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EXPRESSION AND STRUCTURE-FUNCTIONAL STUDIES OF HUMAN APOLIPOPROTEIN CIII

A thesis submitted in accordance with the regulations of the University of London for the degree of Doctor of Philosophy
October 2000

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ABSTRACT

Apolipoprotein (Apo) CIII plays a key role in triglyceride (TG)-rich lipoprotein metabolism and is a risk factor for coronary heart disease (CHD). The study involved in this thesis is the first in vitro structure-functional study using recombinant apoCIII proteins.

The physicochemical properties of recombinant wild type and A23T, a naturally occurring mutation that is associated with apoCIII deficiency and lower plasma TG levels, as well as three site-directed mutants of apoCIII, designed by molecular modelling and implicated in lipid binding (L9T/T20L, F64A/W65A) or lipoprotein lipase (LPL) inhibition (K21A), were compared. Relative lipid binding efficiencies of each apoCIII variants to 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) were: L9T/T20L>WT>K21A>A23T>F64A/W65A with an inverse correlation with size of the discoidal complexes formed. Physicochemical analysis (Trp fluorescence, circular dichroism (CD) and GdnHCl denaturation) suggested that the stability of the resulting apoCIII:DMPC complexes were dependent on their lipid binding properties. The displacement of apoE by apoCIII variants were tested by gel filtration of apoE:dipalmitoylphosphatidylcholine (DPPC) discoidal complexes mixed with the various apoCIII variants. All apoCIII proteins bound to the apoE:DPPC complexes and the capacity to displace apoE from the complex was dependent on their lipid binding affinity. All the recombinant apoCIII proteins inhibited LPL in the presence or in the absence of apoCII, with F64A/W65A displaying the most inhibition, suggesting that apoCIII inhibition of LPL is independent of lipid binding and therefore due to the protein:protein interaction with apoCII and/or LPL.

Taken together, our data suggest that the hydrophobic residues F64 and W65 are crucial for the lipid binding properties of apoCIII and the redistribution of the N-terminal helix of apoCIII (L9T/T20L permutation) can enhance the lipid binding properties of the protein. Additionally, the reduced lipid binding capacity of the naturally occurring mutation A23T could lead to reduced plasma apoCIII and lower plasma TG levels in carriers.
ACKNOWLEDGEMENTS

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To Xiaohai, DouDou, Dad and Mum
Chapter 1 Introduction

1.1 Lipoprotein Metabolism 21
  1.1.1 Lipoproteins and Apolipoproteins 21
  1.1.2 Apolipoprotein Family 24
    1.1.2.1 Amphipathic Helices: the Key Structure of the Apolipoproteins 24
    1.1.2.2 Structural Features of the Apolipoproteins 26
  1.1.3 Lipoprotein Metabolism 27
    1.1.3.1 Exogenous Pathway 27
    1.1.3.2 Endogenous Pathway 28
    1.1.3.3 Reverse Cholesterol Transport 28
  1.1.4 Plasma Lipid Levels and Atherosclerosis 30

1.2 Apolipoprotein (Apo)CIII 32
  1.2.1 ApoCIII: Gene 32
  1.2.2 ApoCIII: Protein 33

1.3 In Vivo Functions of ApoCIII 35
  1.3.1 Clinical Evidence: Can ApoCIII Be a New Tool for Assessing Cardiovascular Risk? 35
1.3.2 Transgenic Animal Studies

