Early Disulfide Bond Formation Prevents Heterotypic Aggregation of Membrane Proteins in a Cell-free Translation System

Mamadi Yilla, Darrell Doyle, and James T. Sawyer

Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York 14260

Abstract. We previously demonstrated that a heterotypic complex of the two rat asialoglycoprotein receptor subunits was assembled during cell-free translation (Sawyer, J. T., and D. Doyle. 1990. *Proc. Natl. Acad. Sci. USA.* 87:4854–4858). We have characterized this system further by analyzing polypeptide interactions under both reducing and oxidizing translation conditions. This report shows that the complex represents a heterogeneous interaction between reduced membrane proteins rather than a specific oligomeric structure. In the reduced state membrane proteins interact in this system to form aggregates of diverse size and composition. The aggregated nascent polypeptides interact with the immunoglobulin heavy chain binding protein but this protein is not an integral component of the aggregate. Aggregation occurs via the exoplasmic domain, rather than the transmembrane domain, and the folding of this domain by the formation of intramolecular disulfides, prevents the interaction from occurring. Additionally, the folded molecules containing intramolecular disulfides lack high affinity binding activity and thus appear to resemble the earliest folding intermediates seen in vivo (Olson, J. T., and M. D. Lane. 1989. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3: 1618–1624). These results lead us to suggest that the formation of intramolecular disulfides during early biogenesis serves to prevent nonspecific associations between nascent polypeptides.

STUDIES of early events along the secretory pathway indicate that misfolded or unassembled proteins are often not transported along their targeted pathways, but are retained instead within the ER (Rose and Doms, 1988; Hurtley and Helenius, 1989). In some cases the retained molecules are found in association with accessory proteins resident in the ER lumen such as the Ig heavy chain binding protein (BiP)[1] (Bole et al., 1986; Hurtley et al., 1989). Retention can also result from the aggregation of misfolded proteins in the absence of significant interaction with an accessory factor (Rose and Doms, 1988). Aggregation is commonly thought to result from the exposure of cysteine residues or hydrophobic domains (Rose and Doms, 1988). Since membrane proteins are in general oligomeric, control of the early folding events leading to the correct assembly is a significant problem with respect to the organization of the cell surface and membrane-bound organelles.

We have been using the mammalian asialoglycoprotein receptor to study this general problem. The asialoglycoprotein receptor is a major endocytic protein of the liver which recognizes and internalizes galactose-terminated serum glycoproteins (Spiess, 1990). Several lines of evidence suggest that this receptor exists on the cell surface as a hetero-oligomer of two or more subunits, depending on the species (McPhaul and Berg, 1986; Sawyer et al., 1988; Bischoff et al., 1988; Shia and Lodish, 1989). The receptor is preferentially expressed on the basal surface of hepatocytes (Matsuura et al., 1982; Geuze et al., 1982) and a similar polarization is evident in transfected MDCK cells (Graeve et al., 1990). Therefore, the asialoglycoprotein receptor should serve as an important model in determining relationships between folding, assembly and vectorial transport.

In previous work, translation of the two subunits of the rat asialoglycoprotein receptor (also known as the rat hepatic lectin [RHL]) in a cell-free system containing pancreatic microsomes resulted in efficient assembly of a heterotypic complex (Sawyer and Doyle, 1990). Assembly was thought to be specific to the RHL subunits (called RHL-1 and RHL-2/3) since a truncated form of an unrelated membrane glycoprotein, dipeptidyl peptidase IV (DPP-IV), was not incorporated into the complex. Further characterization of this system has demonstrated that the interaction of RHL subunits actually represents a nonspecific complex, or aggregate, of inactive molecules lacking disulfide bonds which is generated under reducing translation conditions. These polypeptides interact in a heterogeneous fashion through their exoplasmic domains. When the exoplasmic domain is truncated, or when the molecules are synthesized under oxidizing translation conditions which allow the exoplasmic domain to begin folding, the subunits do not aggregate.

These experiments show that reduced membrane proteins have the intrinsic capacity to interact in a heterogeneous fashion within microsomal vesicles. We suggest that hetero-

1. *Abbreviations used in this paper*: BiP, immunoglobulin heavy chain binding protein; Cx-32, connexin-32; DPP-IV, dipeptidyl peptidase IV; GSSG, oxidized glutathione; PDI, protein disulfide isomerase; RHL, rat hepatic lectin.

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geneous aggregation would represent a dead-end folding pathway in vivo and anticipate that cells have a mechanism controlling nonspecific nascent chain interactions. Since the nonaggregated, oxidized polypeptides resemble early folding intermediates in other systems (Olson and Lane, 1989), we propose that the rapid folding of polypeptides during translocation into the lumen of the ER (Bergman and Kuehl, 1979; Peters and Davidson, 1982; Braakman et al., 1991) serves as a control mechanism preventing nonspecific, heterotypic aggregation.

