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Co-Translational Modification of Nascent Immunoglobulin Heavy and Light Chains

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We have investigated the in vivo co-translational covalent modification of nascent immunoglobulin heavy and light chains. Nascent polypeptides were separated from completed polypeptides by ion-exchange chromatography of solubilized ribosomes on QAE-Sephadex. First, we have demonstrated that MPC 11 nascent heavy chains are quantitatively glycosylated very soon after the asparaginyl acceptor site passes through the membrane into the cisterna of the rough endoplasmic reticulum. Nonglycosylated completed heavy chains of various classes cannot be glycosylated after release from the ribosome, due either to rapid intramolecular folding and/or intermolecular assembly, which cause the acceptor site to become unavailable for the glycosylation enzyme. Second, we have shown that the formation of the correct intrachain disulfide loop within the first light chain domain occurs rapidly and quantitatively as soon as the appropriate cysteine residues of the nascent light chain pass through the membrane into the cisterna of the endoplasmic reticulum. The intrachain disulfide loop in the second or constant region domain of the light chain is not formed on nascent chains, because one of the cysteine residues involved in this disulfide bond does not pass through the endoplasmic reticulum membrane prior to chain completion and release from the ribosome. Third, we have demonstrated that some of the initial covalent assembly (formation of interchain disulfide bonds) occurs on nascent heavy chains prior to their release from the ribosome. The results are consistent with the pathway of covalent assembly of the cell line, in that completed light chains are assembled onto nascent heavy chains in MPC 11 cells (IgG_{2b}), where a heavy-light half molecule is the major initial covalent intermediate; and completed heavy chains are assembled onto nascent heavy chains in MOPC 21 cells (IgG_1), where a heavy chain dimer is the major initial disulfide linked intermediate.

Key words: nascent chains, co-translational modification, glycosylation, polypeptide folding, covalent assembly, heavy and light chains

Abbreviations: H, heavy chain; L, light chain; F_{CL}, constant region kappa light chain fragment; SDS, sodium dodecyl sulfate; IAA, iodoacetic acid; IAM, iodoacetamide; PBS, phosphate-buffered saline.

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Immunoglobulin heavy and light chains undergo several post-translational covalent modifications of their primary sequence prior to attaining the mature form which binds antigens and mediates numerous immunological functions. The immunoglobulin molecule is composed of two disulfide-linked heavy chain glycoproteins, each of which is linked to a light chain by a single disulfide bond [1]. Heavy chains and light chains are composed of four and two sequential domains, respectively. Each 12,500 dalton domain contains a single disulfide loop [2]. A substantial amount of work has been done using the immunoglobulin molecule as a model system for studying these covalent modifications – eg, glycosylation [3–7], intramolecular folding [8–10], and intermolecular assembly [6, 7, 11–17]. However, it is not certain whether such covalent modifications begin during synthesis while the nascent polypeptides are still bound to the polyribosomal complex, or only after the polypeptides have been completed and released from the ribosome into the cisternae of the endoplasmic reticulum.

We have examined the temporal relationship between translation and various post-translational (co-translational) modifications (ie, glycosylation, formation of intrachain disulfide bonds, and intermolecular covalent assembly) by separation of nascent polypeptides from completed polypeptides using ion-exchange chromatography based on the multiple negative charges contributed by the tRNA moiety of the peptidyl-tRNA complex [18].

MATERIALS AND METHODS

Cells

Table I lists the mouse plasmacytoma cell lines used. All cell lines were maintained in suspension in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated horse serum, 2 mM glutamine, and nonessential amino acids.

Cell Labeling, Fractionation, and Nascent Chain Isolation

Cell labeling and isolation of membrane-bound ribosomes and nascent chains were essentially as described previously [18, 30]. To prepare [^{3}H]-iodoacetic acid-labeled nascent chains, the nascent chain fraction was dialyzed extensively vs 10 mM NH_4HCO_3 (pH 7.8), lyophilized, resuspended in 1–2 ml 0.1 M Tris-Cl (pH 7.8)–2.0% SDS, and reduced overnight with 100 mM dithiothreitol at 37°C. The sample was then dialyzed for 4–6 h vs 50 volumes 0.1 M Tris-Cl (pH 7.8)–0.1% SDS, and subsequently alkylated for 20 min at 37°C with 1–2.5 mCi [^{3}H]-iodoacetic acid (IAA) (New England Nuclear, 300 mCi/mmole), with unlabeled IAA added to a total concentration of 10 mM IAA. After alkylation the sample was dialyzed extensively vs 0.1 M Tris (pH 7.4)–0.1% SDS and then specifically immunoprecipitated as described below.

Column Chromatography

For purification of L chain and F_{CL} marker proteins and for size separation of nascent chains, the immunoprecipitated samples were resuspended in SDS-sample buffer and analyzed by Sephadex G200 chromatography (1.5 × 95 cm or 1.5 × 190 cm columns) using a buffer system consisting of 0.1 M NH_4HCO_3 (pH 7.8), 0.05% SDS [31].

For tryptic peptide analysis, samples were precipitated with 20% Cl_3CCOOH, collected by centrifugation, and washed successively with 5% Cl_3CCOOH, ethanol: ether (1:1), and ether. The dried pellet was resuspended in 1.0 ml 0.1 M NH_4HCO_3(pH 8.0)

TABLE I. Mouse Myeloma Cells Used in Analysis of Co-Translational Modification*

Cell line	H chain	L chain	Reference
MPC 11 clone 45.6	γ_{2b}	κ, F_{CL}[b]	[19]
clone 66.2	–	κ, F_{CL}	[20]
clone NP-2	–	–, F_{CL}	[21]
clone M311	γ_{2b}[a]	κ, F_{CL}	[22–24]
MOPC 21 clone PB00.1	γ_1	κ	[25]
clone NSI	–	κ	[26]
MOPC 104E	μ	λ	[27]
S107	α	κ	[28]
MOPC 46 (tumor)	–	κ	[29]

*The cell lines used in these studies and their immunoglobulin synthetic products are indicated.

[a]The M311 clone synthesizes a variant heavy chain with a carboxy terminal deletion comprising approximately the third constant region domain.

