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Evidence for Posttranslational O-Glycosylation of Fetuin†

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ABSTRACT: Fetuin, a major glycoprotein in the serum of fetal calves that contains three N-linked and three O-linked carbohydrate side chains, was found to be synthesized in the liver with an 18 amino acid signal peptide, Met-X-X-X-X-Leu-Leu-X-Cys-Leu-Ala-X-Leu-X-X-Cys-X-X, and to undergo cotransational N-glycosylation. In order to examine O-glycosylation, fetuin peptidyl-tRNA was purified from liver and analyzed for O-linked carbohydrate by quantitating the released [^{3}H]GalNAcitol produced after β-elimination in the presence of NaB^3H_4. Within the limits of the assay, <1.3% of the O-linked chains had been initiated. Additionally, rough microsomes were used to program a cell-free protein synthesis system. A radiolabeled fetuin intermediate was isolated by immunoprecipitation and shown to contain N-linked carbohydrate by binding to concanavalin A and by susceptibility to cleavage by endoglycosidase H. However, this fetuin intermediate was not detectably bound (<1%) by GalNAc-specific lectins, which were shown to bind asialoagalactofetuin. These results suggest that O-glycosylation of fetuin is a posttranslational event.

Many serum proteins are glycosylated, and their carbohydrate side chains are frequently attached through a glycosylamine linkage to specific asparagine residues. It is well established that the formation of these N-linked side chains begins in the rough endoplasmic reticulum (RER) with the cotranslational transfer of a core oligosaccharide unit from a lipid carrier to protein asparagine residues (Presper & Heath, 1983; Hanover & Lennarz, 1981; Staneloni & Leloir, 1982). Carbohydrate attached through a glycosidic linkage to serine or threonine residues occurs in serum proteins less frequently. These O-linked carbohydrate side chains are synthesized by the sequential addition of individual monosaccharide units from nucleotide sugars starting with the attachment of an α-linked GalNAc residue. Although Strous has presented evidence that O-glycosylation is initiated cotranslationally (Strous, 1979), most of the data currently available indicate that O-glycosylation is posttranslational, occurring in the Golgi apparatus [Hanover and Lennarz (1981) and Berger et al. (1982) for reviews].

Fetuin, an abundant serum protein found in fetal calves, has three N-linked and three O-linked carbohydrate side chains (Spiro & Bhoyroo, 1974). Fetuin was found to be a major protein synthesized by the liver and, as for many other secretory proteins, was found to be synthesized with a signal peptide on membrane-bound polysomes and to undergo cotranslational N-glycosylation. Therefore, fetal bovine liver peptidyl-tRNA was analyzed for the presence of O-linked GalNAc by two independent methods of analysis in order to compare the temporal and spatial relationship between polypeptide synthesis and both N- and O-glycosylation.

Experimental Procedures

Materials. Fetuin, purified by the method of Spiro, was purchased from GIBCO Laboratories (Grand Island, NY). Protein A coupled to Sepharose and bovine submaxillary mucin were from Sigma Chemical Co. (St. Louis, MO). Concanavalin A–agarose and endo-β-*N*-acetylglucosaminidase H were purchased from Miles Laboratories, Inc. (Elkhart, IN). The

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lectins soybean agglutinin (SBA),[1] *Ricinus communis* agglutinin II (RCA II), *Dolichos biflorus* agglutinin (DBA), and *Sophora japonica* agglutinin (SJA) coupled to agarose were purchased from EY Laboratories, Inc. (San Mateo, CA). The B_4 lectin from *Vicia villosa* was prepared as described by Tollefsen and Kornfeld (1983) with an affinity column of *N*-acetylgalactosamine coupled to epoxy-activated cross-linked agarose beads (Pierce). The B_4 lectin was coupled to cyanogen bromide activated Sepharose CL-4B (Pharmacia) by the procedure of March et al. (1974). Excess active groups were blocked with 0.5% ethanolamine (pH 8.0). Glutaraldehyde-fixed *Staphylococcus aureus* (Cowan I), IgGSORB, was purchased from the Enzyme Center (Boston, MA). Radiolabeled materials were obtained from either Amersham Corp. (Arlington Heights, IL) or New England Nuclear (Boston, MA) and included L-[^{3}H]alanine (60 Ci/mmol), L-[^{3}H]aspartic acid (15 Ci/mmol), L-[^{35}S]cysteine (1000 Ci/mmol), L-[^{3}H]leucine (140 Ci/mmol), L-[^{35}S]methionine (1000 Ci/mmol), L-[^{3}H]proline (100 Ci/mmol), UDP-*N*-acetyl[1-^{3}H]galactosamine (10.7 Ci/mmol), and UDP-[6-^{3}H]galactose (15.6 Ci/mmol). All other reagents were obtained from commercial sources at the highest available purity.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was done as described by Laemmli (1970) except that the separating gel consisted of a linear gradient of polyacrylamide (8–12%) and of glycerol (0–15%). Stained gels containing proteins labeled with sulfur-35 were dried and directly subjected to autoradiography with Kodak XAR-5 radiographic film. Stained gels containing proteins labeled with tritium were impregnated with sodium salicylate and analyzed by fluorography (Chamberlain, 1979).

Cell-Free Protein Synthesis. Translation of mRNA, RER-enriched microsomes, or polysomes was performed in a micrococcal nuclease treated, cell-free, rabbit reticulocyte lysate protein synthesis system according to Jackson and Hunt (1983) as previously described (Hanley et al., 1983). Translation products were analyzed by measuring the incorporation of radioactivity into 10% trichloroacetic acid (TCA) precipitable material. When [^{35}S]cysteine was used, labeled proteins were carboxyamidomethylated (0.2 M iodoacetamide in 8 M urea, 0.1 N NaOH, and 0.5% 2-mercaptoethanol at 37 °C for 30 min) prior to TCA precipitation.

