Determinants of oligomeric structure in the chicken liver glycoprotein receptor

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The oligomeric state of the chicken liver receptor (chicken hepatic lectin), which mediates endocytosis of glycoproteins terminating with *N*-acetylglucosamine, has been investigated using physical methods as well as chemical cross-linking. Receptor isolated from liver and from transfected rat fibroblasts expressing the full-length polypeptide is a homotrimer immediately following solubilization in non-ionic detergent, but forms the previously observed hexamer during purification. These results are most consistent with the presence of a trimer of receptor polypeptides in liver membranes and in transfected cells. Analysis of truncated receptors reveals that the C-terminal extracellular portion of this type-II transmembrane protein does not form stable oligomers when isolated from the membrane anchor and cytoplasmic tail. The behaviour of chimeric receptors, in which the cytoplasmic tail of the glycoprotein receptor is replaced with the corresponding segments of rat liver asialoglycoprotein receptor or the β-subunit of Na^+,K^+-ATPase, or with unrelated sequences from globin, indicates that the cytoplasmic tail influences oligomer stability. Replacement of N-terminal portions of the receptor with corresponding segments of influenza virus neuraminidase results in formation of tetramers, suggesting that the membrane anchor and flanking sequences are important determinants of oligomer formation.

INTRODUCTION

Many membrane receptors and transport proteins exist as oligomers of one or more types of polypeptides. In many cases, oligomer formation is a prerequisite for exit from the endoplasmic reticulum, so that polypeptides which fail to associate appropriately do not pass on to the Golgi and thence to the plasma membrane (Doms et al., 1988; Singh et al., 1990; Klausner and Sitia, 1990). Changes in oligomeric state of receptors in the plasma membrane are associated with signal transduction in receptors containing tyrosine kinases in their cytoplasmic domains, and with constitutive activation of such receptors upon transformation (Lax et al., 1991; Gullick et al., 1992).

In several mature receptors, the formation of oligomers is essential for generation of high-affinity ligand-binding sites. In the growth-hormone receptor, the formation of a hormone-binding site requires the presence of two identical receptor subunits that bind to two distinct regions of a single hormone polypeptide (de Vos et al., 1992). In a number of receptors that bind sugars, each subunit contains one or more functional saccharide-binding sites, but high-affinity binding of oligosaccharide ligands requires the presence of a cluster of these ligand-binding domains. Examples include the sialic acid-binding influenza virus haemagglutin (Glick et al., 1991) and the mammalian asialoglycoprotein receptor and its avian homologue, the chicken hepatic lectin (CHL) (Loeb and Drickamer, 1988; Rice et al., 1990).

CHL is an excellent model receptor because it contains only one type of subunit, which is small in size (207 amino-acid residues). The polypeptide is arranged in a type-II orientation, with a 23 amino-acid N-terminal cytoplasmic tail, an internal signal-anchor sequence of 25 uncharged residues, an extracellular neck region of 23 amino acids that contains a single site of glycosylation, and a C-terminal globular domain of 136 residues, that has been designated the carbohydrate-recognition domain (CRD) (Chiacchia and Drickamer, 1984). This C-terminal domain is part of a family of protein modules that are similar in amino-acid sequence, and that display Ca^{2+}-dependent carbohydrate-binding activity. Protein domains sharing this sequence motif are referred to as C-type CRDs (Drickamer, 1988).

The three-dimensional structure of one C-type CRD, from a serum mannose-binding protein, has been determined, and can be used as a model for other domains such as those in CHL (Weis et al., 1991). However, the full specificity of saccharide binding by the intact receptor is a function of its oligomeric structure (Loeb and Drickamer, 1987; Piskarev et al., 1990). Hydrodynamic and cross-linking studies of wild-type and modified CHL reported here indicate that regions outside the CRD, including the membrane anchor and flanking portions of the CHL polypeptide, contribute to formation of stable trimers.

MATERIALS AND METHODS

Materials

Restriction enzymes, DNA-modifying enzymes, and linkers were purchased from New England Biolabs. Oligonucleotides were synthesized on an Applied Biosystems model 391 DNA synthesizer. Polyclonal rabbit antiserum raised against CHL has been described previously (Loeb and Drickamer, 1987). Streptavidin and goat anti-(rabbit IgG) antibodies were from Gibco/BRL. Protein A and molecular-mass markers were purchased from Sigma. Radiochemicals and nitrocellulose were products of Amersham. Cross-linking reagents were purchased from Pierce Chemical Company.

Construction of plasmids

Standard recombinant DNA techniques were used in the construction of plasmids (Maniatis et al., 1982). Non-complementary

Abbreviations used: CHL, chicken hepatic lectin; CRD, carbohydrate-recognition domain.