[b]All MPC 11 cell lines synthesize F_{CL}, a constant region kappa light chain gene product with an internal deletion of the variable region; this unusual product is apparently encoded by a separate gene from the gene that encodes the normal kappa light chain expressed in these cells [21, 31].

and digested for 2 h with 250 μg/ml trypsin-TPCK (Worthington) at 37°C. After digestion, the sample was lyophilized, resuspended in 1.0 ml 20% formic acid, and chromatographed on tandem G25-G50 Sephadex columns (1.5 X 96 cm each) equilibrated with 10% acetic acid. Fractions 1.4 ml were collected, dried, and the radiolabeled peptides were located by scintillation counting.

Electrophoresis

Pooled fractions from the Sephadex G25-G50 column were lyophilized, resuspended in a minimal volume of 10% acetic acid, and subjected to high voltage paper electrophoresis in a pH 3.6 pyridine:acetic acid:water (1:10:289 v/v) buffer, for 1.5 h at 3,000 volts. The paper strips were cut into 1.0 cm pieces and counted as described below.

Immunoprecipitated samples were boiled in SDS-sample buffer and electrophoresed in various concentration polyacrylamide gels using a discontinuous SDS-Tris-glycine buffer system essentially as described by Laemmli [32] and Maizel [33]. Cylindrical gels were sliced into 2 mm fractions with a Gilson Aliquogel fractionator and counted as described below. Slab gels were subjected to autoradiography for visualizing the radioactive protein bands.

Immunoprecipitation

Direct immunoprecipitations were performed in antibody excess using appropriate antisera specific for H and L chains and were incubated overnight at 4°C [34]. The immunoprecipitates were collected by centrifugation through 1.0 M sucrose in phosphate-buffered saline (PBS), and washed once with PBS [35].

Radioactivity Measurement

Gel filtration aliquots were dried and counted in a Beckman LS 230 scintillation counter after addition of 0.5 ml of NCS (Amersham/Searle)–H_2O (10:1) and 7.5 ml of toluene containing 0.4% PPO. Crushed polyacrylamide gel samples were counted in a Triton X-100/toluene (1:2) scintillation fluor containing 0.4% PPO and 10% H_2O.

RESULTS

Nascent MPC 11 H Chains Are Quantitatively Glycosylated With a Core Oligosaccharide

Various investigators have shown that the initial glycosylation event involves the transfer of a large molecular weight oligosaccharide (containing N-acetylglucosamine. mannose, and glucose) as a unit from a dolichol lipid intermediate to an asparagine acceptor residue on the polypeptide [44, 45]. Experiments were performed to determine what percentage of nascent MPC 11 H chains are glycosylated prior to completion of H chain translation. The results in Figure 1 indicate that the ratios of [^{3}H]-glucosamine-to-[^{35}S]-Met are very similar for completed H chains (isolated from the rough endoplasmic reticulum) and large nascent H chains, implying that the glycosylation of the nascent H chains is quantitative. The observation that the ratio obtained for the nascent H chains increases from 0.23 to 1.49 in a single fraction (approximately 38,000 daltons) indicates that quanti-

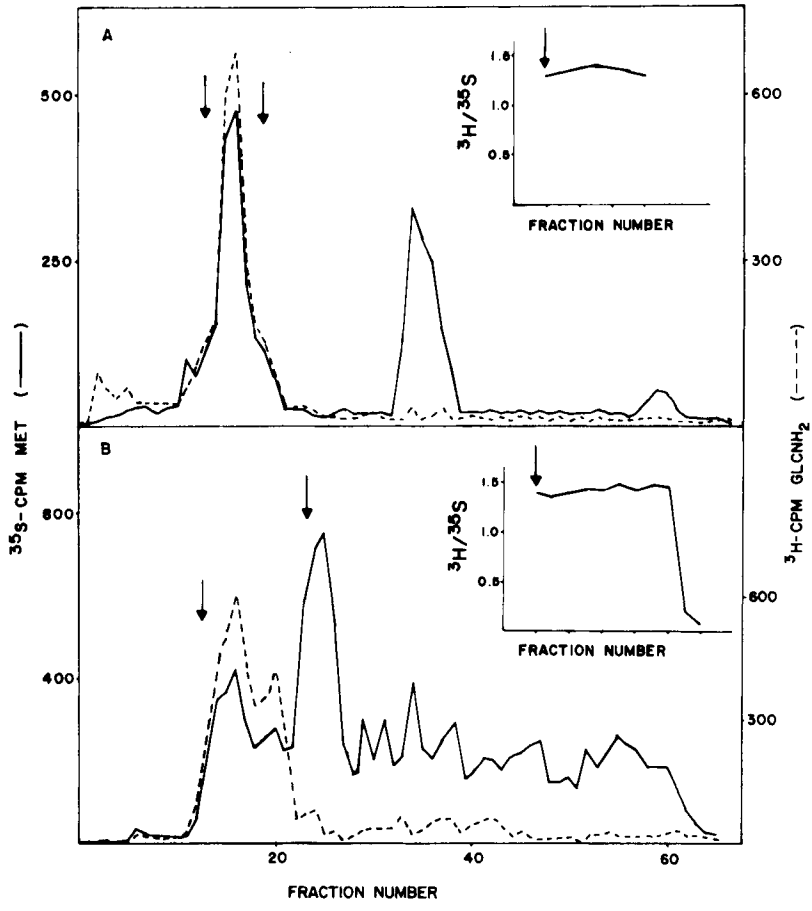


Fig. 1. SDS polyacrylamide gel electrophoresis of MPC 11 completed and nascent immunoglobulin chains. 2 × 10^9 MPC 11 clone 456 cells were collected and labeled for 5 min with [^{3}H]glucosamine and [^{35}S]Met at isotope concentrations of 100 and 15 μCi/ml, respectively, in the presence of medium lacking glucose but containing all amino acids except Met. The isolation of completed and nascent heavy and light chains was as described previously [18, 30]. The samples were electrophoresed on a 10% polyacrylamide gel in SDS buffer. Migration is from left to right. (A) completed chains; (B) nascent chains. Dashed line, [^{3}H]glucosamine; solid line, [^{35}S]Met. The ratio of [^{3}H]glucosamine to [^{35}S]Met for each fraction marked by the arrows was calculated and plotted (insets) [30].

tative glycosylation of the nascent H chain occurs very soon after the asparaginyl acceptor site (residue 291, 32,000 daltons) passes through the membrane into the cisterna of the rough endoplasmic reticulum. The prominent [^{35}S]Met-labeled peak (fractions 23–27, 34,000–37,000 daltons) seen in the nascent chain sample appears to be due to a translational block related to the glycosylation event (E. Harris, L.W. Bergman, and W.M. Kuehl, unpublished data).

