Glycosylation of Human Apolipoprotein E
THE CARBOHYDRATE ATTACHMENT SITE IS THREONINE 194*

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The glycosylation of human apolipoprotein (apo) E was examined with purified plasma apoE and apoE produced by transfected cell lines. The carbohydrate attachment site of plasma apoE was localized to a single tryptic peptide (residues 192–209). Sequence analysis and amino acid analysis of this peptide derived from asialo-, monosialo-, or disialo-apoE indicated that the carbohydrate moiety is attached only to Thr194 in monosialo- and disialo-apoE and that asialo-apoE is not glycosylated. Mammalian cells that normally do not express apoE were transfected with human apoE plasmid expression vectors to test the utilization of potential carbohydrate attachment sites and the role of apoE glycosylation in secretion. Site-specific mutants of apoE, designed to eliminate or alter glycosylation sites, were expressed in HeLa cells by acute transfection. Apolipoprotein E(Thr194→Ala) was secreted exclusively as the asialo isoform, confirming that Thr194 is the site of carbohydrate attachment in these cells and indicating that glycosylation of apoE is not essential for secretion. Apolipoprotein E(Thr194→Asn,Gly198→Ser), which introduces a potential site for N-glycosylation at position 194, was secreted with a higher apparent molecular weight than native, O-glycosylated apoE. Studies with tunicamycin indicated that this apoE was N-glycosylated at Asn194. Stably transfected cell lines expressing human apoE were prepared from wild-type Chinese hamster ovary (CHO) cells and from CHO ldlD cells, which are defective in glycosylation. The transfected wild-type cells secreted multiply sialylated apoE. The transfected ldlD cells also secreted high levels of apoE even in the absence of glycosylation, which confirms that glycosylation is not essential for secretion of apoE.

Apolipoprotein apoE, a protein of M , 34,000, is synthesized primarily by the liver and is an integral part of several lipoproteins. As a ligand for the low density lipoprotein (LDL) receptor, apoE directs the delivery of triglyceride and cholesterol from lipoproteins to cells and also may have a role in the efflux of cholesterol from cells (reverse cholesterol transport) (for review, see Ref. 1). The primary structure of human apoE has been determined by protein and DNA sequencing (2–4). Posttranslational modification of apoE results in variable sialylation: 80–85% of human plasma apoE is in the asialo form, and monosialo and disialo isoforms are present as minor components (5). However, newly secreted apoE is much more highly sialylated. The apoE secreted by fetal liver organ cultures (6) and human hepatoma cells lines (7) consists almost exclusively of sialylated isoforms, and apoE containing up to 6 sialic acid residues has been detected (7). The apoE secreted by mouse macrophages, human monocyte-macrophages, and transfected mammalian cell lines, as well as the circulating apoE present in cerebrospinal fluid, also appears to be sialylated more extensively than plasma apoE (8–11). This difference between plasma apoE and newly secreted apoE suggests that glycosylation may play a role in cellular processing and secretion of this apoprotein.

Because human apoE lacks the Asn-X-Thr/Ser consensus sequence necessary for N-linked glycosylation, O-linked carbohydrate probably accounts for apoE glycosylation (2). This conclusion is supported by the absence of an effect on apoE secretion by tunicamycin, a specific inhibitor of N-glycosylation (12, 13), and by the endoglycosidase H insensitivity of rat apoE, which also lacks N-linkage sites (14, 15). The purpose of this study was to establish the carbohydrate attachment site(s) for human apoE and to determine the role of glycosylation in the secretion of apoE. Our data indicate that Thr194 is the sole carbohydrate attachment site on plasma apoE, that it is the site of glycosylation on newly secreted apoE, and that secretion of apoE does not depend on glycosylation. A portion of these results has been reported in preliminary form (16).

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and G418 (sulfate salt) were obtained from Gibco. Serum-free Hana Biologicals Chinese Hamster