Materials and Methods

Materials

SP6 RNA polymerase, canine pancreatic microsomes, rabbit reticulocyte lysate, nucleotides, RNAsin, and all restriction enzymes except for BalI were obtained from Promega Biotec (Madison, WI); BalI and [^{35}S]methionine (>1,000 Ci/mMol; 1Ci=37 GBq) were from Amersham Corp. (Arlington Heights, IL); cap analogue 7-methylguanosine (5') triphosphoguanosine and protein A–Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ); purified Triton X-100 (Surfact-Amps X-100) was from Pierce Chemical Co. (Rockford, IL); oxidized glutathione (GSSG) was from Sigma Chemical Co. (St. Louis, MO); SDS-PAGE calibration standards were from Bethesda Research Laboratories (Bethesda, MD). RHL and DPP-IV cDNAs were as previously described (Sawyer and Doyle, 1990). cDNA for connexin-32 (Cx-32) was from B. Nicholson (State University of New York at Buffalo).

Antibodies

Monoclonal anti-BiP was a gift from D. Bole (Howard Hughes Medical Institute, Ann Arbor, MI); rabbit polyclonal antibody to protein disulfide isomerase (PDI) was a gift from R. Freedman (University of Kent, Canterbury, England); antibody to Cx-32 was a gift from B. Nicholson; antibody to native RHL and antibody to gel-purified DPP-IV were prepared in the Doyle laboratory by J. Petell and W. Hong, respectively. The RHL-1 and RHL-2/3 carboxy-terminal antisera have been described (Sawyer et al., 1988).

In Vitro Transcriptions and Translations

Full-length transcripts for RHL, DPP-IV, and Cx-32 were synthesized with the Promega Biotec riboprobe system as previously described (Sawyer and Doyle, 1990). Truncated transcripts were made by linearizing cDNAs within the coding sequence as indicated in the results. Translations were as previously described and included four equivalents of pancreatic microsomal membranes as defined by the manufacturer (Sawyer and Doyle, 1990) with the exception that in some experiments the reticulocyte lysate was supplemented with GSSG to induce the formation of disulfide bonds. Levels of GSSG allowing disulfide formation without inhibiting translation were determined in trial experiments. Actual GSSG concentrations are indicated in the legends.

Immunoprecipitations

Translation reactions typically were diluted in 20 vol of TBS containing 1% Triton X-100 and 5 mM iodoacetamide (extraction buffer). In the absence of iodoacetamide, intermolecular disulfide bonds were generated during extraction. The reaction mixture was extracted on ice and the solubilized material immunoprecipitated as described (Sawyer and Doyle, 1990). In some experiments the supernatants of immunoprecipitates were collected and immunoprecipitated a second time to determine various combinations of chain interactions. mAbs were incubated with protein A–Sepharose before immunoprecipitations were carried out. To assay for BiP or PDI association with nascent chains, translation cocktails were depleted of ATP by treatment with apyrase (45 U/ml final concentration) for 2 min at 30°C, before extraction and immunoprecipitation. Immunoprecipitates were analyzed by SDS-PAGE according to Laemmli (1970) and the labeled proteins detected by fluorography (Bonner and Laskey, 1974). Fluorograms were quantitated by excising the desired bands from dried gels and counting their radioactivity by liquid scintillation counting.

Ligand Binding Assays

Translation reactions were extracted with 20 vol 10 mM Tris, 1.25 M NaCl, 50 mM CaCl, 0.5% Triton X-100, pH 7.8 (loading buffer) (Hudgin et al., 1974), and the solubilized material applied to a 1-ml galactose–Sepharose column previously equilibrated with loading buffer. After 60 min, the column was washed three times with a total of 1.5 ml loading buffer and then eluted with 20 mM ammonium acetate, 1.25 M NaCl, 0.5% Triton X-100 pH 5.4 (Hudgin et al., 1974). 0.5-ml fractions were collected, neutralized with 1 M Tris and immunoprecipitated with antibody to native RHL.

Sucrose Gradient Density Centrifugation

Translation reactions were diluted with extraction buffer as described above and applied to a 5-ml, 5–20% continuous sucrose gradient. Gradients were spun in an SW55 Ti Rotor for 7 h at 9,700 *g* at 4°C. 0.5-ml fractions were collected and immunoprecipitated with antibody to native RHL.

Results

RHL Interactions in the Cell-free System Occur Only under Reducing Conditions

Under standard translation conditions, the RHL polypeptides are synthesized in their reduced forms since the commercial lysate is stabilized with 2 mM DTT (Fig. 1, lanes *1*, *2*, *4*, *5*). Supplementing the translation cocktail with GSSG results in the formation of intramolecular disulfide bonds as evidenced by an increased electrophoretic mobility under nonreducing conditions (Fig. 1, lanes *3* and *6*). RHL-2/3 shows an apparent molecular mass difference between the reduced and oxidized states of ~5 kD, whereas RHL-1 shows a difference of ~3 kD. The disulfide structure of native RHL and the cell-free translation products has not been determined. Since both subunits are identical in the number and position of cysteine residues, we expect the pattern of disulfide bonds to be conserved. Therefore, the oxidized RHL-1 and RHL-2/3 products may represent different intermediates in the folding pathway. Alternatively, the discrepancy between the RHL-1 and RHL-2/3 oxidized poly-

