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 (αβ), 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 [Glc_Mant(GlcNac)_3] from a lipid donor to an asparagine residue of an elongating polypeptide chain, this carbohydrate chain may or may or not be subsequently trimmed to the trimannosyl core oligosaccharide [Man_8(GlcNac)_nAsn...], 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 (αβ), 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; Mr 153917) that is proteolytically cleaved after dimerization into α-subunits (735 amino acids; Mr, 84214) and β-subunits (620 amino acids; Mr, 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...
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...YLLAR 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 Ncol, 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 BamHI 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'-BamHI fragment was then cloned into the baculovirus expression plasmid pAc373.2 (Ellis et al., 1988a), by the use of unique Smal and BamHI 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:

\[
\text{Met-Arg-Pro-Met... 5'...ATAAAT ATG GGC CCC ATG...3'}
\]
\[
3'...TATTTA TAC GGC GGG TAC...5'
\]

where the first methionine residue is Met +1 of polyhedrin, the arginine and proline residues are derived from the vector sequence (including the Smal 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 EcoRV [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 Smal 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 BamHI site, which is the unique BamHI 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 of 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 x 10^6 cells/25 cm^2 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 [monolabeled with 125I (90 Ci/μg) from New England Nuclear and unlabeled 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 Siddle (University of Cambridge, U.K.) (Soos et al., 1986).
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 (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 $^{122}$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 $[^{35}S]m$ethionine 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$138000 [as estimated by SDS/polyacrylamide-gel electrophoresis (PAGE) on 10% (w/v) gels], as expected for the glycosylated $\alpha$-subunit precursor (lane 3; the deduced $M_r$ of the precursor is 105119). 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$108000, as expected for the glycosylated $\alpha$-subunit after cleavage of the precursor (lane 2; the deduced $M_r$ of the $\alpha$-subunit is 84214). The difference in size of these two bands, namely $\sim$29000, is about that expected for the glycosylated extracellular portion of the truncated $\beta$-subunit, whose deduced $M_r$ is 20905. 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 $\alpha$-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$135000; as estimated by SDS/PAGE on 5–15% gradient gels) to an $M_r$ ($\sim$110000) close to that deduced for the non-glycosylated precursor (105119), which suggests that all of the N-linked carbohydrate attached to the precursor is of the high-mannose form (Fig. 4; cf. lanes 2 and 4). This change in mobility ($\sim$25000) 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 coreoligosaccharide[$\text{Man}_n$($\text{GlcNAc}_m$Asn)...], with limited accumulation of higher mannose 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 $\text{Man}_n$($\text{GlcNAc}_m$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$120000, a change of $\sim$10000, 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 $\alpha$-subunit ($M_r$ $\sim$106000) has an estimated three N-linked endoH-sensitive chains, as its $M_r$ after digestion is $\sim$95000 (a change of $\sim$10000; Fig. 4, cf. lanes 1 and 3). The small extracellular portion of the $\beta$-subunit, visible in Fig. 4 at an $M_r$ of $\sim$24000, is endoH-resistant (Fig. 4, cf. lanes 1 and 3, lower band). The $\beta$-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-\(\alpha\)-N-acetylgalactosaminidase (O-glycanase), which is specific for
Insulin-receptor ectodomain secreted from insect cells

Fig. 5. Gel-filtration chromatography of the AchiRO1 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 AchiRO1 protein (results not shown). Thus the trimming of the high-mannose oligosaccharide chains initially attached to the intracellular AchiRO1 precursor (see above) is heterogeneous, as has been observed for other glycoproteins synthesized in insect cells (see the Introduction).

The secreted AchiRO1 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 AchiRO1, culture medium harvested 48 h after infection of SF9 cells with the AchiRO1 virus was fractionated by gel-filtration chromatography on a Sephacryl S-300 column (exclu-
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. (1988)]. 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.

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 $\sim 80$ pm. $^{125}$I-insulin (Fig. 8a). The concentration of unlabelled porcine insulin required to inhibit half-maximal binding of labelled insulin is $\sim 10^{-8}$ 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 (1 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 $^{125}$I-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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