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Assembly of a heterooligomeric asialoglycoprotein receptor complex during cell-free translation

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ABSTRACT **We have translated RNAs for the two rat asialoglycoprotein receptor polypeptides together in a cell-free system containing dog pancreatic microsomes and immunoprecipitated the products with antibodies that distinguish the two proteins. In this system the proteins oligomerize, as judged by their coprecipitation with either of the subunit-specific antisera. Oligomerization does not occur between subunits synthesized without microsomes or between subunits synthesized on separate microsomes mixed during detergent solubilization. Thus, oligomerization occurs within the microsomal membrane. We calculate that oligomerization proceeds with an efficiency of ≈85%. The receptor complex appears to represent a specific oligomer because it excludes a third membrane glycoprotein synthesized in the same reaction. Oligomerization of the asialoglycoprotein receptor *in vitro* should provide a useful system to study the assembly of a membrane–protein complex.**

The hepatic asialoglycoprotein receptor of rats, rat hepatic lectin (RHL), consists of three different subunits (1–3). Two of these (RHL-2 and RHL-3) have the same polypeptide chain (RHL-2/3) but differ in carbohydrate modifications, whereas the remaining subunit (RHL-1) is a distinct, but related, polypeptide (4). The receptor binds serum glycoproteins that have carbohydrate chains terminating in galactose, and each subunit has a galactose-binding site (4–6). As in rat, the receptor exists as more than one polypeptide in the mouse, rabbit, and human (7–9). Sequence analysis shows that the two receptor polypeptide subtypes identified in human hepatoma cells are quite similar in their primary structure to those of rat (4, 10).

We have previously reported that all three rat receptor subunits can be immunoprecipitated with antisera reacting specifically with the carboxyl terminus of either the RHL-1 or RHL-2/3 polypeptide (11). Thus, the various rat receptor subunits can form a heterotypic oligomer that may be the principal form found on the cell surface. A heterooligomer of the human polypeptides has also been proposed on the observation that antibodies specific for one of the molecules accelerate the turnover of both (12). In addition, the same antibodies will precipitate a complex of both human subunits after chemical cross-linking (12).

The physical evidence for the heterooligomeric structure of the receptor is corroborated by cDNA transfection experiments. Synthesis in various cell lines of transfected asialoglycoprotein receptor with properties similar to that of the endogenous receptor of hepatocytes occurs only when both polypeptides are expressed in the same cell (13–15). Though each subunit has a galactose-binding site (4–6), the heterooligomeric complex apparently has to be assembled to achieve high-affinity uptake of serum glycoproteins.

Despite the above information, the RHL complex has yet to be well characterized. The carboxyl-terminal antibodies we have generated have been of limited usefulness in this regard because these react with only a fraction of the receptor present in cell extracts. We have found, however, that receptor polypeptides synthesized *in vitro* are quantitatively precipitated by the carboxyl-terminal antisera. In the experiments reported here we show that the heterooligomeric receptor complex can be efficiently assembled during cell-free translation of the subunits. These observations confirm our previous work and provide a model for analyzing the formation of the receptor complex.

MATERIALS AND METHODS

Materials. Critical reagents and materials were obtained from the following suppliers: SP6 RNA polymerase, rabbit reticulocyte lysate, nucleotides, and RNasin from Promega Biotec; cap analogue 7-methylguanosine(5′)triphospho(5′)-guanosine from Pharmacia; dog pancreatic microsomes from Amersham or Promega Biotec; [^{35}S]methionine (>1000 Ci/mMol; 1 Ci = 37 GBq) from Amersham or DuPont/New England Nuclear; endoglycosidase H from Genzyme; Triton X-100 (Surfact-Amps X-100) from Pierce. cDNA encoding RHL-1 was a gift of K. Drickamer (Columbia University) (16). We have reported cloned cDNA for RHL-2/3 (17) and dipeptidyl peptidase IV (DPP-IV; ref. 18). Each cDNA has been previously inserted downstream of the SP6 promoter in either pSP64 (RHL-1), pGEM-3 (RHL-2/3), or pGEM-4 (DPP-IV). The RHL-1 and RHL-2/3 carboxyl-terminal antisera have been described (11). Immunoglobulin fractions were prepared from these by precipitation with 50% ammonium sulfate.

