Modification of Fibrinogen Chains during Synthesis: Glycosylation of Bβ and γ Chains†

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ABSTRACT: Specific immunoprecipitation and affinity chromatography on Con A–Sepharose of in vitro translated products derived from rat liver messenger ribonucleic acid (mRNA), total polysomes, and rough microsomes have been used to determine temporal events of glycosylation of the subunits of fibrinogen. The Aα chain is not glycosylated, whereas both the Bβ and the γ chains have carbohydrate clusters (probably Asn linked). Evidence presented here shows that the γ chain receives its core carbohydrate as an early cotranslational event. The Bβ chain is glycosylated later and likely is glycosylated at the time of polypeptide termination or shortly after it is released from the ribosome into the cisternal space of the rough endoplasmic reticulum.

Rat fibrinogen is a glycoprotein consisting of six polypeptides linked by numerous disulfide bridges. There are two sets of three nonidentical polypeptides, $(A\alpha\text{–}B\beta\text{–}\gamma)_2$, in the molecule (Doolittle, 1973). The rat Aα chain is not glycosylated, whereas the rat Bβ and γ chains are glycosylated as shown by PAS (periodic acid–Schiff) staining (Nickerson & Fuller, 1981). Each human fibrinogen Bβ and γ chain is glycosylated only once at an asparagine acceptor site of the general form N–X–T(S). The human Bβ chain is comprised of 461 residues and has the carbohydrate attached through the asparagine residue at position N-364. Similarly, the human γ chain is 411 residues in length; however, its carbohydrate is located at asparagine residue N-52 (Dayhoff, 1978). It has been previously shown that nonglycosylated rat fibrinogen chains could be obtained by treatment of a rat hepatoma cell line with tunicamycin (Nickerson & Fuller, 1981). This drug has been shown to inhibit asparagine-linked glycosylation of polypeptides by preventing the synthesis of *N*-acetylglucosaminopyrophosphoryldolichol (Tkacz & Lampen, 1975). The inhibition of glycosylation of the rat fibrinogen chain lends support to the idea that glycosylations of human and rat fibrinogen chains are very similar. We were interested in determining precisely when the two fibrinogen chains are glycosylated since several investigations of secretory proteins showed that there is a tight coupling among polypeptide translation, translocation, and core glycosylation (Lingappa et al., 1978; Hortin & Boime, 1980; Rosen & Shields, 1980). In earlier studies (Nickerson & Fuller, 1981) characterizing the messenger ribonucleic acid (mRNA) translation products of rat fibrinogen chains, we suggested that core glycosylation of the rat γ chain may be an early cotranslational event and that core glycosylation of Bβ may be an extremely late cotranslational event or early posttranslational event. In this report, we provide evidence that in vivo the rat γ chain is, in fact, glycosylated while the nascent polypeptide is still bound to the ribosome. The Bβ chain, however, is glycosylated shortly after the polypeptide is completed in the rough microsome or, at the very earliest, when the chain is being terminated. The strategy employed in this study was to translate in a cell-free system rat liver mRNA, total polysomes, or rough microsomes. The elongated chains from the polysomes and microsomes, and the mRNA translation products were chromatographed on Con A–Sepharose to separate glycosylated from nonglycosylated chains. Analysis on sodium dodecyl sulfate–polyacrylamide gels provided a means to identify specific chains in their glycosylated and nonglycosylated states. This protocol provided a means to identify the times at which the Bβ and γ chains were glycosylated during fibrinogen synthesis.

Experimental Procedures

Most of the methods used in this study have been described in detail previously (Nickerson & Fuller, 1981). Total polysomes were prepared as described by Bouma et al. (1975), except that heparin and cycloheximide were omitted. Messenger RNA was prepared from total polysomes by proteinase K treatment, phenol–chloroform extraction (Perry et al., 1972), and oligo(dT)–cellulose chromatography (Aviv & Leder, 1972). In vitro translations by using an mRNA-dependent reticulocyte lysate were carried out essentially as described by Pelham & Jackson (1976). Immunoprecipitations were carried out by using the indirect *Staphylococcus aureus* procedure of Kessler (1975) as described by Nickerson & Fuller (1981); however, ovalbumin carrier protein was omitted. Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis and fluorography were performed as described by Laemmli (1970) and Laskey & Mills (1975), respectively. Growth of and tunicamycin treatment of rat hepatoma cells (Faza) were performed as previously described.