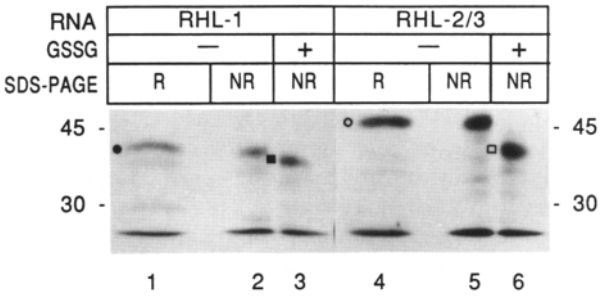

Figure 1. Synthesis of reduced and oxidized RHL polypeptides. RNAs for RHL-1 and RHL-2/3 were individually translated in the presence of canine pancreatic microsomes for 90′ at 30°C. Rabbit reticulocyte lysate was supplemented with 3 mM GSSG where indicated. The products were immunoprecipitated with antibody raised against native receptor. The polypeptides were subsequently analyzed under reducing (*R*) or nonreducing (*NR*) SDS-PAGE conditions. ○, reduced RHL-2/3; ●, reduced RHL-1; □, oxidized RHL-2/3; ■, oxidized RHL-1. Additional RHL translation products have previously been described and include primary translation products, intermediate glycosylation products, and internal initiation products (Sawyer and Doyle, 1990). Numbers in the margins of this and other figures indicate protein standard sizes in kilodaltons.

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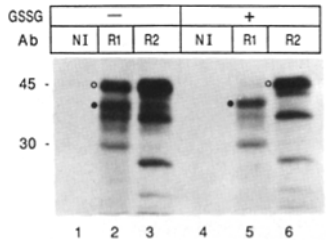

Figure 2. Cotranslation of reduced and oxidized RHL polypeptides. The two RHL RNAs were cotranslated under reducing (−GSSG) or oxidizing (+3 mM GSSG) conditions as indicated. The products were immunoprecipitated with antisera specific to the carboxy terminus of either RHL-1 (*R1*) or RHL-2/3 (*R2*), or with control serum (*NI*). The immunoprecipitates were analyzed under reducing SDS-PAGE conditions. o, glycosylated RHL-2/3; •, glycosylated RHL-1.

peptides may reflect a difference in other conformational features.

As we have previously shown, RHL polypeptides translated under reducing conditions form a heterotypic complex as demonstrated by coimmunoprecipitation with subunit specific antisera (Fig. 2, lanes *1–3*). In typical experiments ~40% of each subunit is coimmunoprecipitated with the heterologous antibody (Sawyer and Doyle, 1990). Consistent with a lack of disulfide bond formation the complex dissociated into monomeric forms under nonreducing SDS-PAGE conditions (data not shown). After translation under oxidizing conditions, however, the RHL polypeptides do not coimmunoprecipitate as a heterotypic complex (Fig. 2, lanes *4–6*). GSSG is commonly found to reduce the yield of translation product through the inactivation of eukaryotic initiation factor 2 (Ernst et al., 1978; Scheele and Jacoby, 1982). Titration of RNA over a 10-fold range indicates that the reduced subunits interact at concentrations much lower than obtained in the presence GSSG (data not shown), indicating the slight decrease in the level of translation products evident in the presence of GSSG (Fig. 2, lanes *4–6*) is not significant with respect to subunit interactions. Therefore, folding of the polypeptide chains as indicated by the formation of intramolecular disulfides prevents heterotypic complex formation. It appears that only reduced RHL molecules interact during cell-free translation.

"Unfolded" RHL Polypeptides Interact Nonspecifically

To determine whether the oxidized polypeptides represent legitimate intermediates in the folding pathway we assayed their functional state by binding to galactose-Sepharose. Drickamer and co-workers have shown that both RHL-1 and RHL-2/3 can bind independently to galactose-Sepharose after cell-free translation (Hsueh et al., 1986; Halberg et al., 1987). As might be expected, each subunit appears completely inactive after translation under standard reducing conditions. All of the product applied to the affinity column is released in the flowthrough and the first wash fraction (Fig. 3, *A* and *D*). Therefore, subunits translated under reducing conditions lack the native tertiary structure as indicated by the apparent absence of a high affinity binding site, and the lack of disulfide bonds. We refer to these as "unfolded" molecules. When translated in the presence of GSSG the molecules show low affinity binding as evidenced by retardation on the column (Fig. 3, *B* and *E*). The fraction of material which is retarded by the column increases after a "chase" with a higher concentration of GSSG which inhibits further