Transcriptions and Translations. Plasmids were prepared for transcription by cleavage with either *Eco*RI (RHL-1), *Bgl* I (RHL-2/3), or *Stu* I (DPP-IV). Typically, 6 μg of plasmid DNA was transcribed according to Promega protocols with the inclusion of 0.5 A_{260} unit of the cap analogue 7-methylguanosine(5′)triphospho(5′)-guanosine. Trial experiments determined the amount of transcription product necessary for convenient translation results. A typical translation used 1/50 of the RNA. Translations were 90 min and were done according to Promega, including 2 μl of pancreatic membranes where indicated. Puromycin was added to 1 mM for 20 min at the end of reactions including truncated DPP-IV RNA (see *Results*).

Immunoprecipitations. In most experiments, translation reactions were treated with 20 vol of Tris-buffered saline containing 1% Triton X-100, sonicated, and incubated on ice for 30 min. Insoluble material was pelleted in a Beckman Microfuge for 5 min, and 100-μl aliquots of the supernatant were treated with antibody by using the equivalent of 1 μl of original antiserum. After 3 hr on ice the samples were incubated overnight with 50 μl of a 10% (wt/vol) suspension of protein A-Sepharose (Pharmacia). In early experiments,

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Abbreviations: RHL, rat hepatic lectin; RHL-1, -2, and -3, polypeptides that compose RHL; RHL-2/3, the common polypeptide backbone of RHL-2 and RHL-3; DPP-IV, dipeptidyl peptidase IV.

the pellets were washed three times with Tris-buffered saline containing 1% Triton X-100, 1% deoxycholate, 0.1% $NaDodSO_4$, and 1% bovine serum albumin and washed three times with Tris-buffered saline containing 0.1% $NaDodSO_4$. We have since found such extensive washing is not only unnecessary but significantly decreases the recovery of antigen. For most experiments, therefore, immunoprecipitates were washed one time with Tris-buffered saline. Only the experiments shown in Fig. 3*B* were done with the earlier more stringent washing procedure. Immunoprecipitates were eluted with $NaDodSO_4$-containing sample buffer for $NaDodSO_4$/PAGE (19) or urea-containing sample buffer for isoelectric focusing (20). In some experiments the supernatants of the immunoprecipitations were precipitated with methanol (21) and analyzed by $NaDodSO_4$/PAGE to determine the amount of antigen not bound by antibody. To determine the extent of glycosylation, washed immunoprecipitates were eluted with 1% $NaDodSO_4$, diluted 2-fold with 100 mM Mes, pH 5.5, and treated with 10 milliunits of endoglycosidase H overnight at 37°C.

Electrophoresis. Immunoprecipitates were analyzed in one dimension by $NaDodSO_4$/PAGE, according to Laemmli (19), and in two dimensions, according to O'Farrell (20). Gels for $NaDodSO_4$/PAGE contained 9% acrylamide. Isoelectric focusing gels contained 3% each of pH 3.5–10, pH 4–6, and pH 9–11 Ampholines (LKB). $NaDodSO_4$/PAGE calibration standards were from Bethesda Research Laboratories, and isoelectric focusing standards were from Pharmacia. The [^{35}S]methionine-labeled proteins were detected by fluorography (22). Fluorograms were quantitated by excising the desired bands from the dried gels and counting their radioactivity in a liquid scintillation counter.

RESULTS

To analyze formation of the heterooligomeric asialoglycoprotein receptor complex, cell-free translations were programmed with RNAs derived from *in vitro* transcriptions.