We have determined that the nascent H chains contain at least glucosamine and mannose, but not galactose [18, 36]. After extensive pronase digestion, the [^{3}H]glucosamine-labeled glycopeptide derived from nascent chains is indistinguishable by P-6 chromatography [24] from the [^{14}C]glucosamine-labeled glycopeptide present on completed H chains isolated from the rough endoplasmic reticulum; but both glycopeptides are smaller than either the glycopeptide derived from completed H chains isolated from the smooth membrane/Golgi fraction or the glycopeptide derived from secreted H chains (L.W. Bergman and W.M. Kuehl, unpublished data).

Nonglycosylated Completed H Chains Cannot be Glycosylated

Although the experiment described above demonstrates quantitative glycosylation of the nascent H chains with a core oligosaccharide at a precise time, we attempted to determine whether it is possible for the cell to glycosylate completed H chains of various classes after release from the ribosome. Non-glycosylated completed H chains synthesized in the presence of excess glucosamine (to block glycosylation) cannot be glycosylated during a chase period in the absence of the inhibitor, although the cells regain the ability to glycosylate newly synthesized H chains by 20 min into the chase [30]. Representative results for MOPC 104E (μ) and MPC 11 (γ_{2b}) are seen in Figure 2. Similar results are found for cells synthesizing γ_1 and α H chains [30]. However, Figure 2 also shows that M311 cells, which synthesize an MPC 11 variant H chain having a carboxy-terminal deletion, glycosylate a completed chain to a near normal extent in a similar experiment as described above. Thus, this result provides evidence that the lack of glycosylation of the wild type H chains is a function of the protein itself and is not due either to spatial separation of the H chain from the glycosylation enzymes or to the general inhibitory effect of the glucosamine.

Rapid Folding (Intramolecular Disulfide Bond Formation) Occurs on Nascent Polypeptides

To study the formation of intrachain disulfide bonds on nascent polypeptides we have chosen the MPC 11 L chain as a model system. Figure 3 summarizes the cysteine residues of the L chain and the size and chromatographic properties of the reduced and alkylated Cys-containing tryptic peptides [38]. Our approach has been to isolate the nascent L chains in the presence of excess unlabeled iodoacetamide (IAM) to block any free sulfhydryls and then to selectively label with [^{3}H]-iodoacetic acid (IAA) only those cysteine residues involved in intrachain disulfide bonds. Figure 4 shows an SDS-polyacrylamide gel of [^{3}H]-IAA-labeled immunoprecipitated nascent L chains that were isolated in the presence or absence of excess IAM. The [^{3}H]-IAA-labeled nascent L chains isolated in the absence of IAM (all Cys residues are labeled) show a heterogeneous size distribution of 25,000 daltons (full size) to approximately 10,000 daltons, while the nascent L chains isolated in the presence of IAM (only nascent L chains containing Cys residues in disulfide bonds are labeled) show a size distribution of 25,000–16,000 daltons. Thus only nascent L chains at least 16,000 daltons in size contain disulfide bonds, whereas nascent L chains may be immunoprecipitable as small as approximately 10,000 daltons.

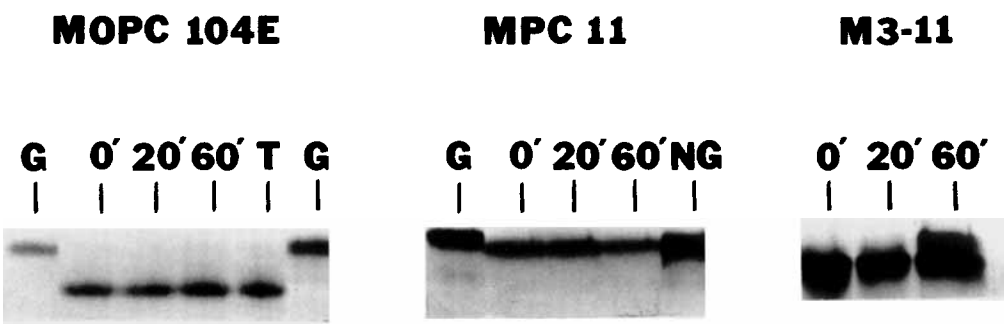
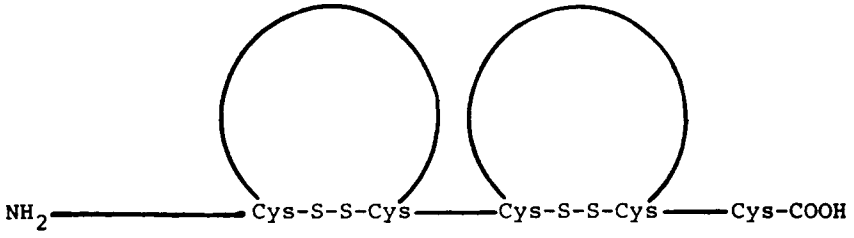


Fig. 2. Effect of glucosamine inhibition on glycosylation of MOPC 104E, MPC 11, and M311 heavy chains. Cells were incubated for 50 min in the presence of 10 mg/ml glucosamine and then labeled for 5 min with [^{14}C]-labeled amino acids in the presence of the inhibitor. Samples were removed at various intervals during a chase in medium containing excess glucose and unlabeled amino acids but lacking glucosamine. The samples were immunoprecipitated and analyzed on 12.5% polyacrylamide slab gels in an SDS buffer as described in Materials and Methods. Column G is glycosylated marker H chain, whereas NG and T are nonglycosylated marker H chains isolated from glucosamine- or tunicamycin-treated cells [37], respectively.



Cys residue No.	35	100	146	206	226
Tryptic peptide (Reduced-alkylated)	V_1	V_2	C_1	C_2	C_3
No. residues in tryptic peptide	6	42	34	11	3
G25-G50 peak	3	1	1	2	4

Fig. 3. Cysteine and cystine residues present in MPC 11 κ light chain. Molecular size and G25-G50 Sephadex chromatographic properties of the reduced and alkylated Cys-containing tryptic peptides are indicated.