1.4 In Vitro Functions of ApoCIII

1.4.1 Inhibition of Lipoprotein Lipolysis by ApoCIII

1.4.1.1 Lipoprotein Lipase (LPL)

1.4.1.2 ApoCII

1.4.1.3 In Vitro and In Vivo Inhibition of LPL Activity by ApoCIII

1.4.1.4 Hepatic Lipase (HL)

1.4.2 Inhibition of Cellular Uptake of Lipid Particles by ApoCIII in Vitro

1.4.2.1 ApoE: the Key Ligand of the Cell Surface Receptors

1.4.2.2 Low density lipoprotein receptor (LDLR)

1.4.2.3 LDL-receptor related protein (LRP)

1.4.2.4 Inhibition of Receptor Mediated Uptake of Lipid Particles by ApoCIII

1.5 Regulation of ApoCIII Expression

1.5.1 Inhibition of ApoCIII Expression through PPARs

1.5.1.1 Peroxisome Proliferator-activated Receptors (PPARs)

1.5.1.2 Inhibition of ApoCIII Expression by Hypolipidaemic Drugs

1.5.2 Inhibition of ApoCIII Expression by Insulin

1.6 Molecular Variants in the Human APOC3 Gene

1.6.1 RFLPs of the APOA1-C3-A4 Gene Cluster and Their Association

1.6.2 APOC3 Deficiency

1.6.3 Rare ApoCIII Mutations

1.6.3.1 Previously Identified ApoCIII Mutations
1.6.3.2 Background to This Study: Identification of the Novel ApoCIII Variant: Ala23Thr

1.6.4 Structure-Functional Relationships of ApoCIII

1.7 Aims of the Thesis

Chapter 2  Materials and Methods

2.1 Materials

2.2 Methods

2.2.1 Plasmid DNA Preparation

2.2.2 Cloning of DNA

2.2.2.1 Restriction Enzyme Digestion

2.2.2.2 DNA Clean up from Agarose Gels

2.2.2.3 Ligation

2.2.2.4 Transformation

2.2.3 PCR

2.2.4 Mutagenesis

2.2.5 Sequencing by Fluorescence Dye Terminator Technology on ABI Sequencer

2.2.5.1 Sequencing Reaction

2.2.5.2 Purification of Sequencing Products

2.2.5.3 Preparation of Sequencing Gel and Loading of Samples

2.2.5.4 Sequencing Analysis

2.2.6 Protein Analysis

2.2.6.1 Protein Analysis by SDS-PAGE
2.2.6.2 Coomassie Blue Staining 65
2.2.6.3 Silver Staining 65
2.2.6.4 Western-blotting 66
2.2.6.5 Detection of Free Protein Concentration 67
2.2.6.6 Enzyme-linked Immunosorbent Assay (ELISA) 67

2.2.7 Expression in Insect Cell Lines 68
2.2.7.1 Cell Culture and Infection with Baculovirus 68
2.2.7.2 Cationic Liposome Mediated Transfection of Insect Cells 68
2.2.7.3 Plaque Purification of Recombinant Virus and the Determination of the Virus Titer 69
2.2.7.4 Amplification of the Virus from the Plaque 69
2.2.7.5 Extraction of Viral DNA 70
2.2.7.6 Time Course of ApoCIII Expression 70
2.2.7.7 Large Scale Expression of Recombinant ApoCIII Proteins in Insect Cells 71
2.2.7.8 Isolation of 6xHis-apoCIII Fusion Proteins from Insect Cells (A) Native Condition 71
(B) Denaturing Conditions: 72

2.2.8 Expression and Isolation of Recombinant ApoCIII Protein from E. coli 73
2.2.8.1 Detection of Small Scale Expression of Recombinant ApoCIII Protein 73
2.2.8.2 Large Scale Expression of Recombinant ApoCIII Protein 74
2.2.8.3 Isolation of Recombinant GST-ApoCIII Fusion Protein from the E. Coli Cells 74
2.2.8.4 Isolation of Recombinant ApoCIII-6xHis Fusion Protein 75
2.2.8.5 Purification of ApoCIII-6xHis Fusion Protein using FPLC 75
2.2.8.6 Cleavage of GST from the GST-ApoCIII Fusion Proteins Using Proteases 75