Isolation of Rat Liver Rough Microsomes. A postmitochondrial supernatant was prepared as described for total polysome preparations (Bouma et al., 1975); however, 3 mM dithiothreitol was included in the homogenization buffer, and heparin and cycloheximide were omitted from all solutions. The postmitochondrial supernatant (9 mL) was layered onto a discontinuous sucrose gradient consisting of (from bottom to top) 4 mL of 2.0 M sucrose, 6 mL of 1.75 M sucrose, 4 mL of 1.5 M sucrose, and 4 mL of 1.0 M sucrose, all in TNM buffer (50 mM Tris, 25 mM NaCl, and 5 mM $MgCl_2$, pH 7.0, at 24 °C). The gradients were poured in polycarbonate centrifuge bottles that had been autoclaved before use. The gradients were centrifuged at 59 000 rpm ($360000g_{max}$) for 6 h at 4 °C in a 60Ti rotor in an L2-65B (Beckman) centrifuge. The 1.75 M sucrose layers, containing the rough microsomes, were pooled and diluted with 1.2 volumes of TNM buffer containing no sucrose. The diluted rough microsomes (11.2 mL) were layered on a discontinuous gradient of 0.5 mL of 2.0 M sucrose and 1.5 mL of 1.3 M sucrose in TNM buffer.

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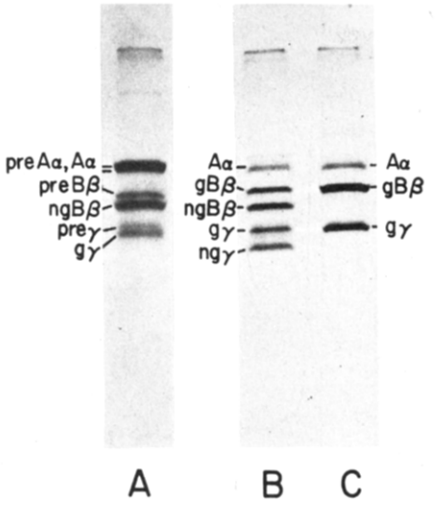

FIGURE 1: Comparison of translation products from total polysomes with fibrinogen chains from hepatoma cells, and with TM fibrinogen (fibrinogen secreted by tunicamycin-treated hepatoma cells). (A) Total polysome translation products [for chain identifications, see Nickerson & Fuller (1981)]; (B) TM fibrinogen; (C) hepatoma fibrinogen. All lanes were immunoprecipitates of [^{35}S]Met-labeled material. The prefix "g" refers to putative glycosylated states and "ng" to nonglycosylated states. The prefix "pre" refers to precursor forms of the chains, containing leader or signal sequences.

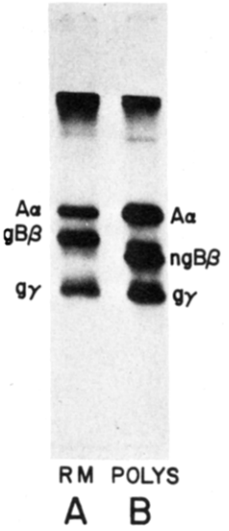

FIGURE 2: Comparison of rough microsome and total polysome translation products. Rat liver rough microsomes (RM) or total polysomes (POLYS) were added to the translation system; subsequently, fibrinogen chains were immunoprecipitated and electrophoresed. There is a substantial difference in the mobilities of the polysome elongated Bβ chains and the rough microsome Bβ chains. The rough microsome product Bβ chain migrated slower than the total polysome Bβ chain by an amount corresponding to about 3000 daltons. This migration was attributed to a glycosylated rough microsome Bβ chain and a nonglycosylated total polysome Bβ chain. (A) Rough microsome products; (B) total polysome products. The "g" refers to the putative glycosylated state, and "ng" refers to the nonglycosylated state.