Protein Sequencing. Amino-terminal sequencing of radiolabeled protein was performed by automated Edman degradation on a Beckman 890C sequenator with a modified 1 M Quadrol program as described (Hanley et al., 1983), except that IgGSORB was used instead of protein A–Sepharose for immune precipitations. The protein was reduced and carboxyamidomethylated as previously described (Grant et al., 1980), and 0.4 mg of sperm whale apomyoglobin was used as a carrier protein. Repetitive yields for carrier apomyoglobin ranged between 91 and 98%.

Immunological Procedures. Antifetuin serum was prepared in rabbits with 5 mg of fetuin in Freund's complete adjuvant at five sites (two intramuscularly and three subcutaneously) at 2-week intervals for 6 weeks. Thereafter, booster injections of 2 mg of fetuin in incomplete Freund's adjuvant were given, 14 and 10 days before ear bleeding. Fetuin-binding immunoglobulins were isolated on a fetuin-derivatized Sepharose CL-4B column as previously described (Goldstein & Heath, 1984). The IgG fraction was isolated on a column of protein A–Sepharose (Hjelm et al., 1972). The procedure of Anderson and Blobel (1983) was used for immunoprecipitation of the proteins derived from an in vitro rabbit reticulocyte cell-free translation system.

Preparation of RNA. Fresh fetal bovine liver was obtained at a local abattoir, immediately frozen in liquid nitrogen, and stored at −80 °C until needed. Total RNA was prepared by the procedures of both Cox (1968) and Chirgwin et al. (1979). The concentration of RNA was estimated by the absorbance of the solution at 260 nm (1 A_{260} unit = 42 μg of RNA/mL). A typical yield was 2 mg of RNA/g of liver. Poly(A)–RNA was obtained by oligo(dT)–cellulose chromatography as previously described (Goldstein & Heath, 1984). A typical yield of 1.6% of the total RNA was obtained as polyadenylated RNA.

Preparation of Polysomes. Fetal bovine liver polysomes were prepared from frozen liver (−80 °C) by the magnesium precipitation procedure described by Palmiter (1974) as modified by MacGillivray et al. (1979). The polysomes obtained were washed by suspension in 2 volumes of polysome buffer [20 mM HEPES–KOH (pH 7.5), 1 mM $MgCl_2$, and 2 mM DTT] and centrifuged through a layer of 0.8 M sucrose in polysome buffer (100000*g*, 4 h). The pellet was suspended in polysome buffer and stored in aliquots at −80 °C until needed. The A_{260}/A_{280} ratio of a typical preparation was 1.7, and the yield was 35 A_{260} units/g of liver.

Preparation of Rough Endoplasmic Reticulum Microsomes. Rough endoplasmic reticulum-enriched microsomes were prepared by the procedure of Scheele et al. (1980) from frozen liver. The rough microsomes obtained were suspended in 1.3 M sucrose in 20 mM HEPES–KOH (pH 7.5), 1 mM $MgCl_2$, and 2 mM DTT, divided into 100-μL aliquots, and stored at −80 °C after rapid freezing in liquid nitrogen. A typical preparation had an A_{260}/A_{280} ratio of 1.3 and yielded 20 A_{260} units/g of liver.

Preparation of Peptidyl-tRNA. Total peptidyl-tRNA was prepared from polysomes as described by Strous and coworkers (Strous et al., 1974). The peptidyl-tRNA preparation obtained from the sucrose gradient (Strous et al., 1974) was dialyzed against 50 mM Tris-HCl (pH 7.4) and 0.15 M NaCl and further purified by chromatography on DEAE-cellulose by a modification of the procedure of Kiely et al. (1976). Approximately 6 A_{260} units of crude peptidyl-tRNA was applied to a 1 × 11 cm column of freshly autoclaved DE-52 (Whatman) equilibrated with 50 mM Tris (pH 7.4). The column was washed with Tris buffer containing sodium chloride in the following concentration increments: 0.15, 0.4, 0.6, and 1 M NaCl. Purified peptidyl-tRNA eluted with the 0.6 M NaCl buffer (A_{260}/A_{280} = 2.0), gave an 80% yield on the basis of A_{260} units applied to the DEAE-cellulose column, and was extensively dialyzed against water, lyophilized, and stored at −20 °C until needed.

The subfraction of the total peptidyl-tRNA, which is bound by an anti-fetuin–IgG–Sepharose column, was prepared by denaturing total peptidyl-tRNA (2.5 A_{260} units) in 2% SDS at 37 °C for 2 min, cooling, adding 4 volumes of dilution buffer [0.05 M Tris-HCl (pH 7.4), 0.19 M NaCl, 6 mM EDTA, and 2.5% Triton X-100], and passing the sample over a 3-mL anti-fetuin–IgG–Sepharose column at room temperature. The column was washed with 2 mL of 0.5% Triton X-100 in TBS (25 mM Tris, pH 7.4, 140 mM NaCl) followed by 4 mL of TBS before the bound material was eluted with 1 M acetic

[1] Abbreviations: SBA, soybean agglutinin; B_4, B_4 lectin from *Vicia villosa*; Con A, concanavalin A; DBA, *Dolichos biflorus* agglutinin; DTT, dithiothreitol; endo H, endo-β-*N*-acetylglucosaminidase H; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; RCA II, *Ricinus communis* agglutinin II; SDS, sodium dodecyl sulfate; SJA, *Sophora japonica* agglutinin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

acid. Fractions of 1.1 mL were collected from the column, and UV absorbance at 260 nM was measured. The peak fractions were pooled and placed on ice. After addition of 0.1 mL of 1 M Tris-HCl (pH 8), the sample was dialyzed against two changes of TBS followed by three changes of distilled water. A control column of nonimmune IgG coupled to Sepharose was also run. Both columns contained 0.4 mg of bound IgG/mL of Sepharose. Yields were 0.112 and 0.050 A_{260} unit for fetuin peptidyl-tRNA and the nonimmune control, respectively.