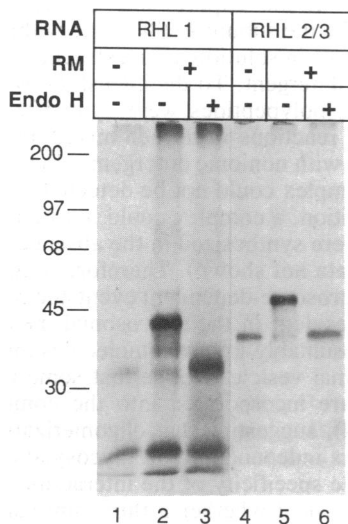

FIG. 1. Identification of RHL translation products. RNA for RHL-1 or RHL-2/3 was translated with or without dog pancreatic microsomes (RM), and the products were immunoprecipitated with antiserum against purified RHL. Immunoprecipitates were either prepared directly for electrophoresis or first digested with endoglycosidase H. Sizes in kDa of protein standards are indicated at left. In addition to the expected translation products, each RNA produces smaller polypeptides, such as that migrating just behind the dye front in lanes 1–3. Their size and reactivity with the carboxyl-terminal antibodies (as shown in other experiments) suggest they originate from initiations at internal methionines.

The translation products of the major RHL form, or RHL-1, have been characterized (16), and our results agree. RHL-1 is synthesized as a 31-kDa polypeptide (Fig. 1, lane 1) consistent with the size predicted by its cDNA sequence (4). When synthesized in the presence of dog pancreatic microsomes, the major product is 40 kDa (Fig. 1, lane 2), which is degraded by endoglycosidase H to 31 kDa (Fig. 1, lane 3). Thus the 31-kDa primary translation product is modified within the microsome by the addition of asparagine-linked carbohydrate chains. In addition to the 31-kDa and 40-kDa products, we consistently find intermediate-sized products of ≈33 and 36 kDa. Presumably these represent the addition of one and two asparagine-linked chains, respectively. The 40-kDa product would then contain three asparagine-linked carbohydrates. The primary translation product of RHL-2/3 is 36 kDa (Fig. 1, lane 4), and this product is also converted by microsomes to a form ≈8- to 9-kDa larger, which is sensitive to endoglycosidase H (Fig. 1, lanes 5 and 6). As with RHL-1, two intermediate products have been seen in some experiments. Thus, both polypeptides appear to receive three asparagine-linked carbohydrate chains, consistent with the presence of three potential asparagine acceptor sites in both sequences (4). In the cell the RHL-2/3 translation product gives rise to two proteins (i.e., RHL-2 and RHL-3) apparently through addition of polylactosamine units to some of the molecules (4).

Each RHL polypeptide is specifically recognized by antiserum directed against its carboxyl terminus (Fig. 2). Quantitative analysis showed ≈60% of RHL-1 and 95% of RHL-2/3 could be recovered in immunoprecipitates (Fig. 2, lanes 2 and 7). The fraction of RHL-1 originally bound to antibody appears greater, however, because the anti-RHL 1 supernatant contained only ≈10% of the input antigen (Fig. 2, lane 3). We conclude that ≈90% of RHL-1 was originally bound to antibody but that a fraction of the bound antigen was removed during the single rinse in Tris-buffered saline. Thus, each antibody recognizes virtually all of the homologous receptor polypeptide, but the two antibodies have different apparent affinities. Neither receptor polypeptide bound detectably to the heterologous antibody, as determined by direct analysis of the immunoprecipitate (Fig. 2, lanes 4 and 9) and by analysis of the supernatant of the immunoprecipitate (Fig. 2, lanes 5 and 10).

When the two RNAs are translated in the same reaction, however, each antibody precipitates both the 40-kDa RHL-1 and the 45-kDa RHL-2/3 receptor polypeptides after solubilization in nonionic detergent (Fig. 3*A*, lanes 1 and 2). If the reactions are solubilized in $NaDodSO_4$, however, only the homologous peptide is immunoprecipitated, confirming the