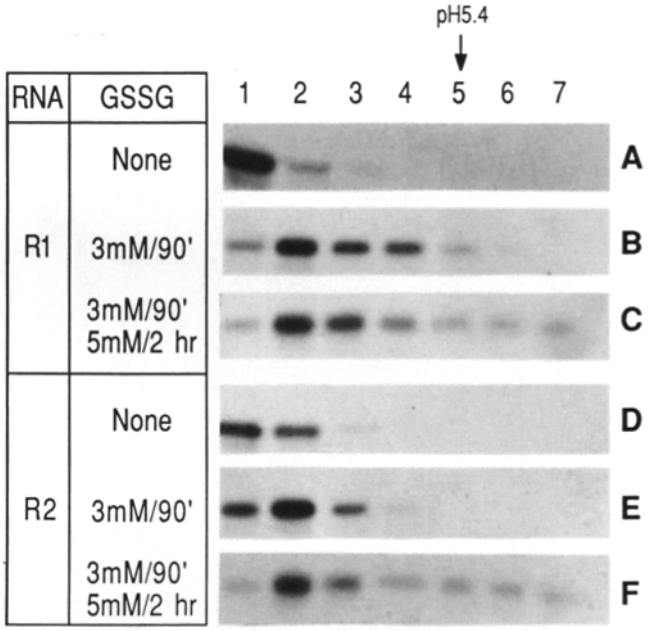

Figure 3. Ligand binding activity of reduced and oxidized RHL molecules. RHL subunits were independently synthesized under reducing or oxidizing conditions, as previously described. In some cases, the reactions were brought to 5 mM GSSG and incubated for 2 h. The translation products were applied to a galactose–Sepharose affinity column and the flowthrough (lane *1*), column washes (lanes *2–4*), and eluate fractions (lanes *5–7*) were immunoprecipitated with antibody to native RHL.

translation (Fig. 3, *C* and *F*). This is indicated by a reduced amount of material in the flow-through and an increase in material eluted at pH 5.4. In the case of RHL 2/3 a small peak of apparently high affinity binding is evident by quantitative analysis of the elution profile (data not shown). The inclusion of GSSG in the initial translation reaction, therefore, induces the formation of intramolecular disulfides but does not result in folding of a high affinity binding site. The "step-wise" effect of GSSG suggests that the oxidized translation products are intermediates in the early folding pathway. The formation of these putative folding intermediates is at the expense of the interaction between the two chains.

In previous work we used a truncated version of DPP-IV to demonstrate subunit specificity in the RHL heterotypic complex (Sawyer and Doyle, 1990). This apparent specificity has been reinvestigated using full-length DPP-IV. Under reducing conditions full-length DPP-IV was coimmunoprecipitated with each RHL subunit (Fig. 4 *a*, lanes *1–3*). Curiously, the interaction between RHL and DPP-IV was not detected with DPP-IV antibody (Fig. 4 *a*, lane *4*). Most likely DPP-IV antibody either displaces RHL polypeptides or, alternatively, does not recognize DPP-IV when complexed with RHL. A similar interaction with RHL could be detected with a second unrelated protein, the gap junction protein Cx-32, which differs from RHL and DPP-IV not only in sequence, but also in its transmembrane topology (i.e., has multiple membrane spanning domains; Paul, 1986). After translation under reducing conditions, a small fraction of Cx-32 cotranslated with RHL-1 is immunoprecipitated with RHL-1 antibody (Fig. 4 *b*, lanes *2* and *3*). Specificity of the RHL peptide antisera was verified when cotranslated prod-

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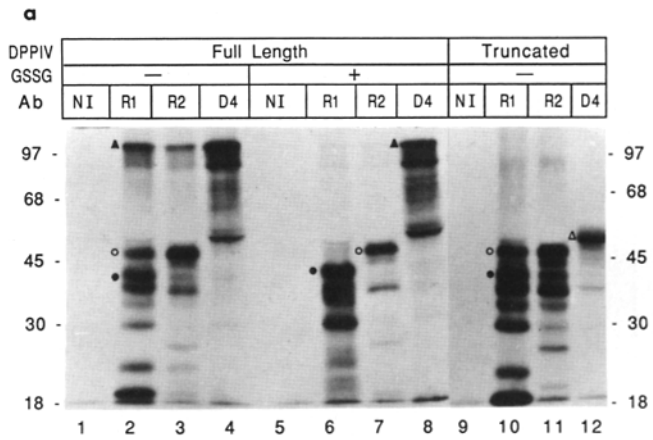

Figure 4. Cotranslation of RHL with DPP-IV and with Cx-32. RHL RNAs were cotranslated with full-length or Stu 1 truncated, DPP-IV (*a*), or with Cx-32 (*b*) under reducing or oxidizing conditions as indicated. The Stu 1 truncation was to a unique site in the exoplasmic domain resulting in the removal of 435 aa residues. The products were immunoprecipitated with carboxy-terminal antisera to each RHL subunit, antibody to gel-purified DPP-IV, carboxy-terminal antisera to Cx-32 or with control serum (*NI*). (○) Glycosylated RHL-2/3; (●) glycosylated RHL-1; (▲) full-length glycosylated DPP-IV; (△) StuI truncated glycosylated DPP-IV; (▼) unglycosylated RHL-1; (**x**) Cx-32; (▽), RHL-1 internal initiation product.

ucts were extracted in SDS. Under these conditions, only the homologous RHL protein was immunoprecipitated (data not shown). After translation under oxidizing conditions the RHL polypeptides do not coimmunoprecipitate with DPP-IV or Cx-32 (Fig. 4 *a*, lanes *5–7*, Fig. 4 *b*, lanes *5* and *6*). Therefore, reduced, but not oxidized, RHL molecules interact not only with each other but with membrane proteins that are unrelated both in sequence and in transmembrane topography. The initial folding of the RHL polypeptide chains serves to prevent the heterogeneous interaction displayed by reduced molecules without producing a high affinity ligand binding site.