Analysis of Peptidyl-tRNA for O-Linked Carbohydrate. Material that was to be assayed for O-linked *N*-acetylgalactosamine (GalNAc) was subjected to β-elimination in the presence of NaB^3H_4 as described by Strous (1979). This reaction cleaves glycosidic bonds between serine or threonine residues and carbohydrate side chains. The linking sugar is labeled as it is converted to an alditol. Briefly, the sample (up to 0.5 A_{260} unit of peptidyl-tRNA, 4.5 mg of fetuin or 20 mg of tRNA) was dissolved in 20–25 μL of 50 mM NaOH containing 0.1 M NaB^3H_4 (11.2 Ci/mmol, New England Nuclear). The reaction was carried out at 20 °C for 16–20 h. The mixture was acidified with 1 volume of 0.5 M HCl and taken to dryness under vacuum. The reaction products were subjected to acid hydrolysis, binding and elution from a cation-exchange column (Dowex-50 W, H^+ form), reacetylation, and final purification on a mixed-bed ion-exchange resin (Bio-Rad AG 501-X8, H^+ and $HCOO^-$ forms) as described previously (Strous, 1979).

Aliquots of the reacetylated amino sugars were spotted on Whatman 1 paper and chromatographed in chromatography system I for 5–16 h. The solvent for descending paper chromatography in system I consists of ethyl acetate–acetic acid–formic acid (90%)–water (18:3:1:4, v/v). Galactosaminitol and *N*-acetylgalactosaminitol (0.6–0.8 μmol) were used as standards, which were detected by periodate and silver staining as described below. Chromatograms were cut into 0.5-cm segments, shaken with 0.5 mL of water for 5 min, and mixed with 5 mL of a Triton X-100 based and toluene-based scintillation cocktail, and the radioactivity content was determined. In order to further examine the material comigrating with *N*-acetylgalactosaminitol in chromatography system I, this material was excised, eluted from the paper with water, and analyzed in paper chromatography system II. Paper chromatography system II (Ogata & Lloyd, 1982) consists of the solvent ethyl acetate–2-propanol–pyridine–water (7:3:2:2, v/v), Whatman 1 paper that has been impregnated with borate by being dipped in a 0.57% $Na_2B_4O_7 \cdot 10H_2O$ solution containing 10 mM NaCl and dried, and descending chromatography for 60–70 h. *N*-Acetylglucosaminitol and *N*-acetylgalactosaminitol (0.6–0.8 μmol) were used as standards, which were detected by periodate and silver staining. If standards were cochromatographed with labeled samples, then the migration positions observed after the silver dip were marked, and the chromatogram was not sprayed with base. The chromatograms were then cut into strips and counted as described for system I. Analysis of fetuin gave an overall recovery of 20% of the starting O-linked GalNAc as *N*-acetylgalactosaminitol in chromatography system II.

Staining Procedures for Paper Chromatography. Reducing sugars separated by descending paper chromatography were located by alkaline silver nitrate staining. The Hough and Jones modification (1962) of the procedure of Trevelyan et al. (1950) was used. Nonreducing sugars were stained by spraying the dried chromatograms with 0.2 M sodium periodate prior to alkaline silver nitrate staining.

Deglycosylation of Glycoproteins. Asialofetuin was prepared by the method of Spiro (1960). Protein concentration was determined with an extinction coefficient at 278 nm ($E^{1\%}$) of 4.10 (Sprio, 1960). Analysis of asialofetuin by gas–liquid chromatography, as previously described (Laine et al., 1972), indicated that desialylation of fetuin with mild acid resulted in the loss of 96% of the sialic acid residues but retention of greater than 90% of the other sugar residues (mannose, galactose, *N*-acetylgalactosamine, and *N*-acetylglucosamine). Asialoagalactofetuin was prepared from asialofetuin by the method of Spiro (1964). Analysis of asialoagalactofetuin by gas–liquid chromatography indicated that, compared to native fetuin, 99% of the galactose and greater than 99% of the sialic acid residues were cleaved and that greater than 90% of the mannose, *N*-acetylgalactosamine, and *N*-acetylglucosamine were retained.

Digestions of immunoprecipitated proteins with endo-β-*N*-acetylglucosaminidase H (endo H) were performed by extracting the bound immunoprecipitate from IgGSORB with 2% SDS at 100 °C. The extracted protein was divided into equal aliquots and diluted with 5 volumes of 1.5 M sodium citrate (pH 5.5) and 40 μg/mL bovine serum albumin. Incubation was carried out at 37 °C with and without three additions of endo H (40 milliunits/mL in the incubation mixture from each addition) given at 0, 6, and 10 h. Incubation was for 20 h in the presence of toluene. The reaction was stopped by cooling to 0 °C and precipitating the protein with 7 volumes of acetone–HCl (acetone–concentrated HCl, 40:1). Samples were centrifuged (16000*g*, 15 min, 4 °C), and the pellets were rinsed with chilled (−20 °C) ethanol, dried under a gentle stream of nitrogen gas, dissolved in SDS sample buffer, and subjected to SDS–polyacrylamide gel electrophoresis as described above.