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ends were made blunt by filling in 5′-protruding ends with the Klenow fragment of DNA polymerase, and exonuclease removal of 3′-protruding ends with T4 DNA polymerase. In order to facilitate manipulation of the CHL cDNA (Mellow et al., 1988), the translation initiation site was modified to create an *Nco*I site (change of base 115 from G to C) and an *Xho*I site was introduced near the neck–CRD junction (change of bases 323–328 from AGCCGG to TCTCGA). These modifications, which do not alter the encoded protein, were effected by substituting synthetic double-stranded DNA fragments for short restriction fragments covering the relevant regions.

For secretion, fragments of the CHL cDNA encoding C-terminal portions of the protein were fused to a preproinsulin cDNA. Vector cDNA cut at a *Hin*cII site two amino-acid residues from the N-terminus of the insulin B chain (Quesenberry and Drickamer, 1991) was fused directly to the CHL cDNA cut with *Bbv*I, or to the cDNA cut with *Sac*I following addition of a custom *Sac*I linker (5′-CTGAGCTCAG-3′). cDNA cut with *Pst*I was ligated into a modified preproinsulin vector (Quesenberry and Drickamer, 1991) containing a short segment of the pSP64 polylinker following the *Hin*cII site. The N-terminal sequences of the resulting proteins are given in Figure 2. A truncated form of CHL lacking the first 18 amino acids was creating by fusing the 3′-untranslated region and codon for the initiator methionine residue of *Xenopus* β-globin cDNA (cut with *Nco*I; Melton et al., 1984) to the CHL cDNA cut at the first *Hpa*II site.

The starting material for chimeric receptors containing sequences from influenza virus neuraminidase was a cDNA clone of the neuraminidase from strain WSN (Hiti and Nayak, 1982), a gift of Dr. Peter Palese, Mount Saini Medical School, New York, NY, U.S.A. Chimera 1 was created by fusing a fragment of the neuraminidase DNA generated by *Bal*31 exonuclease digestion to CHL cDNA cut with *Fnu*4HI. The desired fusion was identified by DNA-sequence analysis (Sanger et al., 1977). Chimera 3 was constructed by addition of a *Bgl*II linker (New England Biolabs, cat. no. 1051) to the modified CHL cDNA cut with *Xho*I. The resulting DNA, cut with *Bgl*II and *Nco*I, was ligated to a *Hin*fI to *Sty*I fragment of the neuroaminidase cDNA. Chimera 2 was generated from chimera 3 by insertion of a synthetic double-stranded DNA (5′-CTTGCAAATAGGAAA-TATTATCTCA-3′) and a *Bbv*I to *Xho*I fragment from the modified CHL cDNA into chimera 3 cDNA cut with *Ssp*I and *Xho*I. The resulting overall structures and fusion sites in the chimeric proteins are illustrated in Figure 4. No extraneous amino acids were introduced at any of the fusion sites.

Chimeric and truncation constructs, created in vector pSP64, were transferred to an expression vector that utilizes the promoter in the left long terminal repeat of Moloney murine leukaemia virus. Depending on restriction sites available, pVcos with a G418-resistance marker inserted (Verrey et al., 1990), or a new vector, pJ603, were utilized. Vector pJ603 is similar to pVNB (Lobel et al., 1989), but without a deletion in the 5′-long terminal repeat. Starting plasmids for these vectors were a gift of John Murphy and Stephen Goff, Columbia University, NY, U.S.A.

Expression in fibroblasts

Stable Rat-6 transfectants were created using pseudovirus generated in the ψ-2 cell line as described previously (Verrey et al., 1990). Cloned G418-resistant cell lines were tested for receptor expression by indirect immunofluorescence and by immunoprecipitation followed by electrophoresis and immunoblotting (Mellow et al., 1988).

Chemical cross-linking

Cells were grown to near confluence on 60-mm diam. culture dishes, and washed three times with PBS. 1,5-Difluoro-2,4-dinitrobenzene was dissolved in ethanol at a concentration of 10 mg/ml, and diluted into PBS before addition to cells. Equivalent amounts of ethanol were added to controls. After incubation at room temperature, cells were again rinsed three times with PBS, harvested by scraping from the plates, and analysed by immunoprecipitation, gel electrophoresis, and immunoblotting as described previously (Mellow et al., 1988).

Receptor solubilization and purification

Receptor was isolated from chicken liver by the method of Kawasaki and Ashwell (1977), using *N*-acetylglucosamine directly coupled to Sepharose 6B as affinity absorbant (Fornstedt and Porath, 1975). For preparation of receptor from transfected cells, five 100-mm diam. plates of cells were grown to confluence, rinsed twice with PBS, and harvested by scraping. The cell pellet was sonicated for 20 s in 2 ml of ice-cold loading buffer (0.5 M NaCl, 50 mm Tris/HCl, pH 7.8, 25 mM $CaCl_2$, 0.05% Triton X-100). The detergent concentration was adjusted to 0.5%, and insoluble material was removed by centrifugation at 150000 *g* for 30 min at 2 °C in a Beckman TLA-100.3 rotor. The supernatant was applied to a column of GlcNAc–Sepharose (1 ml) which was rinsed with 5 ml of loading buffer, and eluted with buffer containing 2.5 mM EDTA in place of $CaCl_2$. Secreted forms of the receptor were purified from 50 ml of culture medium adjusted to a concentration of 25 mM $CaCl_2$ and applied to the same type of column, which was rinsed and eluted with the same buffers but without detergent. Chromatography was performed at 4 °C.