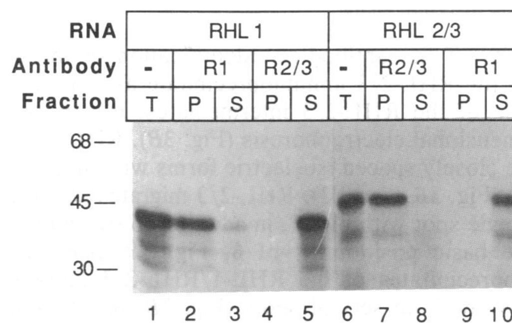

FIG. 2. Immunoprecipitation of RHL polypeptides with carboxyl-terminal antibodies. RNAs were translated in the presence of microsomes. After extraction with Triton X-100, aliquots were either precipitated with methanol (T) or immunoprecipitated with carboxyl-terminal antibodies to RHL-1 (R1) or RHL-2/3 (R2/3). Immunoprecipitation mixtures were separated into their pellets (P) and supernatants (S); the supernatants were then precipitated with methanol. Numbers at left indicate protein-standard sizes in kDa.

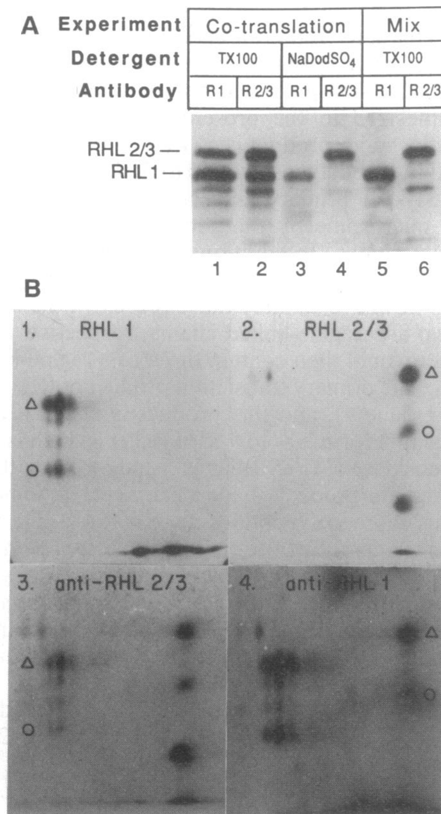

FIG. 3. Formation of a heterotypic oligomer of RHL polypeptides during translation. (*A*) RNAs were translated in the presence of microsomes either in the same reaction (lanes 1–4) or separately (lanes 5 and 6). Aliquots of the cotranslation were solubilized in 1% Triton X-100 or 1% $NaDodSO_4$. Samples treated with $NaDodSO_4$ were diluted 20-fold with 1% Triton X-100 before adding antibody. The reactions for lanes 5 and 6 were combined on ice, immediately treated with Triton X-100, and then handled as for the cotranslation sample. Immunoprecipitations were with the carboxyl-terminal antibodies. (*B*) Two-dimensional analysis of the translation products. RNAs were either translated separately, and the products were immunoprecipitated with their respective antibodies (panels 1 and 2), or the RNAs were translated together and immunoprecipitated as indicated (panels 3 and 4). The basic end of the first-dimension isoelectric focusing gel is at left. ○, Unglycosylated polypeptides; △, glycosylated polypeptides. Note that the presumed internal initiation products are not part of the heterooligomer, as indicated, for example, by absence of the smallest RHL-2/3 spot in panel 4. This result agrees with the suggestion that oligomerization is occurring within the microsome because these polypeptides would lack a membrane insertion signal.

specificity of the antibodies. Identification of the 40-kDa band in the RHL-2/3 immunoprecipitate as RHL-1, rather than one of the RHL-2/3 intermediates, is confirmed by two-dimensional electrophoresis (Fig. 3*B*). RHL-1 migrates as three closely spaced isoelectric forms with an average pI of ≈5.2 (Fig. 3*B*, panel 1). RHL-2/3 migrates as a predominant single spot with pI 4.1, in addition to two minor spots at more basic positions (≈pI 6, Fig. 3*B*, panel 2). The immunoprecipitates of the RHL-1/RHL-2/3 cotranslation clearly show both sets of spots (Fig. 3*B*, panels 3 and 4). Thus, the RHL polypeptides interact to form a complex that is stable, at least under the conditions of immunoprecipitation, and this complex can be detected with either of the carboxyl-terminal antibodies. The complex is dependent on noncovalent interactions, since elution of immunoprecipitates by $NaDodSO_4$ in the absence of reducing agents yields the two polypeptides in their monomeric forms (data not shown).