Use of Lectin Affinity Resins. Batch lectin binding assays using lectins coupled to agarose were performed in the absence or presence of a saccharide that specifically binds to each lectin: 5% methyl α-mannoside for concanavalin A; 0.2 M *N*-acetylgalactosamine for the B_4 lectin from *V. villosa* and for *D. biflorus* agglutinin; 0.2 M lactose for soybean agglutinin, *R. communis* agglutinin II, and *S. japonica* agglutinin. Protein samples to be analyzed for specific binding to lectins were dissolved in 2% SDS–60 mM Tris-HCl (pH 7.4), heated to 100 °C for 2 min, and cooled to 22 °C. The sample was then diluted with 4 volumes of dilution buffer [50 mM Tris-HCl (pH 7.4), 0.19 M NaCl, 6 mM EDTA, and 2.5% Triton X-100]. For Con A binding, the buffers were made without EDTA and were 1 mM in both calcium chloride and manganese chloride. Protein solutions had appropriate saccharides added and were mixed by inversion with immobilized lectins for 3 h at 22 °C. After incubation, samples were centrifuged (10000*g*, 10 s), and the pellets were washed 3 times with at least 20 volumes (with respect to the lectin–agarose pellet volume) of buffer without or with the appropriate saccharide. For Con A–agarose, the wash buffer was 20 mM Tris-HCl (pH 7.5), 0.05% SDS, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and plus or minus 5% methyl α-mannoside. For other immobilized lectins, the wash buffer was 50 mM Tris-HCl (pH 7.4), 0.14 M NaCl, 0.05% Triton X-100, and plus or minus 0.2 M *N*-acetylgalactosamine or lactose. Binding of proteins labeled with iodine-125 was quantitated by direct measurement of the radioactivity on a Beckman Biogamma II. Binding of proteins labeled with sulfur-35 or tritium was quantitated by liquid scintillation spectroscopy. Specific binding is the difference between binding in the absence or in the presence of the competing saccharide. Specifically bound protein was

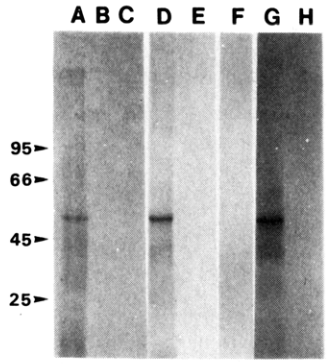

FIGURE 1: Fetuin primary translation product. Total fetal bovine liver RNA (lanes A–F) and the polyadenylated fraction of fetal bovine liver RNA (lanes G and H) were translated in a cell-free rabbit reticulocyte lysate translation system containing either [^{35}S]methionine (lanes A–C) or [^{35}S]cysteine (lanes D–H). The translation products were incubated with either anti-fetuin IgG (lanes A, B, D, E, and G) or nonimmune IgG (lanes C, F, and H). Native fetuin (3 mg/mL) was added to translation products prior to immunoprecipitation of two samples (lanes B and E) to compete for IgG binding to the fetin translation product. The immune precipitates were analyzed by SDS–PAGE and autoradiography. The molecular weight standards are phosphorylase A (95 000), bovine serum albumin (66 000), ovalbumin (45 000), and an IgG light chain (25 000).

eluted either with buffer containing saccharide or with hot 2% SDS.

Protein Assay. Total protein was determined by the procedure of Lowry et al. (1951) with bovine serum albumin as a standard.

Results

Cell-Free Synthesis of Fetuin. Anti-fetuin IgG was used to examine in vitro translation products for molecules immunologically related to fetuin. A cell-free rabbit reticulocyte lysate translation system was programmed with total RNA isolated from fetal bovine liver. The translation products were immunoprecipitated and analyzed by SDS gel electrophoresis and autoradiography. A protein product migrating with an apparent M_r of 49 000 was observed as shown in Figure 1 (lanes A, D, and G). The primary mRNA product could be labeled with either [^{35}S]methionine (lane A) or [^{35}S]cysteine (lanes D and G). This protein was not precipitated by nonimmune IgG (lanes C, F, and H) or when the precipitation was conducted in the presence of excess native fetuin (lanes B and E). This protein was also produced when the polyadenylated fraction of the total RNA was used to program the reticulocyte lysate (lane G) and comprised approximately 11% of the total [^{35}S]cysteine-labeled protein. These data suggest that the mRNA for fetuin is a major mRNA of liver and codes for a polypeptide of 49 kDa (SDS–PAGE).

Partial Sequence Analysis of Primary Translation Product of Fetuin mRNA. In order to obtain a partial N-terminal sequence for the 49-kDa (SDS–PAGE) in vitro translation product, polyadenylated RNA was translated separately with each of six radiolabeled amino acids, either [^{3}H]alanine, [^{3}H]aspartic acid, [^{35}S]cysteine, [^{3}H]leucine, [^{35}S]methionine, or [^{3}H]proline. The product from each in vitro translation was purified by immunoprecipitation and subjected to Edman degradation (Figure 2). The partial NH_2-terminal sequence for the primary translation product was compared to and aligned with the sequence for native fetuin (Alcaraz et al., 1981). As shown in Figure 3, the alignment revealed an overlap with 14 out of the first 25 amino acids. This overlap defined an 18 amino acid signal peptide with the partial amino acid sequence Met-X-X-X-X-Leu-Leu-X-Cys-Leu-Ala-X-Leu-X-X-Cys-X-X. The signal peptide does not contain