Microsomal membranes were prepared from intact liver or from cultured cells by homogenization with a Polytron tissue disruptor (Brinkman Instruments) for two periods of 30 s at full speed in homogenization buffer (0.25 M sucrose, 10 mM Tris/HCl, pH 7.0, 1 mM EDTA). Preparations from liver were subjected to three sequential centrifugations, twice at 2000 *g*, for 10 min and 30 min, and once at 10000 *g* for 30 min in a Sorvall SS-34 rotor. Microsomes were recovered from the final supernatant by centrifugation at 100000 *g* for 60 min in a Beckman type 42.1 Ti rotor, and were stored frozen as a 50% suspension in homogenization buffer. Preparations from cultured cells were subjected to one centrifugation at 1000 *g* for 10 min in a Sorvall SS-34 rotor, after which microsomes were recovered by centrifugation at 350000 *g* for 30 min in a Beckman TLA-100.2 rotor. Solubilization of membrane proteins in loading buffer containing 0.5% Triton X-100 was followed by centrifugation at 130000 *g* for 30 min in a Beckman TLA-100.2 rotor. All steps were performed at 2–4 °C.

Hydrodynamic experiments

Velocity sedimentation, equilibrium density gradient centrifugation, and gel filtration experiments were performed as described by Loeb and Drickamer (1987), except that velocity gradients were centrifuged for 18 h at 4 °C. The receptor content of gradient fractions was assessed by gel electrophoresis or by a dot blot assay. The dot blot assay was performed by suction of aliquots of fractions directly through nitrocellulose in a 96-well apparatus from Bio-Rad Laboratories. For gel electrophoresis, samples were in some cases immunoprecipitated or precipitated with 10% (v/v) trichloroacetic acid before analysis (Holland and Drickamer, 1986). Gels were stained with Commassie Blue or transferred to nitrocellulose (Burnette, 1981). CHL was detected

on dot blots and gel transfers with rabbit polyclonal antiserum raised to CHL, followed by radio-iodinated protein A or biotinylated goat anti-(rabbit IgG) serum followed by iodinated streptavidin (Loeb and Drickamer, 1987; Graeve et al., 1989). Fluorographs were analysed using a Molecular Dynamics model 300A densitometer.

Hydrodynamic calculations

Calculated values of $\bar{v}$ for native, truncated, and chimeric glycosylated CHL polypeptides were obtained using values of 0.725 cm^3/g for protein and 0.622 cm^3/g for carbohydrate combined with the compositions of the proteins (Loeb and Drickamer, 1987). The carbohydrate portion of the receptor expressed in fibroblasts may differ from that of liver-derived material. However, gel electrophoresis of CHL from liver and from fibroblasts indicates that the size of the single N-linked carbohydrate moieties must be quite similar (Mellow et al., 1988). Alternative calculations using sugar compositions for mammalian di- or tri-antennary oligosaccharides do not give $\bar{v}$ values significantly different from those obtained with the composition for CHL derived from chicken liver (Kawasaki and Ashwell, 1977).

In cases where the diffusion coefficient (D) could not be measured directly, it was estimated that D is proportional to $M_r^{-1/3}$, which is equivalent to assuming that all of the protein molecules or detergent–protein complexes have roughly the same, spherical shape. The Svedberg equation can then be rearranged to obtain the relationship:

$$M_r = C \cdot [s/(1-\bar{v}\rho)]^{3/2}$$

where s is the sedimentation coefficient, $\bar{v}$ is the partial specific volume, and ρ is the density. The proportionality constant C was determined from those experiments in which D was measured experimentally combined with published results for globular proteins (ribonuclease A, lysozyme, and BSA). This approach is equivalent to the commonly used assumption that s is proportional to $M_r^{2/3}$ (Cantor and Schimmel, 1980), but includes a small correction for the effect of detergent binding on $\bar{v}$.

When $\bar{v}$ measurements could not be used to establish the detergent content of the complexes, this value was estimated by calculating the expected values of s, assuming different ratios of protein to detergent. Because the partial specific volume of Triton X-100 (0.94 cm^3/g) is close to that of water, the effect of changing the estimates of detergent content tends to cancel out: increasing the amount of detergent increases the estimated size of the complex, but lowers its apparent density, so that the calculated size of the protein portion of the complex is insensitive to this assumption. Values of $\bar{v}$ derived from the experiments with native CHL were used. Assuming more extreme values, ranging from no detergent bound to 70% Triton X-100 in the particle, resulted in no more than 5% deviation from the results obtained with the present assumptions.