Only Cys35 and Cys100 Are Involved in a Disulfide Bond on Nascent Chains

To determine which Cys residues were involved in the formation of the nascent intrachain disulfide bond, nascent L chains were isolated in the presence of IAM from cells labeled for 10 min with [^{35}S] Cys. Figure 5 shows the G25-G50 Sephadex chromatography of a tryptic digest of [^{35}S] Cys-labeled nascent L chains in the presence (panel A) or absence (panel B) of reducing agent (vs reduced and alkylated [^{3}H] Cys-labeled marker L chain in each case). In the presence of reducing agent (panel A), peptides V_2 and/or C_1 (peak 1), peptide C_2 (peak 2), and peptide V_1 (peak 3) are identified. As expected for nascent L

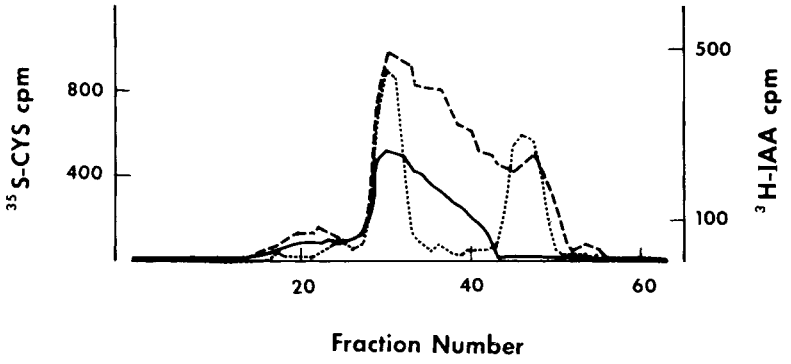


Fig. 4. SDS polyacrylamide gel electrophoresis of nascent L chains. [^{3}H]-IAA-labeled nascent L chains were isolated from MPC 11 clone 662 cells in the presence (——) or absence (– – – –) of excess IAM, as described previously [18, 30] and in Materials and Methods. [^{35}S]-Cys MPC 11 L chain (25,000 daltons) and F_{CL} (11,600 daltons) (. . . .) molecular weight markers were co-electrophoresed with each [^{3}H] cm-Cys sample on 10% polyacrylamide gels in an SDS buffer. Migration is from left to right.

chains, peak 2 (peptide C_2, Cys206) is present in a much lower yield than peak 3 (peptide V_1, Cys35). Peptide C_3 (peak 4), which contains the carboxy-terminal residue of the L chain (Cys226), is completely missing in the nascent chain sample, thereby demonstrating that there is no detectable contamination with completed L chain in the nascent chain sample. In the absence of reducing agent (panel B) there is an additional component (peak X) chromatographing at a larger apparent size than the marker V_2, C_1 peptides (peak 1). Secondly, there is a decreased recovery of peak 3 (peptide V_1) and peak 1 (peptide V_2 and/or C_1), as compared to the sample run in the presence of reducing agent (panel A). The decrease in recoveries of peak 1 and peak 3 in panel B (relative to panel A) may be accounted for quantitatively by the appearance of peak X (peak 1 decreased from 58.7% of the total radioactivity recovered to 36.4%; peak 3 decreased from 32.8% to 9.8% total radioactivity recovered; peak X accounts for 46.0% of the total radioactivity recovered in the absence of reducing agent). Peak X was pooled, reduced, alkylated, and rechromatographed vs [^{3}H] Cys-labeled marker L chain (panel C). It can be seen that reduced peak X contains equal molar quantities of peak 3 (V_1) and peak 1 (V_2 or C_1); in fact, peak 1 isolated from reduced and alkylated peak X contains peptide V_2 and no detectable peptide C_1 (data not shown). Therefore nascent L chains contain some Cys35 and Cys100 in disulfide linkage but no other Cys residues (146, 206, or 226) are present in disulfide bonds (this experiment plus unpublished results).

The First Disulfide Loop (Cys35-S-S-Cys100) Is Formed Rapidly and Quantitatively on Nascent L Chains

To examine the extent and kinetics of disulfide bond formation between Cys35 and Cys100, [^{35}S] Cys-labeled nascent chains were isolated in the presence of excess IAM and subsequently alkylated with IAA to carboxy-methylate only those Cys residues involved in disulfide bonds. The sample was fractionated by G200 Sephadex chromatography and divided into 4 size fractions (25,000– 15,200 daltons). Each fraction was digested with trypsin and chromatographed on a G25-G50 column (profiles are similar to Figure 5). The isolated V_1 peptide was then subjected to high voltage paper electrophoresis at pH 3.6 to resolve the V_1 species that were alkylated with either IAM (Cys present as free sulfhydryl) or IAA (Cys present as disulfide). Table II shows a summary of the data ob-

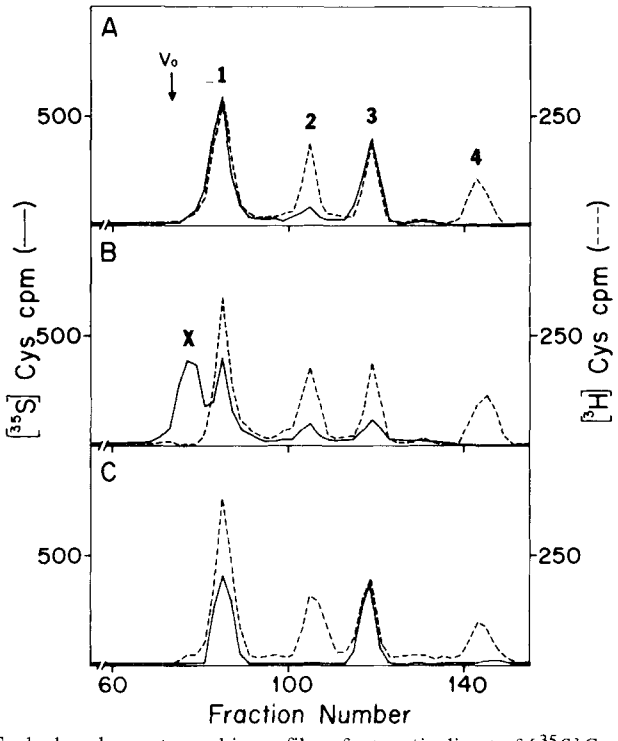


Fig. 5. G25-G50 Sephadex chromatographic profiles of a tryptic digest of [^{35}S]Cys-labeled nascent L chains vs reduced and alkylated [^{3}H]Cys-labeled marker L chain. MPC 11 clone 662 cells were labeled for 10 min with [^{35}S]-Cys. Nascent L chains were isolated, digested with trypsin, and analyzed on tandem G25-G50 Sephadex columns in the presence (panel A) or absence (panel B) of reducing agent. The additional peak (peak X) seen in the absence of reducing agent (panel B) was pooled, reduced, alkylated, and rechromatographed (panel C). Solid line, [^{35}S]Cys-labeled nascent L chains; Dashed line, [^{3}H]Cys-labeled marker L chain. The void volume (Vo) and the tryptic peptide composition of each peak (see Fig. 3) are indicated in panel A.