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1 The abbreviations used are: apo, apolipoprotein; bp, base pair; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DMEM/F12, DMEM and Ham's F-12 medium containing 2 mM glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml; FBS, fetal bovine serum; LDL, low density lipoproteins; MoMLV LTR, Moloney murine leukemia virus long terminal repeat; PMSF, phenylmethylsulfonyl fluoride; PTH, phenylthiohydantoin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SV40, simian virus 40; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
was heat-inactivated (56 °C, 30 min). Radioisotopes were obtained from Amersham Corp. (125I-PICTP, Na125I) or ICN Biomedicals (Tran35S label; Costa Mesa, CA), and Amplify, a fluorographic reagent, was purchased from Amersham Corp. All restriction enzymes, T4 ligase, and the Klenow fragment of DNA polymerase were purchased from either New England Biolabs (Beverly, MA) or Boehringer Mannheim. Additional reagents were obtained from the following sources: aprotinin and phenylmethylsulfonyl fluoride (PMSF), Calbiochem; leupeptin, Vega Biotechnologies (AZ); tunicamycin, Sigma Chemical Co. (St. Louis, MO); and [14C]glycine (ICN Biomedicals, Irvine, CA). Rabbit polyclonal antiserum to human apoE and an affinity-purified preparation Immobiline isoelectric focusing in a vertical slab gel system (18). A portion of each isoform was digested with thrombin as described (19) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The amino sugars glucosamine and galactosamine were quantitated by Beckman 121MB amino acid analyzer (Beckman Instruments) using a modification of the standard three-buffer sodium citrate system: only the second buffer (0.35 N sodium citrate, pH 9.0, 2% iminopropyl) and the 100 °C temperature setting (65 °C) were used. The resolved amino sugars eluted well before the basic amino acids but after phenylalanine (phenylalanine, 19 min; glucosamine, 25 min; galactosamine, 28 min).

Plasmid Construction—A plasmid insert containing a full-length human apoE cDNA fused to the 3' end of the human apoE gene was constructed from previously isolated plasmids. A BalI-SacI fragment containing human apoE cDNA from nucleotide -15 of the 5'-untranslated region to the codon for amino acid 119 of the mature protein was prepared from plH78 (3). In addition, a SacII-BalI fragment containing the coding sequence from amino acid 119 through 69 base pairs downstream from the 3' terminus of the gene was isolated from a human apoE genomic clone (4). The BalI site at the 3' end of the second fragment was modified by the addition of a SpH1 linker. The BalI-SacI and SacII-SphI fragments were treated with DNA ligase, and the resulting 1183-bp fragment of human apoE cDNA sequence was cloned into the vector pUC-19 that had been digested with SmaI-SphI. The sequence of the insert was confirmed by dideoxynucleotide sequencing (20). This clone was designated pHE-1 and contains 18 bp of 5'-untranslated sequence, the entire coding sequence for the E3 allele of human apoE, the 3'-untranslated sequence, and 69 bp of 3'-flanking genomic sequence.

Two expression vectors were constructed using pHE-1. A 1.4-kilobase KpnI fragment from pMT-N (a generous gift of Dr. Eli Gilboa of Princeton University, Princeton, NJ), which contains the Moloney murine leukemia virus long terminal repeat (MoMLV LTR) and approximately 1 kilobase of undetermined mouse genomic DNA (21), was cloned into pHE-1 that had been digested with KpnI. This construct, pML-E1, places the transcription of the human apoE coding sequence in pH-E1 under the control of the MoMLV LTR. To make the second expression vector, a KpnI-HindIII fragment of pH-E1 was cloned into the expression vector pCMV (kindly supplied by Dr. David Russell of the University of Texas, Dallas, TX). This expression vector contains the following sequences, in the 5' to 3' direction, in the plasmid pT718R: the human cytomegalovirus major immediate early gene promoter, bases -737 to +4 (22); a polynucleotide for the insertion of DNA sequences; the 3' end of the human apoE coding sequence, bases -125 to -65; a palindrome for polyadenylation signals; and three segments of the simian virus 40 (SV40) genome, bases 5091-190, 420-479, and 1329-1415 (24), which contain the origin of replication and viral enhancer sequences. A map of the vector with an apoE cDNA insert is shown in Fig. 1. The resulting construct, pCMV-E1, places the transcription of the human apoE coding sequence in pH-E1 under the control of the cytomegalovirus promoter and the SV40 enhancer present in pCMV. The pML-E1 and pCMV-E1 constructs were verified by restriction enzyme mapping. Plasmid DNA was prepared as described above.

Production of Human Apolipoprotein E Site-specific Mutants—The KpnI-HindIII fragment of pH-E1 was cloned into M13mp18, and the single mutant Thr321 to Pro321, or the double mutant Thr321 to Pro321 and Thr324 to Ala324, was constructed in the sequence according to the method of Zoller and Smith (26) with oligonucleotides of the antisense strand. The oligonucleotide 5'-GAGCCCAACGGCGCCCGCGC-3' encompasses amino acid residues 191-197 and was used to change Thr324 to Ala324 (27). The oligonucleotide 5'-CCAGGAGCTCA- CATTGGCGGCCC-3' corresponds to amino acid residues 191-195 and was used to introduce a double mutation Thr321 to Pro321 and Gly324 to Ser324 (28).