2.2.9 Lipid Binding Properties 76

2.2.9.1 Prepare the DMPC MLV 76

2.2.9.2 Lipid Binding Properties of Recombinant ApoCIII 77

2.2.9.3 Preparation of ApoCIII:DMPC Complexes 77

2.2.9.4 Complex Isolation and Characterisation 77

2.2.10 Physicochemical Characteristics of the Native and Lipid Bound ApoCIII 78

2.2.10.1 CD Measurements 78

2.2.10.2 Denaturation 78

2.2.10.3 Fluorescence Measurements 78

2.2.11 Displacement of ApoE by ApoCIII Variants from Discoidal ApoE:DPPC Complexes 79

2.2.11.1 Preparation of Discoidal ApoE:DPPC Complexes 79

2.2.11.2 Displacement of ApoE by ApoCIII Variants from Discoidal ApoE:DPPC Complexes 79

2.2.12 LPL Inhibition by ApoCIII Variants 80

2.2.12.1 Substrate Emulsion 80

2.2.12.2 LPL Assay 80

2.3 Solutions 81

2.3.1 Solutions for Purification of 6xHis-ApoCIII Protein from Insect Cells 81

2.3.1.1 Native Buffers 81

2.3.1.2 Denaturing Buffers 82

2.3.2 Solutions for Purification and Detection of ApoCIII-6xHis Protein from E. coli Cells 83

2.3.3 Solutions for Detection of Recombinant ApoCIII Protein 83
Results and Discussion

Chapter 3  Protein Modelling

3.1  Introduction 87

3.2  Methods 88
   3.2.1  Secondary Structure Prediction 88
   3.2.2  MHP Calculations - 3D Construction of the Peptide 88

3.3  Protein Modelling Results of the ApoCIII Variants 89
   3.3.1  ApoCIII-Ala23Thr (apoCIII-T23) 89
   3.3.2  ApoCIII-Leu9Thr/Thr20Leu (apoCIII-T9L20) 91
   3.3.3  ApoCIII-Lys21Ala (apoCIII-A21) 93
   3.3.4  ApoCIII- Phe64Ala/ Trp65Ala (apoCIII-A64A65) 93

Chapter 4  Expression of ApoCIII Protein in Insect Cells Using Baculoviral Vector

4.1  Introduction 95

4.2  Expression of ApoCIII Using pBlueBacIII as Transfer Vector 96
   4.2.1  Plasmid Construction of pBBIII/WT-apoCIII 98
   4.2.2  Generation of pBBIII/A23T-apoCIII Mutation 99
   4.2.3  Identification of Recombinant 10
Chapter 5  Expression of ApoCIII in *E. Coli*

5.1.1.1 Introduction

5.2  Expression and Purification of Recombinant
    GST-ApoCIII Fusion Protein Using the pGEX2T Vector
    5.2.1 Plasmid Construction  109
    5.2.2 Expression and Isolation of Recombinant GST-apoCIII  110
    5.2.3 Cleavage of GST from the GST-ApoCIII Fusion
    Proteins Using Thrombin  111
    5.2.4 Introducing the Enterokinase/Factor Xa Cleavage
    Site into the pGEX-2T /apoCIII Construct  113
    5.2.5 Cleavage of the GST-apoCIII
    Fusion Protein Using Enterokinase and Factor Xa  115

5.3  Expression and Purification of Recombinant
    ApoCIII Protein Using pET23b Vector
    5.3.1 Plasmid Construction  118
    5.3.2 Generation of ApoCIII Variants  119
    5.3.3 Optimisation of the Expression Conditions  119
Chapter 8  LPL Inhibition by ApoCIII Variants

8.1  Introduction  
8.2  LPL Inhibition by GST-apoCIII Fusion Proteins  
8.3  LPL Inhibition by ApoCIII-6xHis Variants  
8.4  Discussion and Conclusion

Chapter 9  Conclusion and Summary

ApoCIII-Thr23: Explanation for ApoCIII Deficiency and Lower Plasma TG Levels in Carriers  
Site-directed Mutations of ApoCIII