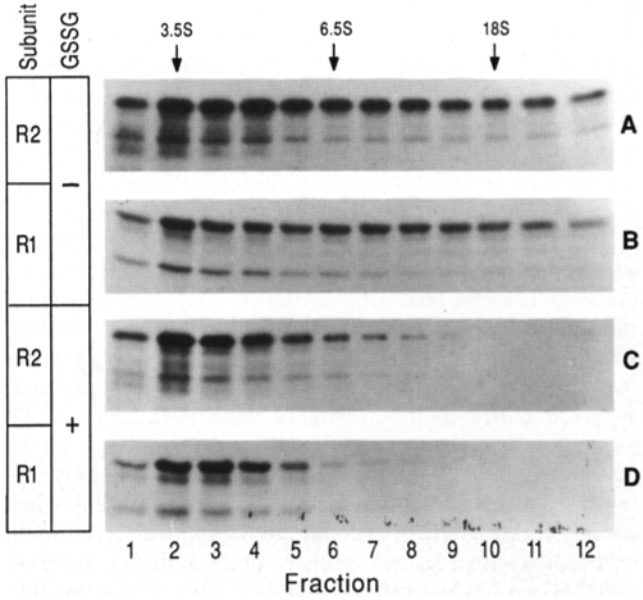

Figure 5. Velocity sedimentation analysis of RHL polypeptides. RHL subunits were translated under reducing or oxidizing conditions and the products were then subjected to continuous sucrose gradient density centrifugation. The gradient fractions were immunoprecipitated with antibody to native receptor. The sedimentation of standards run on a separate gradient, is indicated by arrows above (ovalbumin, 3.5S; immunoglobulin 6.5S; thyroglobulin 18S).

The Nonspecific Chain Interactions Generate Aggregates of Various Sizes and Compositions

To further characterize the interactions of the reduced polypeptides, we sought to determine if distinct sizes and types of complexes were generated. Not surprisingly, we found that both RHL polypeptides were present as a heterogeneous population of sizes which included monomers and a continuum of oligomeric forms when analyzed by velocity sedimentation over sucrose gradients (Fig. 5, *A* and *B*). Though no distinct oligomeric peak was evident, ~50% of the total product was found associated with high molecular weight complexes (Table I). In contrast, the oxidized subunits sedimented largely as monomers (Fig. 5, *C* and *D* and Table I). Thus interactions between the "unfolded" RHL subunits generate complexes of heterogeneous size. Oligomers are not in equilibrium with monomers in the detergent extract as indicated by experiments in which pooled gradient fractions were re-run on a second gradient. Thus, the 3.5S pool (fractions 1–4) was recovered in the 3.5S position when run on a second gradient (data not shown).

Since sedimentation analysis indicated that the reduced polypeptides could interact to generate aggregates of diverse size we expected a diversity of chain composition as well. For example, we expected the interaction of DPP-IV with RHL (Fig. 4 *a*) to generate complexes containing all three chains. Alternatively, DPP-IV might interact with only RHL-1 or RHL-2/3 individually. To distinguish between these alternatives, a cotranslation of DPP-IV and both of the RHL subunits was successively immunoprecipitated with

Table I. Quantitative Analysis of Sucrose Gradients

	RHL-1		RHL-2/3	
GSSG	M	O	M	O
−	48.0 ± 0.31	51.7 ± 1.62	55.1 ± 1.62	44.8 ± 1.62
+	81.7 ± 6.64	18.3 ± 6.64	82.0 ± 3.11	13.0 ± 3.11

Glycosylated bands were excised from gels similar to Fig. 5 and quantitated by liquid scintillation counting. The incorporated radioactivity from individual column fractions was combined into monomeric (*M*) pools (fractions 1–5) or oligomeric (*O*) pools (fractions 6–12). The numbers below represent a statistical average of three separate gradient runs and indicate the percentage of product per fraction relative to total product present.

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Table II. Interactions of DPP-IV with RHL-1 or RHL-2/3

First Ab:	NI	anti-RHL-1	anti-RHL-2/3
Second Ab:			
RHL-1	435 ± 29.7*	44 ± 14.7	249 ± 17.6*
RHL-2/3	250 ± 38.5*	105 ± 12.7*	35 ± 8.1

The product of a cotranslation of DPP-IV, RHL-1 and RHL-2/3 was extracted in the presence of Triton X-100 and the supernatant assayed with peptide-specific RHL antibodies (anti-RHL-1 and anti-RHL-2/3) and with nonimmune sera (*NI*). After removal of immune complexes by adsorption with protein A–Sepharose, the supernatant was assayed with either anti-RHL-1 or with anti-RHL-2/3. The glycosylated DPPIV bands were excised from dried gels and radioactivity incorporated was counted by liquid scintillation counting. Experiments were done in triplicate and the data analyzed according to Dunnett. * P value = 0.0001.

the RHL antibodies. Anti-RHL-1 coimmunoprecipitated an average of 435 cpm in DPP-IV from three experiments (Table II, column *1*). This decreased by ~40% to 249 cpm when preceded by immunoprecipitation with anti-RHL-2/3 (Table II, column *3*). Similarly, RHL-1 immunoprecipitation decreased the pool of DPP-IV complexed to RHL-2/3 by ~50% (Table II, columns *1* and *2*). This demonstrates that approximately one-half of the DPP-IV molecules which interact with RHL-1 also interact with RHL-2/3. Therefore, the complexes can contain all three polypeptides. Based on the lack of specific chain interactions, and the lack of a specific size and chain composition, it appears that the complexes formed under reducing conditions represent the random aggregation of multiple chains.