Oligonucleotides were synthesized on an Applied Biosystems Model 380B (Foster City, CA) and were purified by preparative polyacrylamide gel electrophoresis. The presence of each mutation was confirmed by dideoxynucleotide sequencing (3,4), whereas the double mutation was confirmed by the presence of an extra SacI site. Plasmid DNAs were purified as described above.

Cell Culture—HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. Wild-type Chinese hamster ovary (CHO) and mutant CHO ldlD cells (kindly provided by Dr. Monty Krieger of the Massachusetts Institute of Technology, Cambridge, MA) were cultured in a mixture of equal volumes of DMEM and Ham's F-12 medium containing 2 mM glutamine, 10 units of penicillin/ml, and 100 μg of streptomycin/ml (DMEM/F12) supplemented with 10% FBS. Transfections were performed by the calcium phosphate precipitation method (27), followed by a glycerol shock after 17-18 h. For stable transfections, CHO cells were transfected with 10 μg of plasmid DNA, and apoE mRNA or apoE production was analyzed 2 days later. To produce stable cell lines, CHO or ldlD cells were co-transfected with 10 μg of plasmid pML-E1 plus 0.5 μg of pSVbeta. Stable transfectants were selected by resistance to the neomycin analogue G418, which was added to the medium at 400 μg/ml, and were maintained in DMEM/F12 containing 10% FBS and 200 μg of G418/ml.

Preparation of RNA—Total cellular RNA was isolated from cultured cells by the method of Chirgwin et al. (29). Relative levels of apoE mRNA present in cells were determined by dot blot analysis and 32P-labeled probe purified from Northern blots. Total cellular RNA from HepG2 or Hep3B cells was used as a standard for dot blots and Northern blots. A 1117-bp AatII-HinfI fragment of human apoE cDNA was radioactively labeled by the random priming method (32) and was used as the probe for blot hybridization. Primer extension analysis was performed on RNA from stably transfected cell lines (33).

Gel Electrophoresis and Immunoblotting—Stably transfected cells were plated into 75-cm² flasks or 100-mm petri dishes and cultured for 2-3 days in DMEM/F12 with 10% FBS and without G418. Cell monolayers were washed with phosphate-buffered saline and then refluxed with 8 ml of serum-free medium consisting of Hana Biological Chinese hamster ovary medium supplemented with 2 mM glutamine, 10 mM pyruvate, 20 mM HEPES (pH 7.3), 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 6 μg of aprotinin/ml. Where indicated, 10 μM galactose and 100 μM N-acetylgalactosamine (GalNAc) also were added. Media and cells were collected after 5-24 h. Samples of medium received 1 mM PMSF and were clarified by centrifugation (JS-4.2 rotor, 2000 rpm for 10 min on a J-6B centrifuge, Beckman Instruments). Cell monolayers were chilled, washed with cold phosphate-buffered saline, and then scraped into 5 ml of phosphate-buffered saline (PBS). Samples were sonicated in 1 ml PMSF and pelleted (JS-4.2 rotor, 2000 rpm, 10 min). Cell pellets were solubilized into 0.5 ml of isoelectric focusing sample buffer (1% decyl sulfate, 10 mM Tris-HCl, pH 8, 2% β-mercaptoethanol, 20% sucrose).

For SDS-PAGE, aliquots of medium or of cell homogenate were mixed with Laemmli sample buffer (23), was loaded onto 10% Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 20% glycerol) and electrophoresed on 10 or 12.5% polyacrylamide gels according to the method of Laemmli (34), except...
that 2-amino-2-methyl-1,3-propanediol was substituted for Tris in the buffer system. For isoelectric focusing gels or two-dimensional gels, samples of medium were dialyzed extensively against 5 mM NH₄HCO₃, 0.1% EDTA, lyophilized, resuspended in isoelectric focusing loading buffer, and electrofocused on pH 4–6 isoelectric focusing gel (35). Second dimension SDS-PAGE was performed on 12.5% polyacrylamide gels. The loading buffer consisted of 3% SDS, 30.5 mM Tris-HCl (pH 6.8), 7 mg of diithiothreitol/ml, and 10% glycerol. Samples were transferred to nitrocellulose electrophoretically, and immunoblotting was performed as described (36) using an affinity-purified 115-kDa immunoglobulin G fraction of a rabbit polyclonal antisera to human apoE (0.5 × 10⁶ cpm/ml).

**Results**

The Carbohydrate Attachment Site in Human Plasma Apolipoprotein E—The relative migrations of the asialo-, monosialo-, and disialo-apoE isoforms and their respective thrombolytic activities were compared by SDS-PAGE. Monosialo-apoE migration was slightly retarded compared with asialo-apoE, and disialo-apoE was slightly retarded compared with monosialo-apoE (data not shown). This migration difference was maintained only in the 12-kDa thrombolytic fragments (residues 192–299) and not in either the 22-kDa fragments (residues 1–191) or the 10-kDa fragments (residues 216–299).