Chapter 10  Future Work

APPENDIX I  References

APPENDIX II  Papers Related to This Thesis
List of Tables

Table 1.1 Composition and Physical Characteristics of Major Lipoproteins 22
Table 1.2 Human Apolipoproteins 23
Table 1.3 Phenotype of Hyperlipoproteinaemias 32
Table 1.4 ApoE isoformes 44
Table 1.5 ApoE genotypes and their frequencies 44
Table 1.6 Plasma apoCIII and lipid levels (mg/dl) and APOA1-C3-A4 genotype in Mayan Indians 57
Table 2.1 Primers used to generate the apoCIII construct for the apoCIII expression 84
Table 2.2 Primers used to generate apoCIII variants by the in vitro site directed mutagenesis reactions 85
Table 6.1 Ratio of apoIII bound to DMPC/free protein upon the complex forms 136
Table 6.2 Amphipathicity structure of apoCIII proteins determined by CD in the presence or absence of 50% TFE 139
Table 6.3 Stability of free recombinant apoCIII proteins and the apoCIII:DMPC complexes 140
Table 6.4 Blue shift of the apoCIII variants after bound with DMPC 143
Table 7.1 ApoE/apoCIII ratio (mol/mol) (%) in the apoE:DPPC complex after displaced by apoCIII variants 153
Table 8.1 I_{so} of each apoCIII variants in the presence and absence of apoCII 161
### List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Orientation of the main chain atoms of a peptide about the axis of a $\alpha$ helix</td>
<td>24</td>
</tr>
<tr>
<td>Figure 1.1a</td>
<td>Structural organisation of the human apoAI, AII, AIV, CII, CIII and E genes</td>
<td>26a</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Lipid metabolism schematic diagram</td>
<td>29</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>ApoAI-C3-A4 Gene Cluster</td>
<td>34</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Cartoon of the domains of the LDLR</td>
<td>46</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>The PPAR$\alpha$ signalling pathways</td>
<td>50</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Secondary structure prediction of apoCIII</td>
<td>91</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>HCA plot of the apoCIII sequence</td>
<td>92</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>MHP surfaces around the 15-33 segment</td>
<td>93</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Orientation of the different apoCIII peptides (represented in CPK) at the lipid/water interface</td>
<td>94</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Procedure of Wild Type ApoCIII Expression Using pBlueBacIII as Transfer Vector in Insect Cells</td>
<td>97</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>pBlueBac III vector</td>
<td>98</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>PCR detection of apoCIII insert in isolated recombinant virus</td>
<td>100</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Expression of recombinant WT-apoCIII protein using pBlueBac III in insect cells</td>
<td>101</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>pBlueBacHis 2 vector</td>
<td>103</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Detection of recombinant 6xHis-apoCIII fusion proteins expressed in sf9 cells infected by different MOI</td>
<td>105</td>
</tr>
<tr>
<td>Figure 4.7</td>
<td>Schematic diagram of 6xHis-tag affinity purification system</td>
<td>106</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>pGEX-2T vector</td>
<td>109</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>Purification of GST-apoCIII fusion proteins</td>
<td>111</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>Cleavage of GST-apoCIII fusion protein by thrombin</td>
<td>112</td>
</tr>
</tbody>
</table>
Figure 5.4 Insertion of enterokinase and factor Xa cleavage sites in GST-apoCIII construct by PCR 114

Figure 5.5 Co-digestion of human plasma apoCIII protein and GST-WT-apoCIII fusion protein by enterokinase 116

Figure 5.6 pET-23 vector 119

Figure 5.7 Expression of recombinant wild type apoCIII-6xHis in the different media 121

Figure 5.8 Expression of recombinant wild type apoCIII fusion protein in different E. coli strains 122