The gradients were centrifuged in an SW-40 rotor at 38 000 rpm ($283000g_{max}$) for 50 min at 4 °C. The opaque bands of rough microsomes between the 1.3 M and 2.0 M sucrose layers were pooled and stored in small aliquots at −50 °C. This method is based on the procedure of Jackson & Blobel (1979).

Con A–Sepharose Preparation. Con A (concanavalin A; Sigma) was covalently bound to cyanogen bromide activated Sepharose 4B by using the method of Cooper (1977). A borate buffer (100 mM boric acid, 25 mM sodium tetraborate, and 75 mM NaCl, pH 9.5) was used in the coupling reaction. The conditions for Con A–Sepharose chromatography are described under Results.

Results

Rat liver total polysomes were translated in a reticulocyte lysate system as previously described. The fibrinogen chains were immunoprecipitated and compared with TM fibrinogen (fibrinogen secreted by tunicamycin-treated rat hepatoma cells). At the dose of tunicamycin used, some Bβ and γ chains are glycosylated while others are not. In Figure 1, the γ chain derived from the translation of polysomes (lane A) comigrates with glycosylated γ chain (gγ) derived from TM fibrinogen (lane B) in the $NaDodSO_4$ gel. These two bands migrate slower than the nonglycosylated γ chain (ngγ) from TM fibrinogen. The elongated γ chain (gγ, lane A) from the polysome is likely missing its signal peptide, since signal peptidase removes these N-terminal extensions as an early cotranslational event (Blobel & Dobberstein, 1975). Thus, the difference in molecular weight between the elongated γ chain (lane A) and the nonglycosylated γ chain of TM fibrinogen (ngγ, lane B) should not be attributed to the presence of a signal peptide on the specified elongated γ chain (gγ, lane A) [see Nickerson & Fuller (1981)]. The molecular weight difference can be attributed to the cotranslational glycosylation of nascent γ chains in vivo prior to polysome isolation. It has been shown that core oligosaccharide addition can occur as soon as an asparagine acceptor has crossed into the lumen of the rough endoplasmic reticulum (rER) (Bergman & Kuehl, 1978). If the asparagine acceptor in the γ chain is near the N-terminal end, as it is in humans, it is likely that most nascent γ chains would have already been core glycosylated in vivo prior to the isolation of the polysomes. When these chains are elongated in vitro, they would migrate with a mobility of a glycosylated secreted γ chain, not a nonglycosylated γ chain.

In contrast to the apparent cotranslational glycosylation of the γ chain, the Bβ chain does not appear to be cotranslationally modified. The Bβ chain elongated from total polysomes in vitro (Figure 1, lane A) comigrates with a nonglycosylated Bβ chain (ngBβ, lane B) of TM fibrinogen [for further details on the identification of elongated chains and primary translation products Aα, Bβ, and γ chains, see Nickerson & Fuller (1981)]. If the carbohydrate on the rat Bβ chain is similarly located to that of the human Bβ chain (i.e., near the C terminus), then it follows that few of the appropriate asparagine acceptor sites would be accessible to the core glycosyltransferase before polypeptide termination. The consequence is that elongated Bβ chain would be expected to comigrate with nonglycosylated authentic Bβ chain, and, in fact, the comigration of these two bands is observed on the $NaDodSO_4$ gels (Figure 1, lanes A and B).

In a similar experiment, rat liver rough microsomes, containing attached ribosomes and initiated fibrinogen chains, were added to the in vitro translation system, and the nascent chains were elongated. These products were compared to the total polysome translation products. The rough microsome chains were the same sizes as those of the total polysome products with the exception of the Bβ chains. The Bβ chain elongated in the rough microsome (lane A, Figure 2) was larger than the polysome-elongated Bβ chain (lane B, Figure 2) by about 3000 daltons. This information leads us to suggest that the Bβ chain was being core glycosylated in the lumen of the rough microsome and that little or no glycosylation had occurred on elongated Bβ chains derived from total polysome nascent chains.