This result suggested that the carbohydrate moiety was contained within residues 192–215. In this sequence, there are only two possible sites for O-glycosylation, Thr^194^ and Ser^197^, both of which occur in the same tryptic peptide, residues 192–206. The sequence of this peptide is Ala-Ala-Thr-Val-Gly-Ser-Leu-Ala-Gly-Gln-Pro-Leu-Gln-Glu-Arg (2).

The tryptic peptides of asialo-, monosialo-, and disialo-apoE were isolated as described under “Experimental Procedures.” The 192–206 peptides from the three isoforms were derived from the pCMV-E1 vector, which yields high levels of potential glycosylation sites on apoE. These constructs (Fig. 1) were selected to provide high levels of apoE expression in transfected cells by placing the transcription of an apoE cDNA-gene hybrid under the control of strong heterologous viral promoters. Plasmid pML-E1 uses the MoMLV LTR, and pCMV-E1 is driven by the cytomegalovirus promoter and the SV40 enhancer. Both expression vectors code for the E3 form of apoE.

Expression of Human Apolipoprotein E Site-specific Mutants in Transiently Transfected HeLa Cells—Mammalian cells were transfected with apoE expression vectors to identify the site of glycosylation for newly synthesized apoE and to evaluate the role of glycosylation in the secretion of apoE. The constructs (Fig. 1) were selected to provide high levels of apoE expression in transfected cells by placing the transcription of an apoE cDNA-gene hybrid under the control of strong heterologous viral promoters. Plasmid pML-E1 uses the MoMLV LTR, and pCMV-E1 is driven by the cytomegalovirus promoter and the SV40 enhancer. Both expression vectors code for the E3 form of apoE.

Expression vectors encoding site-specific mutants of human apoE were transfected into HeLa cells to study the utilization of potential glycosylation sites on apoE. These constructs were derived from the pCMV-E1 vector, which yields high levels of apoE expression and thereby allows analyses of apoE isoforms in transient transfections. Transfection with pCMV-E1 produced an apoE mRNA of the appropriate size on Northern blots (data not shown). The level of mRNA for apoE was similar to that observed for Hep3B cells, a human hepatoma cell line that expresses apoE (data not shown). Production of apoE was assessed by metabolic labeling with [35S]methionine, followed by specific immunoprecipitation and gel electrophoresis. After transfection of HeLa cells with

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**Table I**

Amino sugar analysis of peptide 192–206 and of intact apoE

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Galactosamine residues/mol</th>
<th>Glucosamine residues/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asialo</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Monosialo</td>
<td>0.01</td>
<td>0.19</td>
</tr>
<tr>
<td>Disialo</td>
<td>0.85</td>
<td>0.83</td>
</tr>
<tr>
<td>ApoE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asialo</td>
<td>0.04</td>
<td>0.30</td>
</tr>
<tr>
<td>Monosialo</td>
<td>1.02</td>
<td>0.37</td>
</tr>
<tr>
<td>Disialo</td>
<td>0.86</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Values are uncorrected for hydrolytic destruction.

**Table II**

Sequence analysis of the potential glycosylation sites in peptide 192–206

<table>
<thead>
<tr>
<th>Residue</th>
<th>Asialo</th>
<th>Monosialo</th>
<th>Disialo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr^194^</td>
<td>1.08</td>
<td>&lt;0.05</td>
<td>0.22</td>
</tr>
<tr>
<td>Ser^197^</td>
<td>0.73</td>
<td>1.03</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* Yields of PTH derivatives relative to the expected yield (1.00), which was determined from sequencing of other apoE peptides containing both threonine and serine. In all cases, PTH-Thr and PTH-Ser yields were first normalized to that of a stable PTH-amino acid in the peptide sequence.
The transcription start sites in pML-E1 and translation termination sites were determined by site-specific mutagenesis. The human growth hormone (hGH) terminator contains the 3' end of the human growth hormone gene, including the canonical AATAAA polyadenylation site. CMV, cytomegalovirus; SV, simian virus.

Two additional conclusions can be derived from the expression of apoE(Thr<sup>194</sup> → Ala). First, HeLa and CHO cells apparently do not sialylate apoE at alternative O-glycosylation sites even when glycosylation at Thr<sup>194</sup> is prevented. Second, the appearance of asialo-apoE(Thr<sup>194</sup> → Ala) in the medium of transfected cells suggests that glycosylation of apoE may not be necessary for secretion.