"Unfolded" RHL-1 and DPP-IV Interact via Their Exoplasmic Domain

Since DPP-IV truncated to remove 435 aa residues in the exoplasmic domain does not interact with "unfolded" RHL (Sawyer and Doyle, 1990, and Fig. 4 *a*, lanes *9–12*), we examined the role of the exoplasmic domain of RHL-1 in the interaction with RHL-2/3. cDNA to RHL-1 was linearized at various sites within the coding region of the exoplasmic domain. The resulting run-off transcripts were cotranslated with full-length RHL-2/3. Removal of up to 105 residues

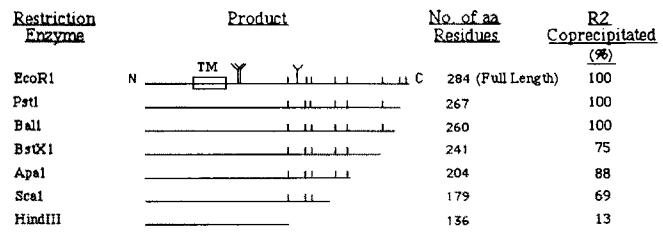

Figure 6. Truncated RHL-1 interactions with RHL-2/3. cDNA for RHL-1 was linearized within the coding region as indicated. The RNAs were individually cotranslated with RHL-2/3 and the reaction terminated by treatment with 1 mM puromycin for 20 min (Adelman et al., 1973). The products were immunoprecipitated with a peptide antisera made to a region 35 aa residues outside the transmembrane domain between residues 90 and 106. Radioactivity in glycosylated RHL-2/3 bands was counted. The amount of RHL-2/3 coimmunoprecipitated with each RHL-1 protein is expressed as a function of that associated with full-length RHL-1. The transmembrane domain of RHL-1 is indicated as TM, N-linked glycosylation sites and cysteine residues are indicated by tall and short lines, respectively.

from the carboxy-terminal domain of RHL-1 is largely without effect on coimmunoprecipitation with RHL-2/3 (Fig. 6). Truncation of an additional 43 residues with HindIII, however, essentially eliminated the interaction with RHL-2/3. Therefore, the nonspecific interactions of both RHL-1 and DPP-IV can be prevented through truncation of the carboxy-terminal exoplasmic domains. In the case of RHL-1, interaction with other "unfolded" proteins minimally involves a 43 amino acid stretch in the exoplasmic domain. The truncations which prevent aggregation remove 10 of 13, and 8 of 8 cysteine residues in the exoplasmic domains of DPP-IV and RHL-1, respectively. This correlates well with the negative effect of the folding of the exoplasmic region via the formation of intramolecular disulfides on aggregation. In addition, this result clearly indicates that the RHL and DPP-IV subunits are not aggregated at their hydrophobic transmembrane domains in this system.

The "Unfolded" Polypeptides do Not Interact through BiP or PDI

Since RHL interactions in this system involve reduced and apparently unfolded polypeptide chains, it seemed likely that the subunits interact with BiP, and that BiP might in fact be an integral part of the complex. The usual conditions of our immunoprecipitations, however, do not favor stable interactions between BiP and other proteins (Munro and Pelham, 1986; Kassenbrock et al., 1988). This was confirmed by immunoprecipitation with BiP mAb (Fig. 7, lane *4*). The reduced RHL polypeptides coimmunoprecipitate with BiP but only after depletion of ATP with apyrase (Fig. 7, lane *6*). Immunoprecipitation of the RHL aggregate, on the other hand, is not dependent on apyrase treatment (Fig. 7, lanes *1–3*). Interestingly, apyrase treatment reveals a nearly quantitative interaction between BiP and RHL implying that BiP associates with both monomeric and aggregated RHL. We also looked for interaction between RHL and PDI but could not detect any coimmunoprecipitation of RHL with PDI antibody (Fig. 7, lanes *5* and *7*). Thus, the RHL interactions are stable under conditions which do not allow coimmunoprecipitation with two major lumenal proteins, BiP and PDI. The RHL subunits will bind to BiP, but BiP is not an integral component of the heterogeneous complex.

Discussion

Heterogeneous Interactions of Membrane Proteins during Cell-free Synthesis

Several studies have strongly suggested that the asialoglycoprotein receptor exists as a heterooligomeric complex of two or more subunits (McPhaul and Berg, 1986; Sawyer et al., 1988; Bischoff et al., 1988; Shia and Lodish, 1989). We previously reported that a heterooligomeric RHL complex was assembled during cell-free translation (Sawyer and Doyle, 1990). A truncated form of an unrelated protein, DPP-IV, was used as a control for specificity in those experiments. When synthesized with the RHL subunits, the truncated DPP-IV was excluded from the RHL complex. In this work we show that under identical translation conditions, full-length DPP-IV coimmunoprecipitates with RHL. Additionally, the gap junction protein Cx-32 also coimmunoprecipitates with RHL. This demonstrates that the interaction iden-

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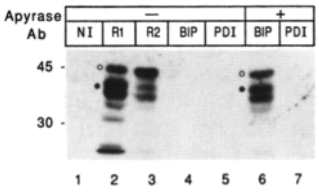