Figure 5.9 Time course of apoCIII-6xHis expression 124

Figure 5.10 Induction of recombinant WT-apoCIII at different temperatures 125

Figure 5.11 Expression and purification of the recombinant WT-apoCIII fusion protein 126

Figure 6.1 Turbidity decrease of DMPC mixed with GST-apoCIII fusion proteins as a function of the temperature 132

Figure 6.2 Turbidity decrease of DMPC mixed with recombinant apoCIII-6xHis fusion proteins as a function of the temperature 134

Figure 6.3 Gel filtration of apoCIII:DMPC complexes in FPLC system 135

Figure 6.4 Regression line between size of the complexes and ratio of apoCIII bound to DMPC/Free protein 136

Figure 6.5 CD spectra of free apoCIII protein (a) and apoCIII/TFE mixtures (b) 139

Figure 6.6 Denaturation of free apoCIII (a) and the lipid bound apoCIII (b) proteins by GdnHCl 141

Figure 6.7 Fluorescence spectra of WT-apoCIII protein and lipid bound WT-apoCIII protein (WT-apoCIII:DMPC complex) 142

Figure 7.1 Displacement of apoE from apoE:DPPC complex by WT-apoCIII 150

Figure 7.2 Protein component in the apoE:DPPC complex
after displaced by WT-apoCIII

Figure 7.3  Distribution of apoCIII and apoE proteins after the displacement of apoE from the apoE:DPPC complex by apoCIII variants

Figure 8.1  Inhibition of LPL by apoCIII variants
## Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AcMNPV</td>
<td><em>autographa californica</em> nuclear polyhedrosis virus</td>
</tr>
<tr>
<td>Apo</td>
<td>apolipoprotein</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolylphosphatate</td>
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<td>CHD</td>
<td>coronary heart disease</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<td>CE</td>
<td>cholesterol ester</td>
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<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
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<td>CHD</td>
<td>coronary heart disease</td>
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<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>dNTP</td>
<td>2'-deoxy-N-5' triphosphate</td>
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<td>DPPC</td>
<td>dipalmitoylphosphatidylcholine</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ETL promoter</td>
<td>early to late promoter</td>
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<td>endoplasmic reticulum</td>
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<td>FCR</td>
<td>fractional catabolic rate</td>
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<td>fetal calf serum</td>
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<td>free fatty acid</td>
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<td>fast protein liquid chromatography</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
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<td>hepatic lipase</td>
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<td>high-performance liquid chromatography</td>
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<tr>
<td>HSPG</td>
<td>heparan sulphate proteoglycan</td>
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<td>IDL</td>
<td>intermediated density lipoprotein</td>
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<td>IEF</td>
<td>isoelectric focusing</td>
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<td>IRE</td>
<td>insulin-responsive element</td>
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<td>isopropyl β-D-thiogalactoside</td>
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<td>kilodalton</td>
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<td>LCAT</td>
<td>lecithin: cholesterol acyltransferase</td>
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<td>LDL</td>
<td>low density lipoprotein</td>
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<td>LDLR</td>
<td>low density lipoprotein receptor</td>
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<td>LMP</td>
<td>low melting point</td>
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<td>LRP</td>
<td>LDL-receptor related protein</td>
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<td>membrane-binding protein</td>
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<td>molecular hydrophobicity potential</td>
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<td>multilamellar vesicle</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
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<td>phospholipid transfer protein</td>
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<td>phenylmethylsulphonyl fluoride</td>
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<td>polh</td>
<td>polyhedrin promoter</td>
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<td>PPAR</td>
<td>speroxisome proliferator-activated receptor</td>
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<td>RAP</td>
<td>receptor-associated protein</td>
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<td>RNA</td>
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<td>SD</td>
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<td>SR-B1</td>
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<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
</tbody>
</table>

Other abbreviations are given in the context of the text.