The second site-specific mutant of apoE consisted of a double substitution: asparagine for threonine at residue 194 and serine for glycine at residue 196. This variant was constructed to generate a potential site of N-linked glycosylation at position 194 by introducing the consensus sequence of Asn-X-Thr/Ser. HeLa cells transfected with this construct secreted a protein that reacted specifically with the antiserum to human apoE but migrated on SDS-PAGE gels with an apparent molecular weight even greater than the O-glycosylated form of native apoE. A similar high molecular weight form of apoE was observed upon transfection of CHO cells with this construct (data not shown). The decreased mobility on SDS-PAGE could result from the addition of N-linked carbohydrate to the Asn<sup>194</sup> residue in the constructed consensus sequence for N-glycosylation.

To confirm that apoE(Thr<sup>194</sup> → Asn,Gly<sup>196</sup> → Ser) was N-glycosylated, the effects of tunicamycin on apoE production were examined. Tunicamycin inhibits synthesis of the dolichol intermediate that is essential for N-glycosylation (37). Various doses of tunicamycin were added during metabolic labeling of HeLa cells that had been transfected with pCMV-E1 (normal apoE) or with the construct that produces apoE(Thr<sup>194</sup> → Asn,Gly<sup>196</sup> → Ser). As expected, tunicamycin had no effect on the extent of O-glycosylation of the normal apoE (Fig. 3, left four lanes). In contrast, increasing concentrations of tunicamycin progressively inhibited the formation of the unique, slower migrating apoE(Thr<sup>194</sup> → Asn,Gly<sup>196</sup> → Ser). Concomitantly, nonglycosylated apoE(Thr<sup>194</sup> → Asn,Gly<sup>196</sup> → Ser), which migrated in the same position as the asialo form of native apoE, was produced (Fig. 3, right four lanes). These data demonstrate that the unique migration of apoE(Thr<sup>194</sup> → Asn,Gly<sup>196</sup> → Ser) in transfected HeLa cells is the result of N-linked glycosylation. Because human apoE does not contain other potential N-glycosylation sites, HeLa cells must use the N-glycosylation site generated at position 194 in apoE(Thr<sup>194</sup> → Asn,Gly<sup>196</sup> → Ser).
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FIG. 3. Effect of tunicamycin on [35S]methionine-labeled apoE and apoE(Thr194 → Asn, Gly196 → Ser) secreted by acutely transfected HeLa cells. Conditions were the same as in Fig. 2, except that the indicated concentrations of tunicamycin were present during metabolic labeling. Equivalent proportions of each sample were analyzed.

FIG. 4. Northern blot of human apoE mRNA in CHO and ldlD cells stably transfected with pML-E1. Cellular RNA preparation and Northern blot analysis were performed as described under "Experimental Procedures." Each lane was loaded with 20 μg of total cellular RNA.

Apolipoprotein E Production by Stably Transfected Chinese Hamster Ovary Cells—Stably transfected cell lines were prepared in order to produce a source of highly sialylated apoE and to confirm that glycosylation of apoE is not essential for secretion. Stable CHO and ldlD cell lines were derived by transfection with pML-E1 and pSV2neo and were selected by resistance to the neomycin analogue G418. While nontransfected CHO cells did not produce detectable apoE mRNA or protein (data not shown), apoE mRNA of the appropriate size (1400 bp) was observed on Northern blots of cellular RNA from the transfected cells (Fig. 4). Primer extension analysis of total cellular RNA confirmed the correct 5' terminus of this mRNA (data not shown). The mRNA level of the selected cell lines was one to eight times greater than that of HepG2, a human hepatoma cell line that expresses apoE. Analyses of apoE protein were performed on s83 (CHO, pML-E1) and s1033 (ldlD, pML-E1), cell lines that expressed the highest levels of apoE mRNA.

To assess apoE production by stable cell lines, cells were incubated in a defined serum-free medium, and then cell extracts and samples of medium were analyzed by SDS-PAGE and immunoblotting. An affinity-purified rabbit polyclonal antibody to human apoE reacted with a single protein in extracts of s83 cells (Fig. 5, second lane from left) that was identical in size to the asialo form of authentic plasma apoE standard. No sialylated apoE was detected in this intracellular compartment. The time course of apoE release into the medium of s83 cells is depicted in the right portion of Fig. 5. Two bands of immunoreactive apoE protein were observed, corresponding to the sialo and asialo forms of plasma apoE. Low amounts of a smaller protein were detected after incubation for 18 or 24 h and presumably are generated by partial proteolytic degradation. These results suggest that there is little intracellular accumulation of sialylated apoE and that sialylation of apoE occurs in the latest stages of the secretion process.