RESULTS AND DISCUSSION

Oligomeric state of CHL before and after purification

In previous studies, CHL purified by two cycles of affinity chromatography was found, by hydrodynamic studies and by chemical cross-linking, to be a hexamer (Loeb and Drickamer, 1987). However, the results reported in Table 1 indicate that the hydrodynamic properties of CHL in detergent solution immediately after solubilization are those of a trimer. The existence of trimers in the membrane was demonstrated previously by chemical cross-linking (Loeb and Drickamer, 1987). It cannot be ruled out that a hexameric form exists in the membrane, but is undetected by cross-linking because of the high concentrations of aminophospholipids and is not stable during the initial solubilization. However, results of radiation-inactivation analysis of CHL are consistent with the existence of a trimer in the membrane and immediately after detergent solubilization (Steer et al., 1990). Interestingly, the major subunit of the human asialoglycoprotein receptor also forms trimers when expressed in the absence of the minor subunit (Shia and Lodish, 1989).

The cause of the association of solubilized trimers to form hexamers is not clear. Rapid purification of CHL from transfected fibroblasts (see below) reveals the presence of some trimers in addition to the predominant hexamers (results not shown), suggesting that formation of hexamers is a function of time in detergent solution. It is possible that tightly bound lipids are displaced slowly, allowing portions of the protein to interact in new ways. Release from the membrane may also remove geometrical constraints, allowing novel interactions between the CHL polypeptides.

Oligomeric state of CHL expressed in fibroblasts

In order to identify portions of the CHL polypeptide that are responsible for trimer formation, structural characterization of modified CHL polypeptides was undertaken using permanent fibroblast lines created by infection of Rat-6 cells with pseudovirus (Verrey et al., 1990). Following treatment of cells expressing wild-type CHL with the bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene, covalently cross-linked dimers and trimers are detected (Figure 1). The pattern observed is very similar to that obtained by cross-linking in liver microsomes (Loeb and Drickamer, 1987). The results of performing sedimentation experiments on purified receptor and on receptor immediately after solubilization from microsomes, summarized in Table 1, also indicate that the expressed protein is a trimer. Small differences between the hydrodynamic values for tissue-derived CHL and for the receptor from fibroblasts are within the uncertainty of the measurements, although they may reflect small differences in glycosylation at the single site of N-linked carbohydrate attachment resulting from expression in rat rather than avian cells.

While the behaviour of CHL expressed in fibroblasts in most respects mimics the behaviour of CHL derived from liver, a fraction (20–30%) of CHL from fibroblasts has a sedimentation coefficient of 2.5 S, corresponding to monomeric polypeptide, in both purified preparations and in receptor analysed directly from solubilized microsomes. Decreased efficiency of oligomerization might be the result of different lipid compositions of rat fibroblast and chicken liver membranes, or it could reflect the effects of slightly different glycosylation on the stability of the receptor trimer.

Oligomeric state of truncated CHL

Previous studies have shown that the C-terminal 136 amino acids of CHL form a subtilisin-resistant domain in the presence of Ca^{2+} (Loeb and Drickamer, 1987, 1988). This fragment of the CHL polypeptide constitutes the CRD, corresponding closely to the minimum CRD of mammalian asialoglycoprotein receptor and serum mannose-binding proteins determined by truncation analysis (Quesenberry and Drickamer, 1991). The free CRD has been shown by cross-linking and gel-filtration analysis to be a monomer (Loeb and Drickamer, 1987), indicating that portions

Table 1 Hydrodynamic properties of CHL

Measured values of $s_{20,w}$, $D_{20,w}$, and $\bar{v}$ were used in the Svedberg equation to calculate M_r for the glycoprotein–detergent complex. The fraction of CHL in the complex was determined from the measured $\bar{v}$ of the complex, the calculated $\bar{v}$ of CHL (0.715 cm^3/g), and the $\bar{v}$ of Triton X-100 (0.94 cm^3/g). M_r of CHL in the complex was then calculated by multiplying this fraction times the M_r of the complete complex. M_r/M_r^0 represents the ratio of the molecular mass of CHL in the detergent complex to the molecular mass of a single glycosylated CHL polypeptide (26250) (Loeb and Drickamer, 1988). The estimated numbers of polypeptides in the complex are indicated in parentheses.