tained. The data indicate that in fractions 1–3 (25,000–18,000 daltons) essentially all the isolated V_1 peptide co-electrophoreses with the carboxy-methylated V_1 marker (as compared to the amido-methylated V_1 marker). In fraction 4 (18,000–15,200 daltons) 85% of peptide V_1 co-electrophoreses with the carboxy-methylated V_1 marker and 15% with the amido-methylated V_1 marker. This indicates that approximately 85% of the nascent L chains between 15,200 and 18,000 daltons have formed the first intrachain disulfide bond, whereas 100% of larger nascent L chains have formed the first intrachain disulfide bond.

Initial Covalent Assembly (Interchain Disulfide Bond Formation) Also Occurs on Nascent H Chains

We have investigated the question of whether intermolecular covalent assembly (ie, formation of interchain disulfide bonds) begins during synthesis while the nascent polypeptides are still bound to the polyribosomal complex, or only after the polypeptide chains have been completed and released from the ribosome. The major initial disulfide linked intermediate in the assembly pathway is H-L for MPC 11 cells and H-H (H_2) for MOPC 21 cells [12, 13]. After a 30-sec pulse-label with [^{35}S]Met, approximately 49% of the MPC 11 completed H chains and 36% of the MOPC 21 completed H chains are assembled

TABLE II. Kinetics and Extent of Cys35–Cys100 Disulfide Bond Formation*

Sephadex G200 fraction	Estimated size of nascent chains	% Carboxy-methylated [^{35}S]-V_1	%Cys 35–Cys100 disulfide
1	25–23.5 × 10^3	100%	100%
2	23.5–21.5 × 10^3	100%	100%
3	21.5–18 × 10^3	100%	100%
4	18–15.2 × 10^3	85%	85%

*Cells were labeled for 10 min with [^{35}S]Cys; nascent L chains were isolated in the presence of excess IAM, then reduced with β-mercaptoethanol, and subsequently alkylated with IAA to carboxy-methylate Cys residues involved in disulfide bonds as described in Materials and Methods and Results. The immunoprecipitated nascent L chains were fractionated G200 Sephadex chromatography (1.5 × 195 cm) and divided into 4 size fractions. Each fraction was digested with trypsin, and peptide V_1 was isolated by G25-G50 Sephadex chromatography (see Figs. 3 and 5). Peptide V_1 was subjected to high-voltage paper electrophoresis at pH 3.6 to resolve the V_1 species that were alkylated with either IAM (Cys present as free sylfhydryl) or IAA (Cys present in disulfide linkage).

covalently into various assembly intermediates [39]. Thus most covalent assembly occurs on completed chains.

To examine further the question of whether some of the initial covalent assembly takes place prior to completion of H chain translation, [^{35}S]Met-labeled nascent chains were isolated from each cell line and analyzed by immunoprecipitation and SDS-gel electrophoresis for the release of completed chains after reduction of the nascent chain fraction. The results in Figure 6A indicate that for MPC 11 cells completed L chain is specifically released by reduction of the nascent chain fraction, whereas a similar analysis of MOPC 21, seen in Figure 6B, reveals that in this case completed H chain (but no completed L chain) is released by reduction of the nascent chains. Thus in MPC 11 cells completed L chains are covalently assembled onto nascent H chains, whereas in MOPC 21 cells completed H chains are covalently assembled onto nascent H chains.

Minimum Size of Nascent H Chain Required for Formation of Intermolecular Disulfide Bonds

To determine the minimum size of nascent MPC 11 H chains that are covalently bound to completed L chains, we have immunoprecipitated the nascent chain fraction (prior to reduction) with an antiserum directed specifically against κ L chains to precipitate only those nascent H chains that are covalently bound to L chains. The results indicate that completed L chains become covalently bound to some nascent H chains of greater than approximately 38,000 daltons [39].

To determine the minimum size of nascent MOPC 21 H chains that are covalently bound to completed H chains, we have immunoprecipitated nascent H chains prior to reduction. Figure 7A shows the sample analyzed on a 7.5% SDS-polyacrylamide gel in the absence of reducing agent (reduced and alkylated [^{3}H] Leu-labeled marker H and L chains have been run as markers).

Radiolabeled material is found migrating with an apparent molecular weight range of 112,000–96,000 daltons (fractions 23–31) [slightly more heterogeneous than H chain dimer (H_2) electrophoresed in a parallel gel] and with a very heterogeneous distribution in size characteristic of nascent H chains – ie, from 55,000 daltons (size of completed H chain) to less than 25,000 daltons. The radiolabeled material in fractions 23–31 was sensitive to partial reduction (conditions which reduce interchain disulfide bonds) in that after