The production of apoE by stable cell line s83 was quantified by densitometric scans of the immunoblots (Fig. 5). The concentration of apoE in the medium increased linearly over the 24-h course of this experiment, reaching 6 μg/ml at 24 h. In several experiments, yields of apoE ranged from 20 to 50 μg/75-cm2 culture flask. Preliminary studies indicate that apoE can be prepared to about 95% purity by heparin-Sepharose chromatography of the cell medium.

The glycosylation pattern of apoE produced by the s83 cells was determined by immunoblots of two-dimensional electrophoresis gels (Fig. 6). The 22-kDa thrombolytic fragment (residues 1–191) of plasma apoE3, included as an internal standard, focused to a position between the asialo-apoE3 and asialo-apoE4 isoforms of plasma apoE. The apoE secreted by s83 cells migrated as asialo-apoE3 and as several sialylated species with both even and odd numbers of sialic acid residues, up to at least tetrasialo-apoE3. This pattern is similar to that reported for apoE produced by fetal liver organ cultures (6) but differs from that observed for HepG2 cells, which secrete primarily the isoforms with even numbers of sialic acid residues (7). These data indicate that apoE can be multiply sialylated even when expressed and secreted in a cell line that does not normally express this protein.

Apolipoprotein E Production by Transfected ldlD Cells—Transfectants of the mutant cell line ldlD were used to

FIG. 5. Immunoblot of intracellular and secreted apoE in CHO cells stably transfected with pML-E1. Confluent s83 cells were incubated with serum-free medium for various times; then aliquots of media and cell extract were subjected to SDS-PAGE and immunoblotting as described under "Experimental Procedures." Purified plasma apoE was used as the standard (far left lane).
pML-E1 and pSV2neo were prepared in parallel with the proceed. Thus, ldlD cells display normal glycosylation when reversible by supplementing the culture medium with galactose.

Experimental Procedures. The 22-kDa thrombolytic fragment of human plasma apoE3 (22-kDa marker) was added as an internal standard. The positions of the asialo- (E3) and sialylated (E3s) apoE produced by the s83 cells are indicated. IEF, isoelectric focusing.

confirm that glycosylation is not required during apoE secretion. These cells were derived from wild-type CHO cells by Krieger et al. (38) and are defective in glycosylation of proteins. In vitro assays demonstrated that ldlD cells lack the 4-epimerase activity necessary for the synthesis of UDP-Gal and UDP-GalNac when glucose is the only sugar source (39). Because UDP-GalNac is essential for carbohydrate attachment of O-linked sugars to the hydroxyl amino acid, its absence prevents O-linked glycosylation. This defect is reversible by supplementing the culture medium with galactose and GalNac, which enter the cell and allow glycosylation to proceed. Thus, ldlD cells display normal glycosylation when cultured in the presence of these sugars (39).

Cell lines of ldlD that had been stably transfected with pML-E1 and pSV2neo were prepared in parallel with the stably transfected wild-type CHO cell lines. The expression of apoE mRNA by the highest expressing transfected ldlD cell line, s1033, was slightly less than that for the transfected CHO cell line s83 (Fig. 4). The production of apoE in s1033 cells was analyzed by immunoblotting of SDS-PAGE gels (Fig. 7). In the presence or absence of added sugars, intracellular apoE was detected as a single band, identical in size to the asialo form of plasma apoE (Fig. 7, second and third lanes from left). In the presence of galactose and GalNac, the s1033 cells secreted sialylated apoE isoforms (Fig. 7, fourth lane from left). Isoelectric focusing gels indicated that s1033 cells secreted multiply sialylated apoE with a glycosylation pattern similar to that produced in s83 cells derived from wild-type CHO cells (data not shown). When glycosylation was prevented by excluding exogenous galactose and GalNac from the medium, s1033 cells continued to produce and secrete apoE (Fig. 7, right lane). The apoE produced in the absence of sugars was exclusively asialo-apoE3, a finding confirmed by isoelectric focusing (data not shown). The absence of the more negatively charged isoforms of apoE when glycosylation was prevented indicates that these isoforms do not result from other types of protein modification. Finally, the total amount of apoE produced was not diminished by the lack of glycosylation. These results confirm that glycosylation is not a prerequisite for apoE secretion by CHO cells.