Source	Preparation	$s_{20,w}$ (S)	$D_{20,w}$ (10^{-7} cm^2/s)	$\bar{v}$ (cm^3/g)	M_r of complex	Fraction CHL	M_r of CHL	M_r/M_r^0
Liver	Purified	6.7	3.3	0.85	326000	0.42	137000	5.2 (6)
	Solubilized microsomes	4.7	3.7	0.87	235000	0.32	75000	2.9 (3)
Transfected cells	Purified	7.1	3.3	0.84	324000	0.44	143000	5.4 (6)
	Solubilized microsomes	4.8	3.7	0.87	239000	0.32	76000	2.9 (3)

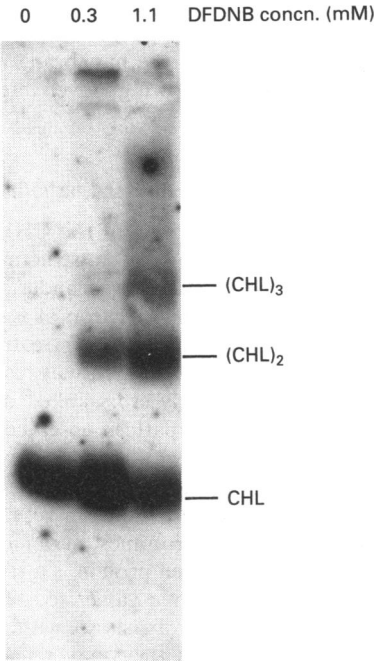

Figure 1 Cross-linking CHL expressed in transfected fibroblasts

Cells were treated with indicated concentrations of 1,3-difluoro-2,4-dinitrobenzene (DFDNB) for 60 min at room temperature, harvested, and analysed by immunoprecipitation, gel electrophoresis, and immunoblotting as described in the Materials and methods section.

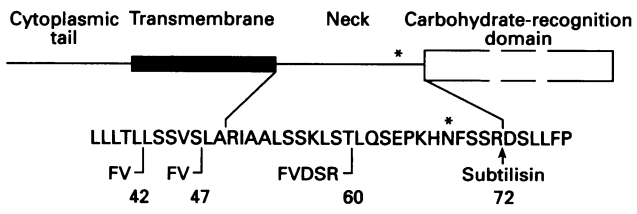

Figure 2 Expression of truncated forms of CHL

Portions of CHL expressed by fusion with the signal sequence of preproinsulin are shown diagrammatically, along with the structure of intact CHL. Asterisks denote site of N-linked glycosylation. The site of limited digestion with subtilisin is also indicated. The sequence of the central portion of the polypeptide is given in the one-letter amino-acid code. Residue numbers for the beginnings of fusion constructs are shown at the bottom. Sequences FV and FVDSR were appended at the positions indicated by fusion with the beginning of the insulin B-chain (Quesenberry and Drickamer, 1991).

Table 2 Hydrodynamic properties of soluble truncated forms of CHL

M_r values were calculated from the measured $s_{20,w}$ and calculated $\bar{v}$ values, assuming that the diffusion coefficient is proportional to the cube root of the molecular mass (see the Materials and methods section). M_r/M_r^0 is the ratio of the measured molecular mass of the expressed fragments to the calculated molecular mass of the constituent glycosylated polypeptide. The estimated numbers of polypeptides in the complex are indicated in parentheses.

Region expressed	$s_{20,w}$ (S)	$\bar{v}$ (cm^3/g)	M_r	M_r/M_r^0
60–207	2.5	0.716	26800	1.1 (1)
47–207	2.6	0.715	28200	1.3 (1)
42–207	2.6	0.715	28200	1.4 (1)

of the protein in the extracellular neck, transmembrane anchor, or cytoplasmic tail must be required to stabilize the oligomer.

Using the fibroblast expression system, it was possible to generate a series of truncated receptor molecules containing part or all of the neck region in addition to the CRD. The structure of these receptors is summarized in Figure 2. The shortest construct is longer than the previously characterized subtilisin-resistant fragment of CHL, including 12 residues from the neck plus five residues from the N-terminus of proinsulin and the linker region of the vector. The longer constructs contain the entire neck region. All three truncated proteins are secreted into the medium under the control of the preproinsulin signal sequence, and can be isolated by affinity chromatography in the absence of detergent. The sedimentation coefficients for each of these proteins (Table 2) indicate that they are monomers. These results show that the extracellular neck alone is not sufficient to form a stable trimer.

The behaviour of CHL contrasts with the behaviour of the cation-dependent mannose-6-phosphate receptor, since the extracellular domain of this protein contains sufficient information to direct dimer formation in the absence of a membrane anchor (Dahms and Kornfeld, 1989). Other proteins in which interactions between the extracellular domains are sufficient to stabilize oligomers include growth-hormone receptor (de Vos et al., 1992), neuraminidase and haemagglutinin of influenza virus (Varghese et al., 1983; Singh et al., 1990), and epidermal-growth-factor receptor, the extracellular domain of which forms oligomers in the presence of the ligand (Lax et al., 1991). Although the β-subunit of the Na^+,K^+-ATPase must be inserted into a membrane domain to associate with the α-subunit, it has been suggested that specificity of oligomer formation lies in the ectodomain of both subunits (Renaud et al., 1991).

