (AchIR01) infected with the AchIR01 virus (●) as compared with its counterpart secreted from a stably transfected mammalian (CHO, Chinese-hamster ovary) cell line [the CHO.hIR01 cells described by Ellis *et al.* (1988b): ▲] and with the wild-type membrane associated hIR synthesized in a stably transfected mammalian (CHO) cell line [the CHO.T cells described by Ellis *et al.* (1986): ■]

^{125}I -insulin binding to each receptor was measured by the solid phase assay in the presence of increasing concentrations of unlabelled porcine insulin (Lily). Results are expressed as a percentage of the ^{125}I -insulin bound in the absence of unlabelled insulin. Individual points represent the average of three determinations of each concentration.

sion-limit $M_r \sim 1.5 \times 10^6$; Fig. 5). The insulin-binding protein is eluted with an M_r equivalent to that of ferritin ($M_r \sim 440\,000$), which is consistent with a dimeric AchIR01 molecule. The chromatographic behaviour of the protein secreted from Sf9 cells is in fact indistinguishable from that of its counterpart secreted from mammalian cells (see Fig. 5 of Ellis *et al.*, 1988b). Covalent cross-linking of the fractions under the Sephacryl S-300 peak with ^{125}I -insulin, followed by SDS/PAGE and autoradiography, reveals a single species of insulin-binding protein that migrates on reducing gels coincident with the processed α -subunit (Fig. 6, lanes 1 and 2; cf. lane 2 of Fig. 3). The secreted non-processed precursor is also a dimer, and is co-eluted with the processed-receptor dimer under the same peak upon gel-filtration chromatography; thus the non-processed secreted precursor does not bind insulin (results not shown).

Further evidence of heterogeneity of attached carbohydrate chains on the AchIR01 protein derives from the behaviour of the Sephacryl S-300 peak material (e.g. that of Fig. 5) on a wheat-germ agglutinin-agarose column (WGA), which specifically binds glycoconjugates bearing *N*-acetylglucosamine or sialic acid residues (Bhavanandan & Katlic, 1979; Monsigny *et al.*, 1980). Sialyltransferases have not been detected in insect cells, and thus insect carbohydrates do not contain sialic acid (Butters *et al.*, 1981). Consistent with these reports, none of the forms of the AchIR01 protein are sensitive to digestion with neuraminidase (acetylneuraminyl hydrolase: Hughes & Jeanloz, 1964; Kiyohara *et al.*, 1974; not shown). The AchIR01 protein specifically binds to WGA and is eluted with a broad trailing peak upon addition of the relevant hapten (i.e. 0.3 M-*N*-acetylglucosamine; Fig. 7). In contrast, the wild-type hIR synthesized in mammalian cells (the CHO.T cells of Ellis *et al.*, 1986) is eluted from such a column as a sharp peak upon the addition of hapten (not shown). This result suggests that the bound material has a graded affinity for the lectin, as would be expected if the trimming of the high-mannose oligosaccharide unit [$\text{Glu}_3\text{Man}_9(\text{GlcNAc})_2\text{Asn}...$] is heterogeneous. Covalent cross-linking with ^{125}I -insulin of the fractions under the WGA peak of Fig. 7 results in the

labelling of a single species that co-migrates on reducing gels with the processed α -subunit of the receptor (Fig. 6, lanes 3 and 4). AchIR01 also binds to concanavalin A (specific for mannose residues), but is eluted with neither hapten nor 1 M-NaCl. The protein does not bind to soybean agglutinin, which is specific for *N*-acetyl-galactosamine residues such as are added to serine and/or threonine residues of some insect glycoproteins (Butters *et al.*, 1981), consistent with the lack of sensitivity to digestion of AchIR01 with *O*-glycanase (results not shown).

The AchIR01 protein binds insulin with high affinity

To assess the affinity of the secreted AchIR01 for insulin, the protein was immunoaffinity-purified by a solid-phase method (Morgan & Roth, 1986; Ellis *et al.*, 1988b) and incubated with increasing concentrations of unlabelled porcine insulin (Lily) in the presence of ~ 80 pM- ^{125}I -insulin (Fig. 8a). The concentration of unlabelled porcine insulin required to inhibit half-maximal binding of labelled insulin is $\sim 10^{-9}$ M, which is quite comparable with that required for similar inhibition of insulin binding by both the hIR01 protein secreted from mammalian cells (Fig. 8a; see Ellis *et al.*, 1988b) and the wild-type membrane bound hIR expressed in mammalian cells (Fig. 8b; the CHO.T cells of Ellis *et al.*, 1986). Analysis of the binding data of Fig. 8(b) by the Scatchard method (Scatchard, 1949; Munson & Rodbard, 1980) illustrates that, like its mammalian counterpart (i.e. hIR01), AchIR01 exhibits a linear Scatchard plot, whereas that of the wild-type membrane bound hIR exhibits a curvilinear plot [Fig. 9; see Ellis *et al.* (1988b) for a discussion].

In summary, these results demonstrate that truncation of the hIR extracellular domain leads to the secretion of a soluble high-affinity insulin-binding protein when expressed in insect Sf9 cells by the use of a baculovirus vector. Despite the relative inefficiency of the proteolytic processing of the precursor, and slow intracellular transit ($t_{1/2} \sim 24$ h), sufficient levels of the protein (milligrams) accumulate in the medium to render feasible its purification for further biochemical and structural studies.

Fig. 9. Analysis of the ¹²⁵I-insulin-binding data of Fig. 8 by the Scatchard method (Scatchard, 1949; Munson & Rodbard, 1980)

(a) Wild-type hIR expressed in CHO, hIR01 cells; (b) Soluble hIR ectodomain secreted from Sf9 cells infected with AchIR01 virus.

Furthermore, this system now provides a rich source of the precursor of the hIR extracellular domain, which does not bind insulin (see Fig. 1b). Parallel studies of this molecule should provide insight into the structural change(s) which result in the constitution of the high-affinity insulin-binding site during biosynthesis of the IR.

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