Table 1. Recovery of RHL subunits in the heterooligomer by immunoprecipitation

		Immunoprecipitation			
		Homologous Ab		Heterologous Ab	
Subunit	Total cpm*	cpm*	Recovery, %	cpm*	Recovery, %
RHL-1	2789 ± 222	1245 ± 63	45	1036 ± 106	37
RHL-2/3	2416 ± 111	1986 ± 105	82	935 ± 107	39

RHL RNAs were translated together as in Fig. 3, and aliquots of the Triton X-100 extract were methanol-precipitated (total) or immunoprecipitated with the carboxyl-terminal antibodies. Homologous Ab and heterologous Ab refer to antibody directed against the subunit indicated or the alternate subunit, respectively. Only the bands corresponding to the glycosylated polypeptides were excised for counting of radioactivity. Results are from two experiments.
*Mean ± SEM; $n = 4$ for total amounts, $n = 4$ for immunoprecipitations with anti-RHL-1, and $n = 3$ for anti-RHL-2/3.

Quantitative analysis of the immunoprecipitates showed that ≈40% of each subunit was coprecipitated by the heterologous antiserum (Table 1). Nominally this suggests that the heterooligomer represents a 1:1 interaction between the subunits. This ratio is, however, probably an underestimate of the extent of oligomerization because the antibodies do not recover all of the homologous subunit. Recovery of the homologous subunit in these experiments is 45% for RHL-1 and 82% for RHL-2/3. These recoveries are less than when the subunits are synthesized individually (as noted above), suggesting that the antibodies recognize the complex less efficiently than the separate subunits. Correction of the recovery of the heterologous subunit for 100% recovery of the homologous subunit suggests that 87% of RHL-2/3 and 45% of RHL-1 is present in the heterooligomer, resulting in a ratio of two RHL-2/3 to one RHL-1. These calculations could be in error, of course, if the antibodies affected the structure of the complex by causing dissociation of the subunits.

The interaction between RHL subunits could be occurring at two points in the experiment, during the translation incubation while the subunits still reside within the microsomal membrane or in solution after extraction of microsomes with nonionic detergent. To distinguish between these possibilities, RHL polypeptides were synthesized in separate reactions. The reactions were then mixed on ice and immediately treated with nonionic detergent. Under these circumstances the complex could not be detected (Fig. 3*A*, lanes 5 and 6). In addition, a complex could not be identified when the subunits were synthesized in the absence of microsomal membranes (data not shown). Therefore, oligomerization is driven by a microsome-dependent event and occurs while the subunits are resident in the microsomal membrane. Oligomerization presumably involves molecules inserted into the same microsomal vesicle. Notice that some unglycosylated polypeptides are incorporated into the complex (Fig. 3*B*, panels 3 and 4), suggesting that oligomerization either precedes or occurs independently of glycosylation.

To assess the specificity of the interaction between RHL monomers we asked whether a third, unrelated membrane glycoprotein, DPP-IV, could compete for the formation of the RHL complex. We have previously shown that a truncated version of DPP-IV lacking about one-half of the molecule from the carboxyl terminus is inserted into microsomes and efficiently glycosylated with asparagine-linked carbohydrate chains *in vitro* (23). This truncated DPP-IV is synthesized as a 38-kDa polypeptide that is modified to 52 kDa by addition of carbohydrate (Fig. 4, lane 1). As with the RHL subunits, several putative glycosylation intermediates are found. After synthesis of the three polypeptides in combination, the RHL complex can be precipitated as usual with the RHL-1 or