Table 3 Hydrodynamic properties of CHL with modified cytoplasmic tails

The M_r of the glycoprotein portion of the solubilized detergent complexes were calculated from the measured values of $s_{20,w}$ by estimating the amount of bound detergent (see the Materials and methods section). Yield represents the fraction of total chimeric protein recovered that sediments in the indicated peak. M_r/M_r^0 is the ratio of the measured M_r of the glycoprotein component to the calculated M_r of the constituent glycosylated chimeric polypeptide. The estimated numbers of polypeptides in the complex are indicated in parentheses.

Cytoplasmic tail	Preparation	$s_{20,w}$ (S)	Yield	M_r	M_r/M_r^0
Truncation	Purified	2.5	0.5	30600	1.3 (1)
(residues 18–207)		> 10	0.5		Aggregate
Asialoglycoprotein receptor	Purified	4.6	0.4	76300	2.7 (3)
		7.1	0.4	146200	5.2 (6)
	Solubilized microsomes	4.8	1.0	81500	2.9 (3)
Na^+,K^+-ATPase β-subunit	Purified	2.5	> 0.6	30600	1.1 (1)
	Solubilized microsomes	3.0	> 0.7	40200	1.5 (1)
Globin	Purified	4.5	> 0.6	73800	2.9 (3)
	Solubilized microsomes	4.8	1.0	81300	3.2 (3)

It has been proposed that the extracellular neck of CHL and other type-II transmembrane proteins containing C-terminal C-type CRDs may form coiled-coils of α-helices (Beavil et al., 1992), that could be important for stabilizing a trimer. While the present results do not rule out the possible presence of such a structure, they do indicate that additional portions of the polypeptide are needed for stable trimer formation.

Role of the cytoplasmic domain in trimer formation

When all but the five membrane-proximal residues of the cytoplasmic domain of CHL are removed, the only discrete peak observed in a sedimentation gradient corresponds to a monomer (Table 3). However, a significant fraction of the truncated receptor appears as an aggregate, sedimenting near the bottom of the gradient. The failure of the truncated receptor to form discrete trimers rules out models in which intersubunit interfaces in the trimer are formed by the extracellular portions of the receptor, but the interactions are only strong enough to stabilize the trimers if the extracellular segment is constrained to the surface of a membrane.

As noted previously, expression levels of the truncation construct are low (Verrey et al., 1990). This result may reflect the fact that altered polypeptides do not insert correctly into the endoplasmic reticulum membrane or do not fold with the full efficiency of native CHL, and are thus subject to degradation in the endoplasmic reticulum (Klausner and Sitia, 1990). Expression levels of intermediate constructs, lacking only part of the cytoplasmic tail, were too low to allow analysis of their oligomerization properties. It is known, however, that removal of the N-terminal 11 amino acids of purified CHL by limited digestion with trypsin does not change the oligomeric state of the purified protein (Loeb and Drickamer, 1987), suggesting that the presence of sequences near the cytoplasmic side of the membrane is sufficient to keep an assembled oligomer stable.

To determine if the requirement for a cytoplasmic tail to achieve normal oligomerization reflects the importance of specific sequences in CHL, the oligomeric state of three proteins containing alternative cytoplasmic tails was examined. In two of these chimeric proteins, the cytoplasmic domain of CHL is substituted by the cytoplasmic tail of the major subunit of the rat asialoglycoprotein receptor and the N-terminal sequence of globin. These sequences are not discernibly related to the sequence of the native CHL tail (see Verrey et al., 1990 for sequence comparisons). For both constructs, trimer formation can be demonstrated by analysis of receptor immediately after solubilization from microsomes (Table 3). These changes affect the rate of formation of hexamers following purification, since the trimer is the predominant species even after purification.

The formation of stable trimers by the globin–CHL chimera indicates that a specific sequence in the cytoplasmic tail of CHL is not essential for trimer formation in the membrane. Thus, the presence of a cytoplasmic tail appears to be a permissive rather than a determining factor in CHL-trimer formation. One possible explanation for this phenomenon is that placement of a peptide segment on the N-terminal side of the transmembrane region may alter the conformation of the transmembrane domain, and thus affect oligomer formation without directly mediating polypeptide associations.

If the cytoplasmic tail of CHL is replaced with the tail of the β-subunit of Na^+,K^+-ATPase, intermediate results are obtained. The β-subunit is a simple type-II transmembrane protein that normally forms part of an $\alpha_2\beta_2$ complex. The Na^+,K^+-ATPase β-subunit tail chimera is predominantly monomeric when analysed immediately after solubilization or after purification (Table 3), although dimers and trimers can be detected by cross-linking in the membrane (Figure 3). Thus, although the chimera may be oligomeric in the membrane, the intersubunit interactions must be weaker than in the native receptor. Specifically, instability of the Na^+,K^+-ATPase chimera may result from formation of a structure on the cytoplasmic side of the membrane that destabilizes the trimers.