Con A–Sepharose Chromatography. For further investigation of whether the γ-chain glycosylation was cotranslational

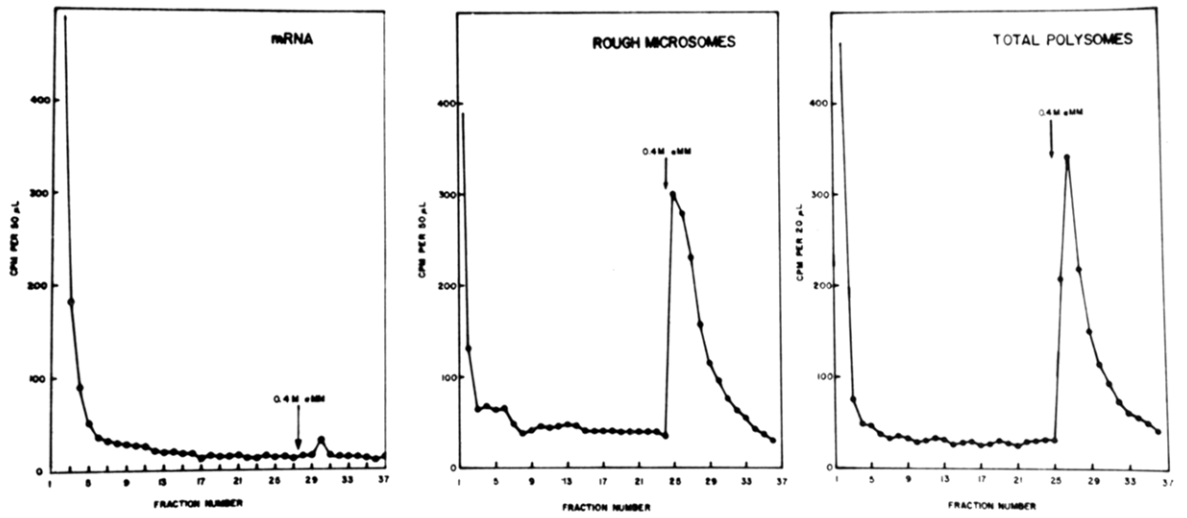

FIGURE 3: Con A–Sepharose chromatography of rat liver mRNA, total polysome, and rough microsome translation products. [^{35}S]-Methionine-labeled fibrinogen products from the indicated sources were immunoprecipitated, solubilized in $NaDodSO_4$, reduced, and alkylated. The resulting chains were diluted and subjected to chromatography on Con A–Sepharose, and the radioactivity in each fraction was determined. α-MM refers to the application of α-methyl mannoside.

and the Bβ posttranslational, liver-derived mRNA, total liver polysomes, and rough microsomes (containing attached ribosomes) were translated and/or elongated in vitro. Glycosylation of the various translation or elongation products was then determined by employing Con A–Sepharose chromatography (Lingappa et al., 1978). Glycosylated polypeptides bind to Con A–Sepharose, while nonglycosylated chains do not. The translation products were immunoprecipitated by using the indirect *S. aureus* technique. The various chains were solubilized by boiling in solubilization buffer [2% (w/v) $NaDodSO_4$, 62.5 mM Tris, 10% (v/v) glycerol, 22 mM DTT, and 0.005% (w/v) phenol red, pH 6.8] and were alkylated by adding 0.1 volume 0.5 M iodoacetamide as described by Blobel & Dobberstein (1975). The samples were diluted to 0.05% (w/v) $NaDodSO_4$ by using buffer N (10 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM DTT, pH 7.5) and were slowly (1–2 mL/h) passed through a 0.3-mL column of Con A–Sepharose (roughly 4 mg of Con A/mL of packed beads). The column was washed with 25 mL of buffer A (0.05% $NaDodSO_4$ plus buffer N) at 10 mL/h. The specifically bound fractions were eluted with 0.4 M α-methyl mannoside in buffer A at the same flow rate. Aliquots of either 20 or 50 μL from each 1-mL fraction were counted in Aquasol to determine the radioactivity profile. Figure 3 shows the profiles of Con A–Sepharose chromatography, i.e., translation products from mRNA, total polysomes, and rough microsomes. The unbound fractions and specifically eluted fractions were dialyzed against 6 L of H_2O for 6 h at 24 °C and lyophilized. The pools were dissolved in solubilization buffer and electrophoresed on $NaDodSO_4$–polyacrylamide gels. In Figure 3, it can be seen that virtually no radioactivity was eluted from the Con A–Sepharose column by α-methyl mannoside from mRNA translation products. On the other hand, both total polysome and rough microsome fibrinogen translation products contained some material that was retained by the Con A–Sepharose column and was specifically eluted by α-methyl mannoside. Both unbound and specifically eluted material were identified by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4). The total polysome specifically eluted material migrates as a glycosylated γ chain (Figure 4, lane F); however, neither Aα nor Bβ chains were retained by the Con A–Sepharose (Figure 4, lane E). Thus, we conclude that neither the Aα nor the Bβ chain is core glycosylated as a cotranslational event, that is, while these