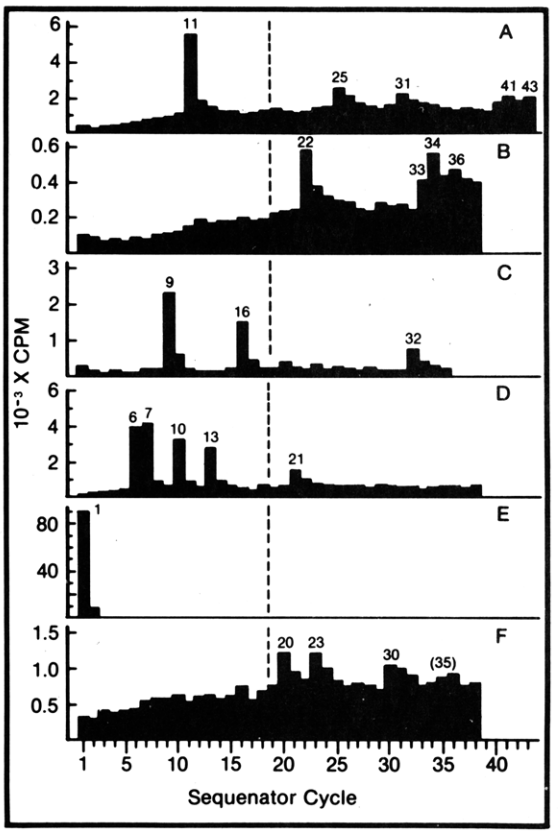

FIGURE 2: Partial amino-terminal sequence analysis of prefetuin. Polyadenylated RNA from fetal bovine liver was used to program a cell-free rabbit reticulocyte lysate protein synthesis system containing either [^{3}H]alanine (panel A), [^{3}H]aspartic acid (panel B), [^{35}S]cysteine (panel C), [^{3}H]leucine (panel D), [^{35}S]methionine (panel E), or [^{3}H]proline (panel F). The translation products were immunoprecipitated with anti-fetuin IgG, reduced, carboxyamidomethylated, and subjected to automated Edman degradation for 18 or more cycles. The radioactivity content of the 2-anilino-5-thiazolinone derivatives released at each cycle was determined in a liquid scintillation spectrometer.

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1                5                10                15
Met- X - X - X - X -Leu-Leu- X -Cys-Leu-Ala- X -Leu- X - X -

                 20                25                30
Cys- X - X - X -Pro-Leu-Asp-Pro- X -Ala- X - X - X - X -Pro-
              Ile-Pro-Leu-Asp-Pro-Val-Ala-Gly-Tyr-Lys-Glu-Pro-