There is no simple correlation between levels of receptor expression and oligomeric state. For example, the level of expression of the tail truncation and the globin-substitution constructs are both low, but only the latter forms trimers. Similarly, the level of expression of the Na^+,K^+-ATPase chimera is close to that of wild-type CHL, but the sedimentation results are clearly different. In addition, analysis of cell lines expressing different levels of wild-type and modified CHL produced indistinguishable results for extent of oligomer formation.

In previous studies, it was shown that each of the fusion proteins mediates uptake of glycoprotein ligands, and that uptake occurs through clathrin-coated pits (Verrey et al., 1990). However, the efficiency of coated-pit localization and ligand uptake is significantly lower for the Na^+,K^+-ATPase-tail construct than for native CHL. The activity of the globin and asialoglycoprotein receptor constructs is closer to the wild type. The correlation between the stability of oligomers and efficiency of endocytosis suggests that oligomerization may be essential for the receptor to interact with components of the endocytic machinery.

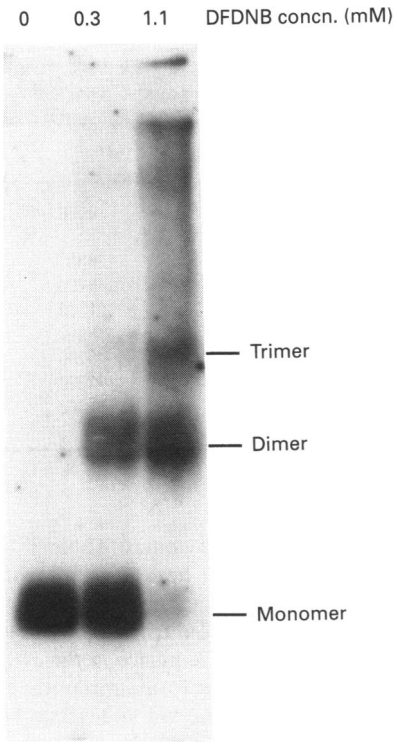

Figure 3 Cross-linking of CHL with a modified cytoplasmic tail

Cross-linking, following the procedure described in Figure 1, was performed on cells expressing CHL in which the cytoplasmic domain has been substituted with the cytoplasmic tail of the β-subunit of Na^+,K^+-ATPase. Concentrations of 1,3-difluoro-2,4-dinitrobenzene used are indicated at the top of each lane.

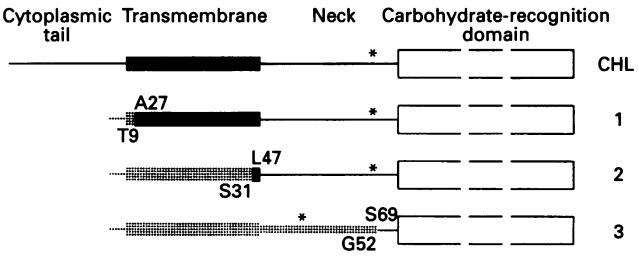

Figure 4 Construction of chimeric proteins containing portions of CHL and influenza virus neuraminidase

Asterisks denote sites of N-linked glycosylation. Portions derived from neuraminidase are stippled. Sites of fusion are indicated by the one-letter amino-acid code and residue number.

The membrane-anchor domain and oligomer formation

Since the extracellular portion of CHL alone is not sufficient to organize the protein into trimers, and since variation in the sequence of the cytoplasmic tail is tolerated, it seems likely that the membrane-spanning hydrophobic segment of the polypeptide contributes in a significant way to formation of trimers. This hypothesis was tested by creating chimeras containing segments of the neuraminidase of influenza virus (Figure 4). This type-II transmembrane protein is a homotetramer (Varghese et al., 1983). Following expression in fibroblasts, it was possible to determine sedimentation coefficients for these proteins, and in two cases direct measurements of partial specific volumes were made (Table 4).

Replacement of the entire tail, membrane anchor, and neck of CHL with the corresponding portions of the neuraminidase (chimera 3) results in formation of a tetramer. Expression of this construct was sufficiently high to allow confirmation of the oligomeric state after affinity purification, as well as directly from solubilized microsomes. These results indicate that the predominant signals for oligomer formation in this chimera come from the neuraminidase portion of the molecule. The high degree of amino-acid-sequence similarity between the CRDs of CHL and mannose-binding protein suggests that the dimensions of the CRD in CHL are probably similar to those of mannose-binding proteins: $25 \times 25 \times 4$ nm (40 Å) (Weis et al., 1991), with the long dimension pointing away from the membrane. The subunits of the neuraminidase measure $25 \times 25 \times 6$ nm (60 Å), again with the long dimension perpendicular to the membrane (Verghese et al., 1983). Therefore, it is possible to imagine that portions of the neuraminidase near the membrane hold the CRDs in an arrangement very similar to the normal packing of the neuraminidase catalytic domains. Any CRD–CRD interactions present in the normal CHL trimer can be overcome by the interactions between the neuraminidase tail, transmembrane, and neck regions.