Figure 7. Immunoprecipitation of RHL translation products with anti-BiP and anti-PDI. RHL RNAs were cotranslated under reducing conditions and the reaction was treated with apyrase where indicated to deplete ATP before extraction. The products were immunoprecipitated with the carboxy-terminal RHL antisera, mAb to BiP, polyclonal antibody to PDI, or with control serum (*NI*), ○, glycosylated RHL-2/3; ●, glycosylated RHL-1.

tified here is not chain specific. Furthermore, the interaction requires elements within the exoplasmic domain as indicated by carboxy-terminal truncations of both DPP-IV and RHL-1. Therefore, it was the use of the truncated version of DPP-IV that led to the apparent specificity in the previous work. This truncation, which lacks 435 aa residues in the COOH terminus was used instead of the full-length DPP-IV because it is more readily glycosylated in the cell-free system. Further characterization demonstrates that these nonspecific chain interactions involve "unfolded" polypeptides and generate aggregates of diverse size and chain composition. Since these aggregates form under reducing conditions preventing disulfide formation, the chains interact noncovalently.

A number of molecular chaperones or polypeptides chain binding proteins are thought to bind nascent chains in the ER lumen and other cellular compartments (Pelham, 1989; Rothman, 1989; Ellis and van der Vies, 1991). We chose to look for the presence of two obvious candidates, BiP and PDI in the nonspecific aggregate. Anti-BiP, but not anti-PDI, coimmunoprecipitates reduced RHL in our experiments in an ATP-sensitive fashion. Since the aggregate is stable under conditions that dissociate BiP–aggregate interactions, BiP is not an integral component of the complex. Therefore, the nascent polypeptides do not interact by indirect contact through BiP or PDI. The polypeptides most likely interact by direct contact, or alternatively, the nascent chains could be bridged through other unidentified lumenal proteins.

It appears from our data, that aggregation involves the exoplasmic surface, and not the transmembrane domain. This conclusion stems from the observation that carboxy-terminal truncated DPP-IV or RHL-1, two type II membrane proteins, do not aggregate. A series of truncations in the exoplasmic region of RHL-1 revealed that removal of a segment of 43 aa residues between the HindIII and ScaI sites prevented the interaction from occurring. It is unlikely that this domain contains a determining recognition sequence for aggregation, since the interaction occurs between diverse polypeptide chains. In all likelihood, this region contains simple structural determinants conducive to aggregation that represent a minimal requirement for interaction.

Small regions of hydrophobicity as modeled by Chou-Fasman predictions, extend through the COOH-terminus of RHL. DPP-IV contains a large hydrophobic segment in the COOH-terminal region that does not appear to be embedded in the membrane (Hong and Doyle, 1988). These hydrophobic regions could serve as the locales for aggregation. Alternatively, a second mode of interaction is suggested by the placement of cysteine residues with respect to the carboxy-terminal truncations that prevent aggregation. These truncations each remove the bulk of the exoplasmic cysteine residues, 10 of 13 in DPP-IV and all 8 in RHL-1. Additionally, Cx-32 has three cysteine residues in each of its two exoplasmic loops. It is possible that these cysteine residues interact via hydrogen bonding. Disulfide formation could prevent aggregation by burying hydrophobic regions or alternatively, by preventing sulfhydryl-mediated hydrogen bonding. In suggesting a model of early folding versus aggregation, Cx-32 presents a dilemma because it contains numerous transmembrane domains with limited exoplasmic sequence. Whether the exoplasmic domain of Cx-32 "folds" or how it would otherwise regulate aggregation sites remains intriguing.

Relevance of the Cell-free System

It has been suggested that oligomerization of membrane proteins fails to occur during cell-free translation because of a lack of multiple insertions into microsomal vesicles (Anderson and Blobel, 1981; Kvist et al., 1982). The nonspecific aggregates described here contain multiple polypeptides, implying that many insertions into microsomal membranes do occur in these experiments. This suggests that the failure of the oxidized RHL polypeptides to oligomerize must be due to some other factor. Studies in other systems have shown that oligomerization of membrane proteins commonly occurs in the ER (Hurtley and Helenius, 1989). Evidence that the asialoglycoprotein receptor also oligomerizes in the ER is provided in studies which demonstrate that one subunit can be rescued from rapid degradation in the ER when coexpressed with the second subunit in transfected cells (Shia and Lodish, 1989; Graeve et al., 1990). Hence we assume that microsomal vesicles should promote RHL oligomerization during cell-free translation. Since the oxidized RHL polypeptides lack high affinity ligand binding activity, later folding events may not be occurring in the microsomes. As has been suggested in other systems, oligomerization might be a late event occurring between completely folded subunits (Gething et al., 1986; Copeland et al., 1988; Doms et al., 1988). Therefore, we suggest that a lack of complete folding in this system causes failure of RHL to oligomerize. Alternatively, there may be specific cellular factors necessary for oligomerization lacking in this system.