                 35                40
Ala-Cys-Asp-Asp-Pro-Asp- X - X - X -Ala-Ala- X -Ala-
Ala-Cys-Asp-Asp-Pro-Asp-Thr-Glu-Gln-Ala-Ala-Leu-Ala-
```

FIGURE 3: Comparison of amino-terminal sequences of prefetuin and native fetuin. The radiosequencing results obtained for the amino terminus of prefetuin, from Figure 2 (upper line), are compared to the amino-terminal sequence of native bovine fetuin (lower line; Alcaraz et al., 1981). Amino acids are numbered from the amino terminus of prefetuin. The N-terminal amino acid of native fetuin aligns with residue 19 of prefetuin.

proline or aspartic acid and is typical in that it contains methionine at position 1 and several hydrophobic residues (alanine at position 11 and leucines at positions 6, 7, 10, and 13). These data confirm the identity of the 49-kDa (SDS–PAGE) protein as the primary translation product for fetuin.

N-Glycosylation of Fetuin. In order to study the intermediates of fetuin biosynthesis that are found in the rough endoplasmic reticulum (RER), a cell-free rabbit reticulocyte lysate protein synthesis system containing [^{35}S]cysteine was programmed with rough microsomes or polysomes as sources of message. Completion of the partially synthesized fetuin nascent chains in the in vitro system labels their C-terminal portion. The translation products were immunoprecipitated

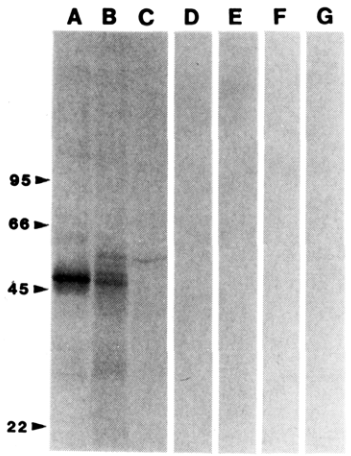

FIGURE 4: Analysis of fetuin intermediates obtained from rough endoplasmic reticulum. Polyadenylated RNA (lane A), polysomes (lanes B, D, and E), and rough microsomes (lanes C, F, and G) from fetal bovine liver were translated in a cell-free rabbit reticulocyte lysate translation system containing [^{35}S]cysteine. The translation products were precipitated either with anti-fetuin IgG (lanes A–D and F) or with nonimmune IgG (lanes E and G). To two samples (lanes E and G) native fetuin (3 mg/mL) was added to compete for IgG binding to the translation product. Immunoprecipitates were analyzed by SDS–PAGE (8–14% acrylamide) and autoradiography. The molecular weight standards are phosphorylase A (95 000), bovine serum albumin (66 000), ovalbumin (45 000), and soybean trypsin inhibitor (22 000).

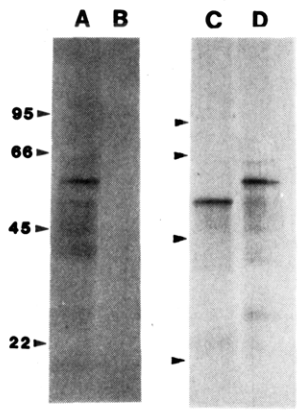

FIGURE 5: Characterization of fetuin processing intermediates. A cell-free rabbit reticulocyte lysate protein synthesis system containing [^{35}S]cysteine was programmed with fetal bovine rough microsomes. The translation products were immunoprecipitated with anti-fetuin IgG, solubilized with 2% SDS at 100 °C, and diluted. Immunoprecipitated translation products were incubated with concanavalin A–Sepharose in the absence (lane A) and presence (lane B) of 5% methyl α-D-mannoside. The Con A–Sepharose was washed, and the bound material was eluted and analyzed by SDS–PAGE and autoradiography. Additionally, immunoprecipitated translation products were incubated 16 h with (lane C) or without (lane D) endo H. The products were then analyzed by SDS–PAGE and autoradiography. The molecular weight standards are the same as in Figure 4.

and analyzed by SDS gel electrophoresis and autoradiography (Figure 4). The 49-kDa product obtained from translation of polyadenylated RNA is shown in lane A. The major fetuin species obtained from a rough microsome runoff translation (lane C) migrated with an apparent molecular mass of 58 kDa (SDS–PAGE). Translation of polysomes (lane B) gave products with apparent molecular masses of 49, 52, 55, and 58 kDa (SDS–PAGE). The 49-kDa (SDS–PAGE) protein corresponds to the primary mRNA product. The 52-, 55-, and 58-kDa (SDS–PAGE) products presumably represent molecules in which the signal peptide has been removed and one, two, or three N-linked core carbohydrate side chains have been attached, respectively. None of these proteins were precipitated by nonimmune IgG (lanes E and G) or when immunoprecipitations were done in the presence of excess native fetuin (lanes D and F). Fetuin intermediates were found to represent approximately 10 and 13% of the [^{35}S]cysteine-labeled proteins obtained from runoff translation of rough microsomes and polysomes, respectively.

It was anticipated that the 58-kDa (SDS–PAGE) runoff translation product would contain N-linked carbohydrate. To test this possibility, the lectin concanavalin A, which binds α-D-mannosyl residues characteristically present in N-linked core oligosaccharides, was utilized. Rough microsome runoff translation products labeled with [^{35}S]cysteine were heated to 100 °C in 2% SDS, diluted, and incubated with Con A–agarose in the absence or presence of the competing saccharide methyl α-D-mannoside. The Con A–agarose was then washed thoroughly and eluted with 2% SDS. The total Con A bound material was immunoprecipitated and analyzed by SDS–PAGE and autoradiography. As shown in Figure 5, the 58-kDa (SDS–PAGE) protein is bound by Con A (lane A), and the presence of methyl α-D-mannoside inhibits this binding (lane B). These results indicate that the 58-kDa (SDS–PAGE) protein contains core high-mannose glycosylamine-linked carbohydrate.

To further substantiate the presence of N-linked carbohydrate cores on the 58-kDa (SDS–PAGE) polypeptide, the enzyme endo-β-*N*-acetylglucosaminidase H (endo H), which cleaves the di-N-acetylchitobiose bond of glycosylamine-linked core carbohydrate side chains, was used. Rough microsome runoff translation products labeled with [^{35}S]cysteine were immunoprecipitated, solubilized with 2% SDS, and diluted. The 58-kDa (SDS–PAGE) fetuin intermediate was then incubated overnight with endo H and analyzed by SDS–PAGE and autoradiography. As Figure 5 shows, digestion with endo H resulted in the loss of approximately 6 kDa from the 58-kDa (SDS–PAGE fetuin polypeptide (lane C vs. lane D). These data substantiate the presence of N-linked carbohydrate on the 58-kDa (SDS–PAGE) protein and argue for cotranslational N-glycosylation of fetuin. This conclusion is in agreement with the results obtained for many other secretory proteins (Hanover & Lennarz, 1981; Presper & Heath, 1983; Staneloni & Leloir, 1982) and supports use of fetuin for comparison of the temporal aspects of N- and O-glycosylation as a valid model protein.