If only the cytoplasmic domain and a small portion of the transmembrane anchor of CHL are replaced with corresponding portions of neuraminidase (chimera 1), the predominant oligomer is again a tetramer immediately after solubilization (Table 4). However, this structure is not stable, as mostly monomer is detected following purification. The fact that a stretch of just nine amino-acid residues is sufficient to affect the oligomeric state of the protein means that the cytoplasmic end of the transmembrane anchor of neuraminidase must play an important role in determining the nature of the oligomer formed. The results described in the preceding section indicate that CHL forms a trimer in the apparent absence of specific sequence information in the cytoplasmic domain. Formation of tetramers by the neuraminidase chimera indicates that the neuraminidase-derived signal for tetramer formation is dominant over the information in the CHL portion of the chimera that would otherwise direct trimer formation. However, the dissociation of chimera 1 during purification indicates that additional interactions are required for full tetramer stability.

An intermediate construct containing only the tail and transmembrane portions of neuraminidase and the entire extracellular portion of CHL (chimera 2) was expressed at significantly reduced levels and could only be detected after purification. It sedimented with a lower apparent molecular mass than the other two neuraminidase–CHL fusion proteins, and its properties lie between those of a trimer and a tetramer. It is difficult to interpret this result definitively. The intermediate sedimentation value may reflect instability of a tetramer on the gradients. This possibility is consistent with the observation that this protein is expressed at low levels, probably also reflecting its instability. Alternatively, the presence of the CHL neck sequence may shift the balance of oligomerization signals in favour of trimer formation.

There are several other receptors in which the membrane domain has an important influence on oligomer formation. These include the oncogenic form of the transforming protein neu (Gullick et al., 1992) and the T-cell-antigen receptor (Manolios et al., 1990; Blumberg et al., 1990). Oligomerization of several type-II transmembrane proteins has been studied. In addition to the β-subunit of the Na^+,K^+-ATPase and influenza

Table 4 Hydrodynamic properties of chimeric proteins containing portions of CHL and influenza virus haemagglutinin

M_r values for detergent–chimera complexes were calculated based on the assumption that the diffusion coeffient is proportional to the cube root of the molecular mass (see the Materials and methods section). The fraction of the complex that is glycoprotein was calculated as in Table 1, using the value of $\bar{v} = 0.86$ cm^3/g, experimentally measured in two cases, for all of the calculations. The similarity of this value to that for wild-type CHL provides explicit demonstration that no significant change in detergent binding accompanies changes in the hydrophoblic domain. Yield and M_r/M_r^0 are defined as in Table 3. The estimated numbers of polypeptides in the complex are indicated in parentheses.

Chimeric protein	Preparation	$s_{20,w}$ (S)	$\bar{v}$ (cm^3/g)	Yield	M_r of chimera	M_r/M_r^0
1	Solubilized microsomes	5.2	–	0.8	94000	3.8 (4)
	Purified	2.2	–	0.6	25900	1.1 (1)
2	Purified	4.8	0.86	1.0	83400	3.4 (4?)
3	Solubilized microsomes	5.4	–	0.7	99500	4.0 (4)
	Purified	5.3	0.86	0.7	96700	3.9 (4)

virus neuraminidase which were used in the present studies, the invariant chain of the class II histocompatibility antigens forms hetero-oligomers with the α- and β-subunits, as well as homotrimers (Kvist et al., 1982; Marks et al., 1990) and the transferrin receptor forms homodimers (Omary and Trowbridge, 1981). A chimeric protein consisting of the cytoplasmic and transmembrane domains of the influenza virus neuraminidase and the extracellular portions of the transferrin receptor has been shown to form tetramers (Kundu et al., 1991). This result, like those reported in the present study, suggests that oligomerization signals in the membrane and cytoplasmic domains are dominant over those in the extracellular domains of these chimeras. It is possible that this pattern is common to type-II receptors. Interestingly, the transmembrane domain and membrane-proximal portion of the cytoplasmic tail are encoded by a single exon in the CHL gene (Bezouska et al., 1991).

This work was supported by grant GM 42628 from the National Institutes of Health and by a grant from the Irma T. Hirschl Foundation. F.V. is recipient of a Senior Postdoctoral Fellowship from the Swiss National Science Fouccation. K.D. is recipient of a Faculty Salary Award from the American Cancer Society.

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Received 27 July 1992/18 November 1992; accepted 9 December 1992