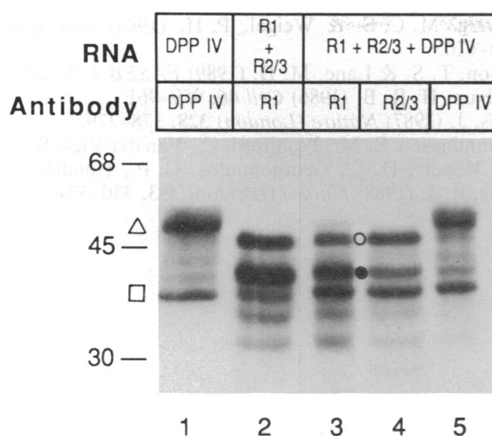

FIG. 4. The RHL oligomer assembled *in vitro* is a specific complex. RNAs for RHL and a truncated form of DPP-IV were translated with microsomes in the combinations indicated. Immunoprecipitations were with the RHL carboxyl-terminal antibodies or with antibody to gel-purified DPP-IV (18). □, Unglycosylated DPP-IV; △, glycosylated DPP-IV; ●, glycosylated RHL-1; ○, glycosylated RHL-2/3. Because DPP-IV unglycosylated and intermediate products are difficult to resolve from some RHL products, emphasis is on the glycosylated DPP-IV molecules. Numbers at left indicate protein standard sizes in kDa.

RHL-2/3 antibodies (Fig. 4, lanes 3 and 4). DPP-IV, however, is precipitated only by anti-DPP-IV (Fig. 4, lane 5). The exclusion of another membrane-bound glycoprotein suggests that formation of the RHL complex represents a specific interaction between RHL-1 and RHL-2/3, rather than a generalized aggregation of membrane glycoproteins.

DISCUSSION

We have demonstrated with subunit-specific antisera that a heterooligomeric RHL complex assembles when the subunits are inserted into microsomes during translation in a cell-free system. Furthermore, another membrane protein, DPP-IV, synthesized in the same reaction is excluded from the RHL oligomer. We propose that the immunoprecipitated heterooligomer represents a specific and direct interaction between the RHL subunits. Alternatively, the newly synthesized RHL monomers may interact with endogenous microsomal proteins, as has been shown for other membrane-bound and secreted proteins (24–26). At present, we do not know whether or not such interactions occur.

The stoichiometry of the complex assembled *in vitro* can be implied from the relative amount of coprecipitated heterologous subunit in each immunoprecipitate (both proteins contain three methionines (4), so corrections for isotope incorporation are not required). Equivalent amounts of the two subunits are recovered in the heterologous immunoprecipitates. After correction for recovery of the homologous subunit, however, it appears that RHL-2/3 is roughly twice as abundant as RHL-1 in the heterooligomer. Thus, the direct results suggest the heterooligomer contains an equimolar amount of each subunit, but we calculate that it is actually composed of two RHL-2/3 molecules for every RHL-1. These results also indicate that oligomerization in the cell-free system is quite efficient because up to 85% of RHL-2/3 appears to be present in the complex in a typical experiment.

Given that the rat hepatocyte contains RHL-1 in excess of RHL-2/3 (1, 27), our model predicts that a proportion of RHL-1 in the cell exists as free subunit. Our previous immunoprecipitation results are consistent with this prediction (11). In contrast to our results, Herzig and Weigel (28) have recently proposed that RHL-1 interacts with RHL-2/3 in the ratio 2:1. The critical difference is their model assumes all RHL-1 polypeptides to be present in the heterooligomeric complex. This distinction between the models remains to be rigorously tested.

Formation of the RHL complex is driven by insertion into microsomal vesicles and, therefore, probably occurs shortly after translocation across the endoplasmic reticulum in the cell. Because the complex is sensitive to $NaDodSO_4$, a particular conformation of the polypeptide chain may be required. We suggest that translocation across the microsomal membrane influences folding of the monomers, so as to expose sequences involved in oligomerization. This interpretation is, in part, based on results of Hsueh *et al.* (6) that indicate active RHL forms during *in vitro* synthesis only after membrane insertion. That membrane translocation might be critical to the formation of the proper disulfides and, therefore, to the folding of nascent chains into active proteins has been suggested (6, 29). Rather than having direct effects on folding of the nascent polypeptide chain, the microsomes might be providing accessory proteins that control oligomeric assembly (30–32). In general, regulation of subunit interactions in oligomeric membrane proteins is poorly understood. The efficiency with which the RHL subunits oligomerize during cell-free synthesis suggests this system as a useful model.

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