Analysis of early events in assembly indicate that membrane and secretory proteins begin to fold and form intramolecular disulfides immediately upon insertion into the ER lumen (Bergman and Kuehl, 1979; Peters and Davidson, 1982; Braakman et al., 1991). This rapid, even cotranslational, formation of intrachain disulfides results in receptor molecules lacking ligand binding activity. Activity is then acquired over a period of time in the ER (Olson and Lane, 1987; Olson et al., 1988). This lag between early disulfide bond formation and acquisition of ligand binding is thought to involve rearrangement of mispaired disulfide bonds formed during, or immediately after, translation (Olson and Lane, 1989). The rearrangement of disulfides appears to be part of the mechanism determining differential rates of export from the ER (Lodish and Kong, 1991).

Translation in vitro in the presence of GSSG mimics the early folding stages in vivo by producing molecules which have acquired intramolecular disulfides but not yet attained high affinity binding capability. When RHL is synthesized in the presence of low concentrations of GSSG, there is an observable effect on its ability to bind to a galactose–Sepharose column. The apparent binding affinity increases with a

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longer incubation at higher GSSG levels. This effect is not due to new synthesis since translation is inhibited at the higher concentration of GSSG (data not shown). The "stepwise" effect of GSSG in increasing the apparent affinity of ligand binding leads us to propose that the oxidized molecules represent intermediates in the folding pathway of RHL. Presumably, the effect of longer incubations with GSSG would be to allow correct, stably folded structures to form via disulfide rearrangement. Hille et al. (1989) reported a similar increase in ligand binding ability of the 46-kD mannose 6-phosphate receptor, dependent on the concentration of GSSG during cell-free translation, which was associated with conformational changes. Hence we believe the oxidized RHL polypeptides are receptor molecules in the early stages of folding.

Nascent polypeptide chains are presumably maintained in an unfolded state during translocation across the ER membrane as for other cellular membranes (Eilers and Schatz, 1986; Randall and Hardy, 1986). The consequence of the in vitro reducing condition appears to be to passively maintain nascent chains in this unfolded state. Therefore, the initial folding events which occur rapidly in vivo can be regulated through manipulation of the redox conditions in the cell-free system. Providing a reducing environment in vitro has revealed an unproductive assembly pathway for nascent chains.

Regulation of Chain Interactions during Biogenesis

It has been suggested that aggregation occurs as an alternative to entry into a proper folding pathway (Jaenicke, 1987). Inactive aggregates of soluble proteins are a common occurrence in in vitro folding experiments with purified enzymes (King, 1989; Gatenby and Ellis, 1990). Membrane proteins have also been shown to aggregate in vivo, in the absence of proper folding processes. For example, newly synthesized chains of the temperature-sensitive vesicular stomatitis virus G protein mutant, ts045, aggregate noncovalently at the nonpermissive temperature and display aberrant folding (Doms et al., 1987; deSilva et al., 1990). These aggregates of membrane and soluble proteins have generally represented homotypic interactions. In contrast, the aggregates described in this report include heterotypic chain interactions. Additionally, the experiments described here lack the overt denaturation common to in vitro refolding experiments. Rather, the reducing environment of the cell-free system passively captures nascent chains in an "unfolded" state as they are exposed in the microsomal lumen which results in heterotypic aggregation of the exoplasmic domains. Do nascent chains aggregate in vivo as an intermediate during early biogenesis? We are not aware of any experimental data to support this model. It is more likely that heterotypic aggregation represents an alternative, dead-end pathway which deviates from the normal pathway essentially upon exposure of the nascent chains in the ER/microsomal lumen. Thus, our experiments infer a mechanism operating in vivo on nascent chains to suppress the unproductive pathway. Once segregated, nascent chains can develop characteristic folds which are the basis for specific chain interactions.

Much current interest has focused on a role for accessory factors or molecular chaperones for such a function (Gatenby and Ellis, 1990). The data presented here suggest that any such factors present in pancreatic microsomes are not sufficient to segregate reduced chains. In fact one of these factors, BiP, apparently binds the aggregated forms. Thus, interaction with BiP does not prevent reduced RHL chains from aggregating. Alternatively, the immediate oxidizing environment permitting folding may be a necessary condition in preventing nonspecific interactions. Since it has been demonstrated that the absence of accessory factors can result in a lack of folding even when the redox environment is conducive (Bullied and Freedman, 1988), both accessory factors and an oxidizing environment may be necessary for inhibiting heterotypic aggregation without either being sufficient. In our system it could be argued that BiP recognizes the aggregated polypeptides as aberrant, binds in an effort to correct the aberration, but is unable to do so due to the reducing environment of the in vitro microsomal lumen. Therefore, we propose that it is not chaperone binding per se which controls aggregation, but the rapid folding of the polypeptide upon exposure to the lumen of the ER.

We propose a working model whereby conditions favoring the rapid formation of intramolecular disulfides have been selected to counteract an intrinsic capacity of membrane proteins to aggregate with one another. In this model the earliest folding events are interpreted as a control mechanism which "chooses" a pathway resulting in properly folded and oligomerized proteins for export from the ER. We believe our model explains the otherwise curious feature of the apparent "mispairing" of cysteines which occurs in some membrane and secretory proteins during biogenesis as a result of cotranslational disulfide formation. These mispaired disulfides, which have to be reshuffled to achieve the active conformation, are tolerated because their formation prevents the presumably dead-end pathway of heterotypic aggregation. The model predicts that if disulfide formation in the ER is inhibited, nascent membrane, and perhaps secretory, proteins would interact with unrelated proteins to form nonspecific aggregates.

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