DISCUSSION

Human apoE has two discrete structural domains (40, 41), and these are represented in its predicted secondary structure (Fig. 8). Protease sensitivity and guanidine denaturation curves have shown that the amino-terminal domain (residues 20–165) and the carboxyl-terminal domain (residues 225–299) have different properties (40, 41). The amino-terminal domain is represented by the 22-kDa thrombolytic fragment of human apoE (residues 1–191). It is globular and monomeric in solution, can associate with lipid preparations, and has full receptor binding activity when incorporated into phospholipid discs. The carboxyl-terminal domain is represented by the 10-kDa thrombolytic fragment (residues 216–299) and is predicted to have a high content of amphipathic helical structure. It is tetrameric and is probably responsible for the tetrameric self-association of intact apoE. The carboxyl-terminal domain is probably the major lipid binding region and may be neces-

FIG. 8. The predicted secondary structure of human apoE. The carbohydrate (CHO) attachment site at Thr359 is within the hinge region of the predicted secondary structure of apoE (modified with permission from The Journal of Biological Chemistry (40)).
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We have identified Thr\(^{214}\) as the sole carbohydrate attachment site in plasma apoE and the site of de novo glycosylation for apoE produced by HeLa and CHO cells. Threonine 194 falls within the predicted random coil or “hinge” region between the two domains of apoE. Thus, it may be readily exposed for glycosylation. The accessibility of this region also is suggested by the observation that N-glycosylation occurred when Thr\(^{214}\) was converted to a potential site for N-glycosylation by site-directed mutagenesis.

The specificity of glycosylation of Thr\(^{214}\) is also apparent. As determined by analysis of plasma apoE and by the expression of apoE(Thr\(^{214}\) → Ala) in cultured cells, there is no indication of O-glycosylation at Ser\(^{217}\), located only 3 residues from the actual site of glycosylation. Disialo-apoE contains only a single GalNAC residue, suggesting a single attachment site. The presence of a GlcNAC residue implies that disialo-apoE has a branched carbohydrate structure, whereas the absence of significant amounts of GlcNAC from monosialo-apoE indicates that the carbohydrate structure of monosialo apoE is simpler than that of disialo-apoE. The monosialo isoform apparently lacks not only a sialic acid residue in comparison with the disialo isoform, but also any neutral sugar residue(s) of the branch at and beyond the GlcNAC.

The observed relationship between the carbohydrate structures of the plasma apoE isoforms (asialo, monosialo, disialo, branched; asialo, branched) cannot be generated simply by removal of sialic acid residues. Two explanations are possible: either a large proportion (80%) of the circulating plasma apoE is actually secreted without being glycosylated, or an unusual processing system acts on the carbohydrate of circulating apoE to generate discrete intermediate monosialo and asialo products that lack both sialic acid and neutral sugar residues.

The first suggestion is contradicted by the data of Zannis et al. (6, 7). The liver is the major site of apoE synthesis, accounting for roughly 80% of total apoE production (30, 42), and newly secreted hepatic apoE from fetal liver organ cultures (6) or from the human hepatoma cell line HepG2 (7) is almost exclusively sialylated. However, the apoE secreted by the adult liver in situ could be glycosylated differently than that produced by fetal liver or HepG2 cells. The more complex processing scheme required for the second possibility has not been demonstrated. It would presumably involve highly specific glycosidases to which circulating apoE has access. However, Ghiselli et al. (43) showed that there is little conversion of disialo-apoE to monosialo- or asialo-apoE during in vivo turnover studies in humans. The observation that apoE is often more highly glycosylated in type I hyperlipoproteinemic patients who are deficient in lipoprotein lipase (43) could indicate that carbohydrate processing is concurrent with or secondary to lipase action. The alterations of very low density lipoproteins during lipolytic processing could expose this site more readily to carbohydrate processing.

The relationship between the newly secreted, multiply sialylated (having greater than 3 sialic acids) apoE and the plasma forms (asialo, monosialo, and disialo) is not clear. The hypothesis that the plasma isoforms are generated from highly glycosylated forms has not been proven, and the data of Ghiselli et al. (43) do not address the possible conversion from these more highly sialylated forms. The lack of multiple sialylation on plasma apoE could be reconciled with the lack of conversion of disialo-apoE to asialo- and monosialo-apoE if newly secreted apoE contained sialylation at multiple sites, with Thr\(^{214}\) as a minor site of glycosylation but the site most resistant to removal of carbohydrate. The use of the Thr\(^{214}\) → Ala mutant of apoE in our experiments suggests that this is not the case since Thr\(^{214}\) is the major site of de novo glycosylation of apoE produced by HeLa and CHO cells. However, the possibility that glycosylation at Thr\(^{214}\) is a prerequisite for glycosylation at other sites cannot be precluded by the use of the Thr\(^{214}\) → Ala mutant. The detection of multiple glycosylation sites on normal apoE produced by these transfected cells would require isolation of a multisialylated species (e.g., tetrasialo-apoE) and determination of its glycopeptide(s) directly. If this apoE is glycosylated at only a single site, as our data suggest, then the multisialylated structures would be unusual, since O-linked sugars rarely contain more than 2 sialic acids.