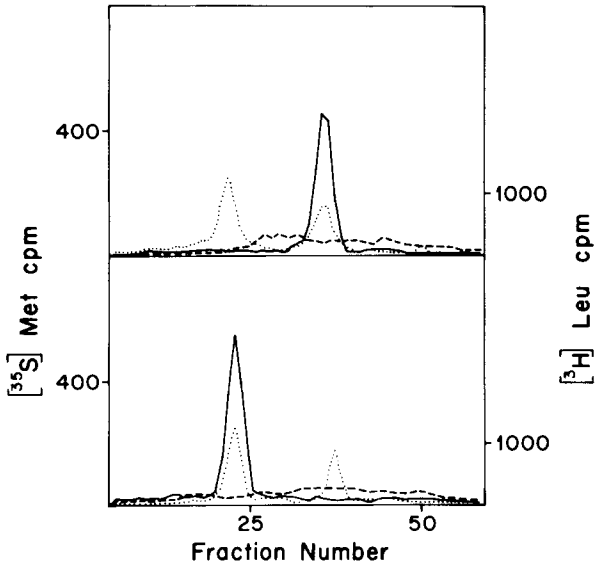


Fig. 6. SDS polyacrylamide gel electrophoresis of completed MPC 11 L chains and completed MOPC 21 H chains released by reduction of nascent chains. Nascent chains were isolated by 2 cycles of QAE-Sephadex chromatography from MPC 11 clone 456 or MOPC 21 cells labeled for 10 min with [^{35}S]Met as described previously [18, 30, 39]. The purified nascent chain fraction was divided into two aliquots, and each aliquot was subjected to a 2 h incubation in elution buffer at 37°C after addition of 0.15 M β-mercaptoethanol to one of the two aliquots. The reduced and nonreduced samples were subjected separately to a third cycle of QAE Sephadex chromatography. The flow-through fraction was collected, dialyzed extensively vs PBS, specifically immunoprecipitated, reduced, and then co-electrophoresed on 10% polyacrylamide gels in an SDS buffer with [^{3}H]Leu-labeled marker H and L chains from the respective cell line. Electrophoretic migration is from left to right. Solid line, immunoprecipitate of flow-through fraction from reduced aliquot; dashed line, immunoprecipitate of flow-through fraction from unreduced aliquot; dotted line [^{3}H]Leu-labeled marker H and L chains. Panel A, MPC 11 cells. Panel B, MOPC 21 cells.

partial reduction no radiolabeled material migrates with an apparent molecular weight larger than the marker H chain (55,000 daltons) (data not shown). The material in fractions 23–31 (Fig. 7A) was pooled and analyzed on a 12.5% SDS-polyacrylamide gel after complete reduction and alkylation (Fig. 7B). Figure 7B reveals that the sample, upon reduction, contains heterogeneous material with an apparent molecular weight range of 55,000–44,000 daltons (ie, contains radiolabeled material co-migrating and migrating slightly faster than the marker H chain). Although approximately 38% of the radioactivity present is due to completed H chains that have covalently assembled to nascent H chains, the heterogeneity present in the reduced sample indicates that nascent H chains of at least 44,000 daltons in size may covalently bind either to a completed H chain or to a second nascent H chain of at least 44,000 daltons in size.

DISCUSSION

Using the mouse plasmacytoma system, we have attempted to determine the temporal relationships between translation and various co-translational modifications. Nascent immunoglobulin heavy and light chains, covalently attached to tRNA molecules, have been separated from completed polypeptides by ion-exchange chromatography based on the multiple negative charges contributed by the tRNA of the peptidyl-tRNA complex. We have examined the glycosylation, folding (formation of intrachain disulfide bonds), and covalent assembly (formation of interchain disulfide bonds) of immunoglobulin polypeptides as they occur on nascent polypeptides.

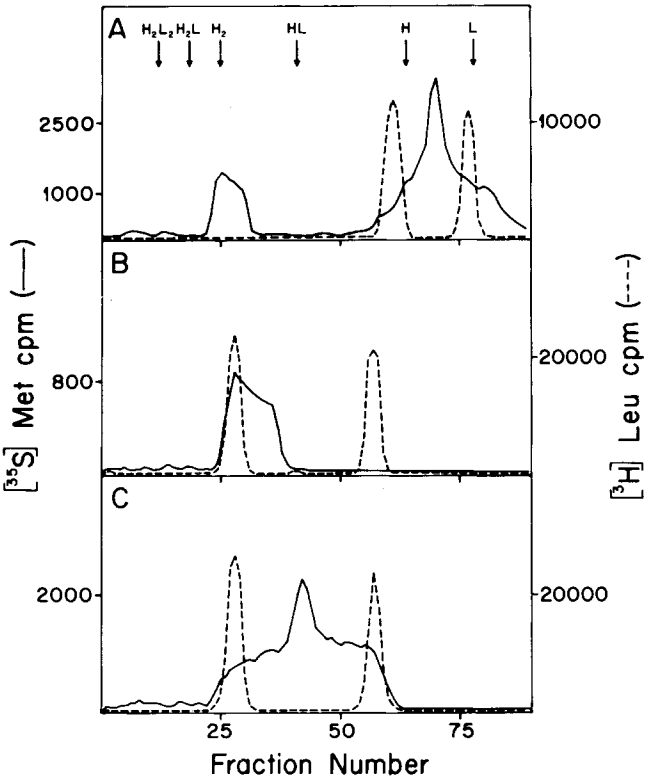


Fig. 7. SDS polyacrylamide gel electrophoresis of MOPC 21 nascent H chains that are covalently attached to completed H chains. MOPC 21 cells were labeled for 15 min with [^{35}S] Met, and nascent chains were isolated by two cycles of QAE-Sephadex chromatography as described previously [39]. The nascent chain fraction was dialyzed extensively vs PBS, subjected to specific immunoprecipitation in the presence of excess unlabeled MOPC 21 κ L chain (prepared from the light chain producer clone NSI), and analyzed by SDS-gel electrophoresis on 7.5% gels in the absence of reducing agent vs [^{3}H] Leu-labeled reduced and alkylated marker H and L chains (panel A). The migration of the covalent assembly intermediates (indicated by arrows) of MOPC 21 immunoglobulin were determined by electrophoresis on a parallel gel. Fractions 23–31 and fractions 61–75 were pooled separately, reduced, and co-electrophoresed on 12.5% polyacrylamide gels in an SDS buffer with [^{3}H] Leu-labeled marker H and L chains as seen in panel B and panel C, respectively. Solid line, [^{35}S] Met-labeled MOPC 21 nascent chains; Dashed line, [^{3}H] Leu-labeled marker H and L chains. Electrophoretic migration is from left to right.

The data presented in Figure 1 and Results indicate that nascent MPC 11 H chains are quantitatively glycosylated with a core oligosaccharide when the chain reaches a size of 38,000 daltons – ie, very soon after the asparaginyl acceptor residue (approximately residue 291, 32,000 daltons) passes through the membrane to the lumen of the rough endoplasmic reticulum and becomes available for glycosylation. Rothman and Lodish [40], using a synchronized wheat germ cell-free synthesizing system in the presence of exogenous pancreatic rough endoplasmic reticulum membranes, have shown a precise temporal sequence of glycosylation and translation of vesicular stomatitis virus G protein. However, in their studies, the observed translation times (23–43 min) are 20–30 times longer than in vivo translation times. Our results provide in vivo evidence that glycosylation occurs quantitatively at a precise interval during translation and that this period may be the only time that glycosylation of the heavy chain can occur (see below).