O-Glycosylation of Fetuin. If O-glycosylation of fetuin occurs cotranslationally, then RER or polysomes isolated from fetal calf liver should contain fetuin species in which *N*-acetylgalactosamine (GalNAc) has already been incorporated in vivo. In an effort to obtain direct evidence for cotranslational O-glycosylation, fetuin peptidyl-tRNA was examined for the presence of GalNAc linked to Ser or Thr residues present in the nascent polypeptide. Peptidyl-tRNA was prepared from fetal bovine liver polysomes and analyzed for O-linked GalNAc as described under Experimental Procedures. Briefly, the purified total peptidyl-tRNA was subjected to β-elimination in the presence of NaB^3H_4. The reaction products were hydrolyzed with acid, bound to a cation-exchange resin, eluted, reacetylated, passed through a mixed-bed ion-exchange resin, and subjected to descending paper chromatography in system I. The material that comigrated with *N*-acetylgalactosaminitol was excised, eluted, and subjected to paper chromatography in system II, which separates *N*-acetylgalactosaminitol from *N*-acetylglucosaminitol (Ogata & Lloyd, 1982). Although β-elimination and analysis of fetuin resulted in substantial radioactivity comigrating with *N*-acetylgalactosaminitol in chromatography system II (Figure

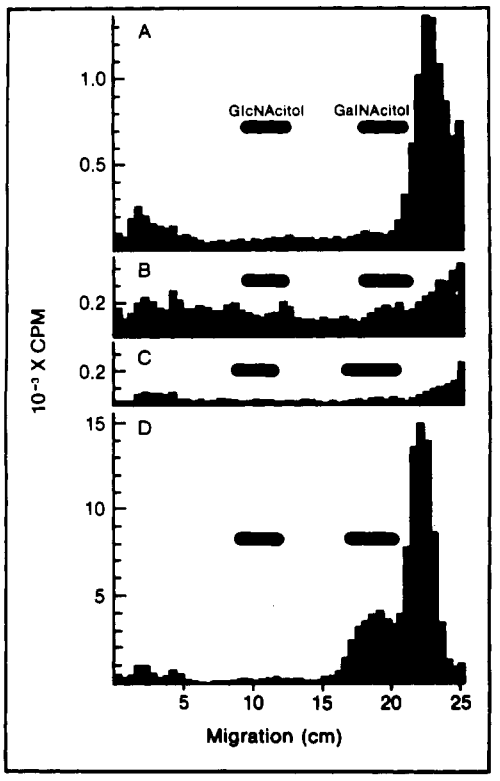

FIGURE 6: Paper chromatography of ^{3}H-labeled linking sugars derived from total peptidyl-tRNA, tRNA, and fetuin. Total peptidyl-tRNA from fetal bovine liver (0.24 A_{260} unit, panel A), rabbit liver tRNA (0.24 A_{260} unit, panel B), a distilled water blank (panel C), and fetuin (2.25 μg, panel D) were subjected to β-elimination in the presence of NaB^3H_4 (11.2 Ci/mmol). Labeled products were hydrolyzed with acid, bound to a cation-exchange resin, eluted, reacetylated, passed through a mixed-bed resin, chromatographed on paper in system I, eluted, and chromatographed on paper in system II. After detection of standards that cochromatographed with each sample, the chromatogram from system II was cut into 0.5-cm strips, and the radioactivity was determined by scintillation spectroscopy.

6, panel D), a similar peak of radioactivity was not derived from total fetal bovine liver peptidyl-tRNA (panel A). Additionally, the control analyses of tRNA (panel B) and the blank (panel C) did not yield any significant peak of radioactivity comigrating with *N*-acetylgalactosaminitol in the final chromatographic system. The control analysis of 139 pmol of O-linked GalNAc attached to native fetuin (2.25 μg) resulted in 24 000 cpm of [^{3}H]-*N*-acetylgalactosaminitol above the base-line radioactivity in chromatography system II. On the other hand, analysis of 170 pmol[2] of potential O-glycosylation sites on the fetuin peptidyl-tRNA present in 0.29 A_{260} unit of total peptidyl-tRNA did not reveal a detectable amount of labeled *N*-acetylgalactosaminitol, suggesting that fetuin peptidyl-tRNA does not contain O-linked carbohydrate.

In order to substantiate the results obtained with total peptidyl-tRNA, fetuin peptidyl-tRNA was isolated by fractionating total peptidyl-tRNA on an affinity column comprised of anti-fetuin IgG coupled to Sepharose as described under Experimental Procedures. To account for leaching of IgG (with its O-linked carbohydrate) and for nonspecific adsorption, a nonimmune control column was also examined. Fetuin peptidyl-tRNA and the control sample were subjected to β-elimination and analysis as previously described. The *N*-acetylgalactosaminitol region of the chromatogram from system II was not significantly different between the analyses for fetuin peptidyl-tRNA and the nonimmune control. These results imply that less than 1.3% of the potential O-glycosylation sites have been glycosylated in fetuin peptidyl-tRNA.

Direct evidence for cotranslational O-glycosylation of fetuin could also be obtained by demonstrating that fetuin nascent polypeptide chains present in the RER (on fetuin peptidyl-tRNA) bind to a lectin that specifically recognizes *N*-acetylgalactosamine. For this reason, rough microsomes were used to program an in vitro cell-free protein synthesizing system containing [^{35}S]cysteine. The major product of the runoff translation, a 58-kDa (SDS–PAGE) protein, was previously shown to contain N-linked carbohydrate side chains (Figure 5). This labeled, glycosylated fetuin species was examined for binding to lectins that specifically bind *N*-acetylgalactosamine.

Of the examined lectins that bind *N*-acetylgalactosamine, the B_4 lectin from *V. villosa* and soybean agglutinin (SBA) specifically bound 94 and 65% of ^{125}I-labeled asialoagalactofetuin, respectively. Other lectins examined (RCA II, DBA, and SJA) specifically bound 10% or less ^{125}I-labeled asialoagalactofetuin. Therefore, the B_4 lectin and SBA were used to examine the 58-kDa (SDS–PAGE) fetuin polypeptide derived from the RER. Total rough microsome runoff translation products (1.1×10^6 cpm of [^{35}S]cysteine-labeled protein/assay) were heat-denatured in 2% SDS, cooled, diluted, and incubated with either B_4–agarose, SBA–agarose, or Con A–agarose in the absence or presence of a competing saccharide. After incubation, the immobilized lectin was washed extensively and extracted 3 times with 2% SDS at 100 °C. The extracted material was then immunoprecipitated and analyzed by SDS–PAGE and autoradiography. Neither of the two lectins that bind GalNAc (B_4 and SBA) bound a significant amount of the applied radioactivity, less than 0.1% in both cases. Since fetuin species represent approximately 10% of the applied labeled protein, these results suggest that less than 1% of the fetuin polypeptides isolated from the RER contain terminal GalNAc. Immunoprecipitation of the trace amount of labeled proteins eluted from B_4–agarose and SBA–agarose, followed by SDS gel electrophoresis and autoradiography, did not result in the isolation of any fetuin intermediates from these samples. Con A–agarose, serving as a positive control, was found to specifically bind 40% of the radioactive protein, and 5% of these labeled proteins were specifically precipitated by anti-fetuin IgG. Therefore, the N-linked side chains of fetuin intermediates isolated from a runoff translation of rough microsomes are readily bound by concanavalin A. Under the same conditions, lectins that bind single GalNAc residues attached to the O-glycosylation sites of asialoagalactofetuin are unable to bind a detectable quantity of fetuin intermediates.

To extend this observation, RER runoff translation products were immunoprecipitated, reduced, and carboxyamidomethylated. Reduced fetuin runoff translation products that had not been carboxyamidomethylated were also analyzed. The immunoprecipitated 58-kDa (SDS–PAGE) fetuin intermediate (4000 cpm of [^{35}S]cysteine-labeled protein/assay) was subjected to binding analysis by immobilized B_4 and SBA

[2] To estimate the average molar amount of fetuin peptides present in the fetuin peptidyl-tRNA preparation, the extinction coefficient for fetuin peptidyl-tRNA at 260 nm was determined. The absorbance of a 10 μM solution of fetuin at 260 nm was determined to be 0.132 for a 1-cm light path. The extinction coefficient for fetuin peptidyl-tRNA was calculated on the basis of an average bovine cytosolic tRNA M_r of 23 800 (Sprinzl & Gauss, 1984) and an average absorbancy of 22 at 260 nm for 1 mg/mL tRNA (Holley, 1967). These values indicate that for cytosolic bovine tRNA the $E^{10\mu M}$ (260 nm) is approximately 5.2 and for fetuin peptidyl-tRNA the average $E^{10\mu M}$ (260 nm) is approximately 5.3 for a 1-cm light path.