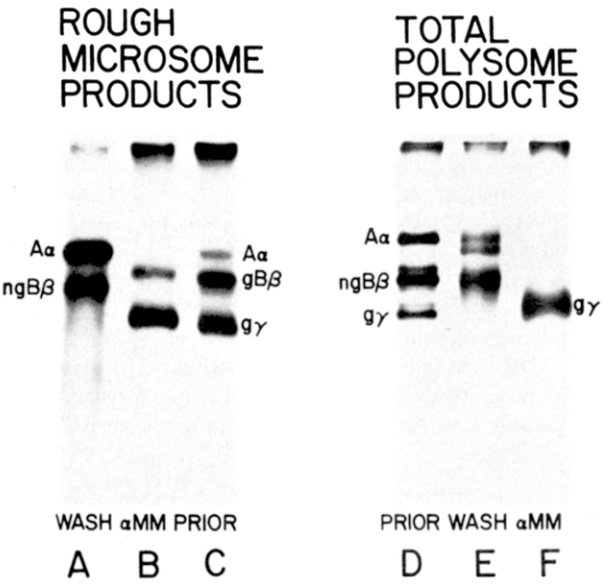

FIGURE 4: Con A–Sepharose chromatography of rough microsome and total polysome translation products. Rough microsomes or total polysomes from rat liver were added to the translation system, and fibrinogen products were immunoprecipitated. The fibrinogen chains were chromatographed on Con A–Sepharose. Unbound chains (WASH) and specifically eluted chains (α-MM; 0.4 M α-methyl mannoside) were electrophoresed with material that was not chromatographed (PRIOR). Lanes A, B, and C were derived from rough microsomes, and lanes D, E, and F were derived from total polysomes. The "g" refers to glycosylated, and the "ng" refers to nonglycosylated.

chains are still bound to the polysomal complex. On the other hand, the γ chain was glycosylated while it was still bound to the ribosome, a cotranslational event. The γ chain was undoubtedly glycosylated soon after the acceptor asparagine crossed into the lumen of the rER.

That core glycosylation of Bβ occurred in rat liver rough microsomes was demonstrated by Con A–Sepharose chromatography and $NaDodSO_4$ gel electrophoresis of the specifically eluted fraction. Both the Bβ and the γ chains were retained by the Con A–Sepharose and were specifically eluted by α-methyl mannoside (Figure 4, lane B). A band of apparently nonglycosylated Bβ chain was detected in the unbound fraction (lane A). The nonglycosylated Bβ chain migrated faster in the $NaDodSO_4$ gel, corresponding to a molecular weight roughly 3000 smaller than that of the gly-