The possibility that glycosylation can occur on completed H chains after release from the ribosome was investigated by inhibiting the transfer of the core oligosaccharide to the nascent H chain with high concentrations of glucosamine. We have shown that non-glycosylated completed γ_{2b}, γ_1, α, and μ H chain (results for μ and γ_{2b} H chains are shown in Figure 2) synthesized in the presence of the inhibitor cannot be glycosylated during a chase period in the absence of the glucosamine, although the cells have regained the ability to glycosylate newly synthesized H chains by 20 min into the chase [30]. A similar experiment using an MPC 11 variant H chain reveals that in this case glycosylation of the completed chain can occur in that there is the appearance of the glycosylated form of the molecule by 20 min into the chase (experiments with M311 in Figure 2). This result provides evidence that the lack of glycosylation of the wild-type H chains is a function of the protein molecule itself and is not due either to spatial separation of the H chain molecules from the glycosylation enzymes or to the general inhibitor effect of the high levels of glucosamine. The variant M311 H chain includes only 38,000 daltons of amino acids due to a deletion, starting soon after the asparaginyl acceptor site, of the carboxy-terminal region of the molecule. This deletion may affect the conformation of the oligosaccharide acceptor site, thereby allowing glycosylation to occur after release of the completed protein from the ribosome.

Two possibilities are suggested for the inability of the cell to glycosylate completed H chains: 1) intramolecular folding (secondary and tertiary structure) and/or 2) intermolecular assembly (quaternary structure). In support of the first possibility, experiments from the laboratory of Lennarz have demonstrated that the core oligosaccharide can be added in vitro only to denatured ovalbumin and RNase A but not to the native forms of these proteins [41, 42]. Therefore, nascent polypeptides may be providing the asparaginyl acceptor site with little secondary or tertiary structure, allowing glycosylation to occur before the protein folds and the acceptor site becomes inaccessible for the glycosylation enzymes. In support of the second possibility, our experiments with MOPC 46B cells indicate that the completed L chain can be glycosylated if it is in a monomeric form but not if it has been assembled into a dimer [30]. Crystallographic studies by Davies and co-workers have suggested that the oligosaccharide moiety may play a central role as the principal contact between the second constant region domains of the H chains in intact immunoglobulin [43]. At this time, we cannot distinguish these two possibilities for the lack of glycosylation of completed H chains, due to the very rapid intramolecular folding and intermolecular assembly of H chains, as discussed below. However, we propose that the cell has evolved a system for efficiently glycosylating nascent chains as they pass through the membrane, thus circumventing the formation of secondary, tertiary, or quaternary structures in which the asparaginyl acceptor site would be unavailable for glycosylation. Apparently the core oligosaccharide is available for processing on completed chains [44, 45], and the type of processing may be determined by the environment of the core oligosaccharide on the completed chain.

We have demonstrated the formation of the initial intrachain disulfide bond on some nascent MPC 11 L chains (see Figure 3 for summary of L chain Cys residues) by isolating the nascent L chains in the presence of excess IAM to block free sulfhydryl groups and then selectively labeling with [^{3}H] IAA only those Cys residues that have previously formed a disulfide bond (see Fig. 4).

To determine which Cys residues are involved in the disulfide bond present on nascent L chains, we have isolated [^{35}S]Cys-labeled nascent L chains and analyzed the G25-G50 Sephadex chromatographic profile of a tryptic digest chromatographed in the presence or absence of reducing agent. In the absence of reducing agent (Fig. 5B), there

is an additional component (peak X) present (which is absent when the tryptic digest is chromatographed in the presence of reducing agent (Fig. 5A). After reduction and alkylation this additional peak yields equal molar quantities of peptide V_1 and peptide V_2 (Fig. 5C; Results). SDS-gel analysis indicates that only nascent L chains of 16,000–25,000 daltons contain Cys residues in disulfide linkage (see Fig. 4). Assuming that approximately 40–50 residues are needed to span the ribosome and membrane of the rough endoplasmic reticulum [46], our results indicate that some nascent L chains have formed the initial intrachain disulfide bond (Cys35-Cys100) as soon as Cys100 enters the cisterna of the rough endoplasmic reticulum. The second intrachain disulfide bond (Cys146-Cys206) present on the MPC 11 L chain is not formed on nascent L chains because Cys206 is only 20 residues from the carboxy-terminus and cannot pass through the membrane before the L chain is completed and released from the ribosome.

The extent and kinetics of formation of the initial disulfide bond on nascent L chains was examined by determining the fraction of Cys35 that was carboxy-methylated with IAA (Cys involved in a disulfide bond) vs the fraction of the Cys residue that was amidomethylated with the excess unlabeled IAM (Cys present as free sulfhydryl) during the isolation of the [^{35}S] Cys-labeled nascent L chains. Table II indicates that for nascent L chains of 25,000–18,000 daltons essentially all the isolated V_1 peptide co-electrophoreses with the carboxy-methylated V_1 marker, indicating that 100% of the nascent L chains in these size fractions have formed the first intrachain disulfide bond. In the smallest size fraction (fraction 4; 18,000–15,200 daltons) approximately 85% of nascent L chains have formed the initial intrachain disulfide bond, using the criteria cited above. Therefore assuming a translation time of 30 sec for L chain [47], these results indicate that the initial disulfide bond is formed quantitatively in less than 4 sec after Cys100 passes through the membrane into the cisterna of the endoplasmic reticulum.

Despite a total lack of corresponding in vivo studies, the kinetics of intrachain disulfide bond formation has been extensively studied in vitro in a number of protein model systems [49–57]. In most of these cases more than one disulfide bond is present in the native protein, and the formation of intermediates with incorrectly paired disulfides occurs during in vitro refolding [58–61]. We have not detected any incorrect disulfide bonds on the nascent L chain. However, it must be mentioned that the Cys residues involved in the intrachain disulfide bonds of L chain are co-linear along the peptide chain. This situation favors formation of the correct disulfide bond because the two Cys residues (Cys35, Cys100) forming this disulfide bond are present in the cisterna of the rough endoplasmic reticulum for a substantial time before other Cys residues (eg, Cys146, Cys206) enter the cisterna. Experiments are now underway to correlate the results presented here with in vitro refolding studies of the MPC 11 L chain, and also to examine the in vivo folding of MPC 11 H chain where the Cys residues involved in interchain disulfide bonds could potentially form an incorrect intrachain disulfide linkage as the protein is being synthesized (see Fig. 8).