lectins. No significant binding was observed with either lectin, less than 0.3% in all cases. Therefore, although B_4 and SBA lectins bind asialoagalactofetuin, these lectins do not bind detectable amounts of the 58-kDa fetuin species obtained from an RER runoff translation. These data indicate that less than 1% of the radioactivity in the [^{35}S]cysteine-labeled 58-kDa (SDS–PAGE) fetuin polypeptide has O-linked *N*-acetylgalactosamine.

Discussion

The de novo synthesis of fetuin, a major fetal bovine serum protein, was investigated in order to examine the temporal relationship of O-glycosylation to other temporally well-defined processing events involved in the biosynthesis of a secretory glycoprotein. The primary translation product for fetuin was identified as a 49-kDa (SDS–PAGE) polypeptide containing an 18-residue signal peptide: Met-X-X-X-X-Leu-Leu-X-Cys-Leu-Ala-X-Leu-X-X-Cys-X-X. Runoff translation of either rough microsomes or polysomes isolated from fetal bovine liver yielded a 58-kDa (SDS–PAGE) fetuin species that can be bound to concanavalin A and is susceptible to cleavage by endoglycosaminidase H (Figure 5). Since the 58-kDa (SDS–PAGE) fetuin species is produced by runoff translation of polysomes that cannot catalyze N-glycosylation and since the 58-kDa protein contains N-linked carbohydrate side chains, it is therefore concluded that N-glycosylation of fetuin occurs in the RER as a cotranslational event like numerous other secretory and membrane proteins [reviewed in Presper and Heath (1983), Hanover and Lennarz (1981), and Staneloni and Leloir (1982)].

Since fetuin biosynthesis follows the same pathway involving a signal peptide and cotranslational N-glycosylation as other secretory and membrane proteins, the biosynthesis of fetuin is a good model system for comparing the temporal and spacial sequence of events that occur in O-glycosylation to the well-characterized processes of signal peptide cleavage and N-glycosylation. Two sensitive and independent methods were used to determine the relative amount of O-glycosylation that occurs cotranslationally. Fetuin peptidyl-tRNA (or total fetal bovine peptidyl-tRNA) was analyzed by β-elimination in the presence of NaB^3H_4 followed by chromatographic analysis of the products. It was found that less than 1.3% of the O-linked side chains of fetuin have been initiated in fetuin peptidyl-tRNA. An important aspect of this observation was the utilization of the chromatography system of Ogata and Lloyd (1982), which, in addition to separating *N*-acetylglucosaminitol from *N*-acetylgalactosaminitol, was found to resolve *N*-acetylgalactosaminitol from an uncharacterized radiolabeled substance that comigrated with *N*-acetylgalactosaminitol in chromatography system I (Figure 6). Additionally, lectin-binding analysis of the 58-kDa (SDS–PAGE) fetuin species, which contains cotranslationally attached N-linked carbohydrate cores, indicated that less than 1% of the [^{35}S]cysteine label in the 58-kDa (SDS–PAGE) fetuin intermediate protein is associated with a molecule that has been O-glycosylated. This analysis was done with GalNAc-binding lectins, which were shown to specifically bind ^{125}I-labeled asialoagalactofetuin. Furthermore, the analyses of total peptidyl-tRNA and total RER runoff translation products did not detect a significant amount of any O-glycosylated molecules in fetal calf liver RER. The lack of detectable O-linked GalNAc on fetuin intermediates present in the RER can be explained in two ways: either, all three O-glycosylation sites are on the extreme C-terminus of the protein, and therefore, a negligible proportion of the in vitro RER species have available O-glycosylation sites, or the three O-glycosylation sites are not clustered on the C-terminus, but O-linked oligosaccharide biosynthesis is initiated after the nascent polypeptide enters the Golgi apparatus. Therefore, it is concluded that O-glycosylation of fetuin is most likely a posttranslational event that occurs in the Golgi apparatus.

The apparent contradiction between the posttranslational O-glycosylation of fetuin and the cotranslational O-glycosylation reported by Strous (1979) for gastric epithelial glycoproteins may be accounted for by several possible explanations. O-Glycosylation may occur cotranslationally in gastric epithelial cells but not in liver cells. Alternatively, O-glycosylation may not occur cotranslationally in gastric epithelial cells. The putative O-linked GalNAc detected by Strous (1979) in his peptidyl-tRNA preparation may be the consequence of contaminating glycoproteins, or it may be due to the same uncharacterized radiolabeled substance that comigrated with *N*-acetylgalactosaminitol in chromatography system I, which Strous also used, but was fully resolved by chromatography system II.

The conclusion that fetuin undergoes posttranslational O-glycosylation is consistent with several recent studies. Hanover et al. (1980) have shown that in oviduct there is a much higher specific activity of the UDP-GalNAc:polypeptide *N*-acetylgalactosamine transferase in smooth endoplasmic reticulum and Golgi membranes than in the rough endoplasmic reticulum. The labeling experiments of White and Speake (1980) showed that inhibition of protein synthesis immediately inhibits N-glycosylation in mammary gland explants but that O-glycosylation continues for 20 min. Cytochemical localization studies of Roth (1984) using *Helix pomatia* lectin bound to colloidal gold revealed that the *cis* Golgi and not the rough endoplasmic reticulum is the site of O-glycosylation in the intestinal goblet cell.

Studies (Hanover et al., 1980, 1982; Cummings et al., 1983) comparing the processing of N-linked side chains to the biosynthesis of O-linked side chains indicated that elongation of O-linked side chains occurs concomitantly with the processing of N-linked side chains. Cummings et al. (1983) have shown that O-glycosylation of the low-density lipoprotein receptor occurs prior to entry into the α-mannosidase I region of the Golgi, thus indicating that O-glycosylation is an event occurring in an early region of the Golgi apparatus.

A model of fetuin biosynthesis consistent with these results is that fetuin, like other hepatic secretory proteins, is initially synthesized on membrane-bound polyribosomes as a precursor protein. Signal-sequence cleavage and core N-glycosylation occur cotranslationally in the rough endoplasmic reticulum. Additional processing events necessary to produce native fetuin, including O-glycosylation, occur posttranslationally in the Golgi apparatus.

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