Stable transformants of CHO and ldlD cells have been developed that express human apoE under the control of the MoMLV LTR and allow examination of de novo glycosylation of apoE. The apoE produced by stable CHO lines is glycosylated similarly to the apoE produced by fetal liver cultures (6) or Hep3B cells (7) in that both odd and even numbers of sialic acid residues are observed in progressively smaller amounts of each isoform, up to tetrasialo-apoE. Stable ldlD cell lines produce similarly glycosylated apoE when cultured with exogenous sugars. This contrasts with HepG2 cells, in which even numbers of sialic acid residues predominate (7). However, isoforms with odd numbers of sialic acid residues also have been observed in apoE from other sources, including cerebrospinal fluid and cultured macrophages (8–11).

The lack of sialylation of intracellular apoE is consistent with O-linked glycosylation occurring in the latest stages of secretion within the Golgi apparatus and suggests that the transfected CHO cells do not maintain an intracellular pool of apoE in secretory vesicles. Similar observations were made for human chorionic gonadotropin (44–46); intracellular accumulation of O-glycosylated proteins is often absent, suggesting that O-glycosylation is a late posttranslational event, occurring immediately before secretion. The addition of complex oligosaccharides and subsequent secretion may occur rapidly after a slower rate-limiting step in the processing pathway (44). Conversely, N-glycosylation often occurs in the endoplasmic reticulum co-translationally. For human chorionic gonadotropin in cultured cells and in transfected CHO or ldlD cells, the intracellular form acquired N-linked high-mannose sugars but no complex sialic acid-containing or O-linked carbohydrate, and the mature (secreted) forms did not accumulate intracellularly (44–46). In contrast, sialylated apoE accumulates intracellularly in HepG2 hepatoma cells, although the extent of sialylation is less than for secreted apoE (12). This difference from apoE expression in CHO cells may reflect processing of apoE for lipoprotein assembly in HepG2 cells that may increase the accumulation of sialylated apoE within compartments of the secretory pathway.

The function of apoE glycosylation remains unresolved. The possibility that glycosylation of apoE is essential for secretion has been disproven by our study and that of Zanni et al. (47). Both studies have found that apoE secretion is unaffected in glycosylation-defective ldlD cells. Furthermore, the Thr\(^{214}\) → Ala substitution prevents O-linked glycosylation at residue 194 but allows secretion of asialo-apoE, a finding consistent with other studies on secreted proteins that have shown that glycosylation may not be essential for secretion. For example, tunicamycin did not affect secretion of LDL (an apoB-containing lipoprotein) or transferrin from cultured hepatocytes (48), and the absence of O-glycosylation in ldlD cells did not affect subunit assembly and secretion of human chorionic gonadotropin (46) or the secretion of vesicular stomatitis virus (39).

Most studies on the interaction between glycosylation and
function have examined various cell surface receptors. A direct role for O-glycosylation in maintaining protein stability has been demonstrated by studies of LDL receptor expression in IdlD cells (49). Although constructs of the LDL receptor with the glycosylation region removed can display receptor function in transfected cell lines (50), full function of the LDL receptor is not achieved in IdlD cells even if only O-glycosylation is blocked (38). The O-linked sugar-deficient form of the LDL receptor is transported to the cell surface but is unstable because of proteolytic cleavage of the receptor in the region of clustered O-glycosylation sites (49). In human apoE, the hinge region is the most sensitive to proteolytic enzymes (40). The location of the carbohydrate attachment site in this region may protect apoE from proteolysis.

Apolipoprotein E has two characterized functions: association with lipoproteins and binding to lipoprotein receptors. To date, there is no direct evidence that glycosylation of apoE modulates either of these functions. It has been suggested that glycosylation of apoE prevents re-uptake and catabolism of newly secreted apoE lipoproteins (12, 51). However, there is no apparent difference in the receptor binding activity of disialo- and asialo-apoE as measured by competition of apoE-phospholipid complexes for LDL binding to human fibroblasts (16) or by apoE turnover rates in humans (43). It is possible that more sialylation of apoE or glycosylation at additional sites in apoE, or both, could affect receptor binding. Another possible function for apoE glycosylation is that it may target apoE to specific lipoprotein classes. Thus, glycosylation may be different for apoE incorporated into very low density lipoproteins than for other lipoprotein-apoE synthesized extraparenchymally.

We have demonstrated that human apoE is glycosylated at Thr\(^\text{64}\) and that this glycosylation is not essential for secretion. However, we do not yet know the mechanism of apoE processing that results in the different extent of sialylation found in nascent versus circulating apoE nor whether differences in apoE glycosylation affect apoE function. A resolution of these questions is now possible with the availability of the apoE-producing stable transformants described in this study.

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