Our results provide rigorous in vivo evidence for the independent folding of the domains of a polypeptide, as been proposed from in vitro studies [62–63]. First, although we have provided evidence that the initial intrachain disulfide bond is formed quantitatively on nascent L chains, it seems unlikely that the formation of this disulfide bond is itself the earliest folding event on the nascent chain. Rather, it is likely that a noncovalent nucleation event [64–67] brings Cys35 and Cys100 into close proximity with subsequent formation of the disulfide bond. Additional noncovalent folding may occur after formation of the disulfide bond [56]. Second, the intrachain disulfide loop of the first or variable region domain of L chain is formed quantitatively by the time the molecule is 18,000 daltons in

size, which is before a significant part of the second or constant region domain (a domain in immunoglobulin is approximately 12,500 daltons) has been synthesized and passed through the membrane into the cisterna of the rough endoplasmic reticulum. This result indicates that in vivo proteins initiate folding sequentially from the amino terminal end to the carboxy terminal end as growing nascent chains prior to release from the ribosome, although the entire molecule may be required for complete folding of the molecule [68].

In view of the fact that the amino-terminal regions of a nascent polypeptide have a longer time to fold than the more carboxy-terminal regions, it is likely that folding of a denatured completed polypeptide in vitro is not a reliable model of in vivo folding. This deficiency of the in vitro model may, in fact, explain why it is not possible to achieve correct folding of some denatured, completed polypeptides in vitro.

The initial step of intermolecular covalent assembly of immunoglobulin molecules involves formation of H–L or H–H disulfide bonds. From the results presented here we conclude that this initial step of covalent assembly occurs – to a substantial extent – on nascent H chains, as well as on completed H chains, as demonstrated previously by others [12, 13]. Our results demonstrate that in MPC 11 cells (see Fig. 6A) completed L chains are assembled covalently onto nascent H chains since L chains are specifically released by reduction of the isolated nascent chain fraction. This finding is consistent with the H–L

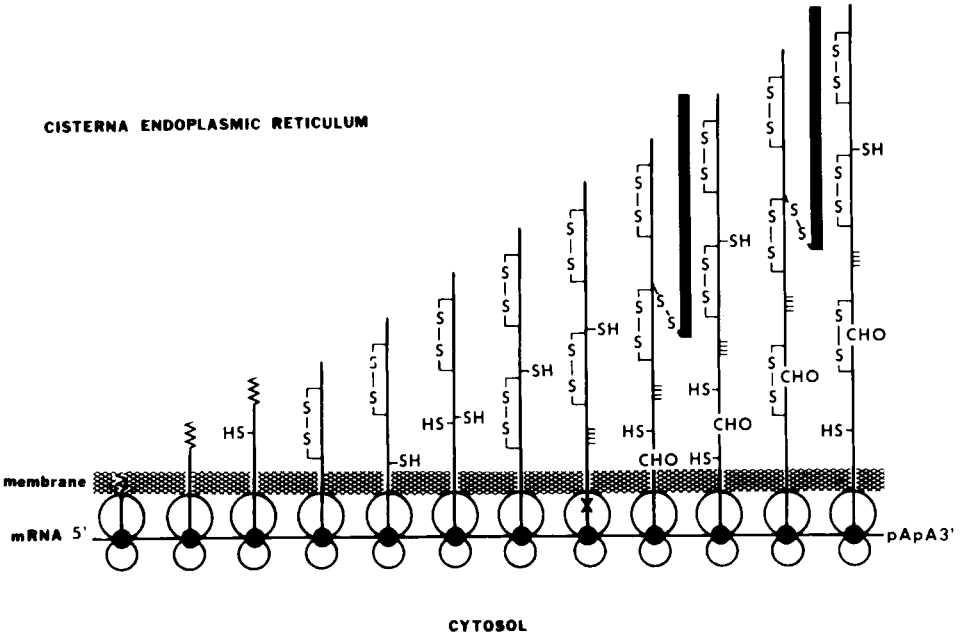


Fig. 8. Model of MPC 11 nascent heavy chain polyribosomal complex showing various co-translational modification events: (1) cleavage of amino-terminal leader or signal sequence (∿∿∿) ([46]; E. Harris and W.M. Kuehl, unpublished data); (2) transfer of core oligosaccharide (CHO) to asparaginyl acceptor residue (X); (3) formation of intrachain disulfide bonds; and (4) formation of interchain disulfide bond to complete light chain (indicated by thick line). Approximately half of the nascent heavy chains form an interchain disulfide bond with a complete light chain before heavy chain completion and release from the polyribosomal complex.

half molecule being the major intermediate in the assembly of the mature MPC 11 molecule [12]. In contrast, by the criteria stated above, completed H chains are covalently assembled onto nascent H chains in MOPC 21 cells (see Fig. 6B) where an H chain dimer is the predominant intermediate in MOPC 21 covalent assembly [13].

We have determined the minimum size of nascent H chains that are covalently bound to completed L chains for MPC 11 and to completed H chains for MOPC 21. The results indicate that the nascent H chains must be approximately 38,000 daltons before completed L chains become covalently bound in MPC 11 [39], whereas the nascent H chain in MOPC 21 must be approximately 44,000 daltons prior to covalent assembly of a completed H chain (see Fig. 7). The H chain Cys residue involved in the interchain disulfide bond with the L chain is residue 131 of the MPC 11 H chain [69], and the residues involved in H–H disulfide bonds in MOPC 21 are Cys221, 224, and 226 [70]. These residues would be expected to enter the intracisternal space when the nascent H chain has achieved a size of approximately 20,000 daltons and 31,000 daltons for MPC 11 and MOPC 21, respectively (assuming 50 amino acid residues to span the ribosome and rough endoplasmic reticulum membrane [46]). This suggests the possibility that the nascent H chain must first achieve some specific secondary or tertiary structure before intermolecular covalent assembly to a completed L or H chain can occur. However, we are unable to determine when non-covalent association of the completed L or H chain with the nascent H chains occurs. Finally, it should be noted that, although a significant amount of initial covalent assembly (H–L in MPC 11 cells and H_2 in MOPC 21 cells) occurs on nascent chains, some H–L and H_2 assembly occurs on completed chains and all further covalent assembly occurs on completed chains [12, 13].

Figure 8 shows a model of a nascent H chain polyribosomal complex and various co-translational modifications (amino-terminal signal sequence removal, glycosylation, folding, and assembly) that we have shown to occur on nascent immunoglobulin H and L chains in vivo.

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