cosylated Bβ chain. This band of nonglycosylated Bβ could represent a Bβ chain that was not translocated or a pool within the lumen of the rough microsomes of nonglycosylated (or not yet glycosylated) free Bβ chain. Within the limits of detection, it appears that rat Bβ chain is glycosylated after this chain is terminated. However, this observtion should be correlated with data from human Bβ chain. The asparagine acceptor residue is N-364, 97 residues from the C terminus. The approximate length of the ribosomal core (30–50 amino acid residues) plus the transmembrane pore complex (30–40 residues) totals 60–90 amino acids. When the nascent Bβ chain is released, the acceptor asparagine might be 7–37 amino acids "into" the lumen, suggesting that *at most* one nascent polypeptide could be glycosylated on the Bβ-synthesizing polysome. Hence, it is anticipated that very few, if any, of the acceptor asparagines would be available or recognizable by the core glycosyltransferase while Bβ was nascent in vivo. However, after release from the ribosome, we suggest that the acceptor site becomes available in the rER lumen and is core glycosylated within the lumen of the rough microsome. The conclusion can be drawn that core glycosylation of Bβ occurs when the Bβ chain is being terminated or shortly afterward within the rER while γ-chain core glycosylation is an early cotranslational event. Second, both chains are core glycosylated in the rER.

Discussion

This study dealt with the glycosylation of the Bβ and γ polypeptides. The idea that most γ chains on total polysomes were glycosylated was shown by in vitro elongation of these chains, showing that most, if not all, of the γ chains could bind to Con A–Sepharose. This information is an indication that even very short (incomplete) nascent chains are glycosylated in vivo. If short nascent γ chains are glycosylated, then it seems likely that the asparagine acceptor residue must be near the N terminus. This prediction correlates with the amino acid sequencing data of the human γ chain, which showed that the Asn acceptor is N-52, near the N terminus.

The corresponding prediction, based on the location of the human Bβ chain Asn acceptor at N-364, was that very few, if any, Bβ nascent polypeptides would be glycosylated. Since no detectable Bβ chain from total polysome translation products bound to Con A–Sepharose, it is thought that glycosylation is, at the very earliest, a very late cotranslational event, and, more likely, it is an early posttranslational event, occurring within the lumen of the rER.

We have shown that the Bβ chain does become glycosylated within rat liver rough microsomes. The nascent fibrinogen chains initiated in vivo in the rough microsomes were elongated in vitro, and a substantial fraction of the Bβ chains then becomes capable of binding to Con A–Sepharose. Thus, it appeared that the Bβ chain was glycosylated in vitro. Presumably, the newly synthesized fibrinogen chains are sequestered within the microsome on which they were synthesized. Even though some microsomes derived from Golgi or smooth endoplasmic reticulum (sER) probably contaminate the rat liver rough microsomal fraction which was added to the in vitro translation system, it seems unlikely that a particular microsome capable of synthesizing and translocating nascent fibrinogen chains (a microsome presumably derived from rER) could also contain Golgi or sER activities. These latter activities should be solely confined to separate microsomes derived from Golgi or sER. There should be virtually no microsomes that are fusion products of a Golgi or sER microsome and and rER-derived microsome. Hence, it would appear that synthesis and glycosylation of the Bβ chain in a rough microsomal fraction in vitro are a true reflection of a normal in vivo occurrence in the rER. In conjunction with the information that Bβ is not glycosylated cotranslationally, the assignment of the rER as the site of core glycosylation implies that glycosylation of Bβ is an early posttranslation event. The location of a newly synthesized secretory protein in the rER is generally thought to be an early event, preceding the subsequent transfer to sER, Golgi, and secretory vesicles prior to secretion. No further refinement of the time of glycosylation of Bβ can be made, based on the available data. Nonetheless, this determination of the time of glycosylation of the Bβ chain is very specific. Core glycosylation of Bβ is confined to a time window of no earlier than Bβ-chain termination and no later than transfer of Bβ out of the rER compartment. This last result also verifies that isolated rat liver rough microsomes are capable of glycosylating at least one polypeptide, the fibrinogen Bβ chain.

In summary, we have demonstrated that both the Bβ and the γ chains of rat fibrinogen are glycosylated via asparagine-linked moieties, and Aα chains are not glycosylated. The γ chain is glycosylated as an early cotranslational event. The Bβ chain is glycosylated at or slightly after Bβ termination.

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