**One and three letter codes for amino acids:**

<table>
<thead>
<tr>
<th>One letter code</th>
<th>Three letter code</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>N</td>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>C</td>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>Q</td>
<td>Gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>H</td>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>L</td>
<td>Leu</td>
<td>leucine</td>
</tr>
<tr>
<td>M</td>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>P</td>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>T</td>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>Y</td>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>R</td>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>D</td>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>E</td>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>G</td>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>I</td>
<td>Ile</td>
<td>isoleucine</td>
</tr>
<tr>
<td>K</td>
<td>Lys</td>
<td>lysine</td>
</tr>
<tr>
<td>F</td>
<td>Phe</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>S</td>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>W</td>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>V</td>
<td>Val</td>
<td>valine</td>
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</tbody>
</table>
Chapter 1
Introduction

Apolipoprotein (apo) CIII is a protein that plays an important role in lipoprotein metabolism. In order to assess the structure-functional relations of apoCIII, it is necessary for us to understand lipoprotein metabolism and the roles played by apoCIII.

1.1 Lipoprotein Metabolism

1.1.1 Lipoproteins and Apolipoproteins

Lipids, which are not soluble in aqueous plasma, are carried in the bloodstream and transported in blood between different tissues of the body as lipoproteins. Apolipoprotein, the protein component of these particles, plays a part in stabilising lipoproteins and directing their metabolism.

Lipoproteins are spherical particles mainly made up of lipids (including cholesterol, triglyceride (TG) and phospholipids) and apolipoproteins. The core of the lipoprotein consists of the non-polar lipids: TG, cholesteryl-esters (CE) and fat-soluble vitamins, they are surrounded by more polar lipid molecules: cholesterol and phospholipids, which forms a surface monolayer which includes the apolipoproteins. Lipoproteins are similar in structure but differ in size, density, lipid composition and amount and type of proteins. They are commonly divided into different classes determined on the basis of density by ultracentrifugation and each lipoprotein class is heterogeneous in composition. The density ranges and average composition of the major lipoprotein classes is given in Table 1.1.

Lipoproteins serve not only as carriers for lipids in plasma, but also play an important function in the regulation of in vivo lipid metabolism. They cannot be synthesised and secreted from liver or intestine without the corresponding structure
## Table 1.1 Composition and Physical Characteristics of Major Lipoproteins (Durrington, 1989).

<table>
<thead>
<tr>
<th>lipoprotein class (origin)</th>
<th>density range (g/ml)</th>
<th>particle diameter (nm)</th>
<th>apolipoproteins</th>
<th>percentage composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>protein</td>
</tr>
<tr>
<td>chylomicron (intestine)</td>
<td>0.92-0.95</td>
<td>100-1000</td>
<td>AI, AIV, B-48, Cl, CII, CIII, E</td>
<td>2</td>
</tr>
<tr>
<td>VLDL (liver)</td>
<td>0.95-1.006</td>
<td>30-80</td>
<td>B-100, Cl, CII, CIII, E</td>
<td>5-10</td>
</tr>
<tr>
<td>IDL (intestine/liver)</td>
<td>1.006-1.019</td>
<td>25-30</td>
<td>B-100, Cl, CII, CIII, E</td>
<td>15-20</td>
</tr>
<tr>
<td>LDL (liver)</td>
<td>1.019-1.063</td>
<td>18-25</td>
<td>B-100</td>
<td>20-25</td>
</tr>
<tr>
<td>HDL (liver)</td>
<td></td>
<td></td>
<td></td>
<td>40-55</td>
</tr>
<tr>
<td>HDL2</td>
<td>1.063-1.125</td>
<td>9-12</td>
<td>AI, AII, AIV, Cl, CII, CIII, E</td>
<td>40-55</td>
</tr>
<tr>
<td>HDL3</td>
<td>1.125-1.210</td>
<td>5-9</td>
<td>AI, AII, AIV, Cl, CII, CIII, E</td>
<td>40-55</td>
</tr>
</tbody>
</table>

22
Table 1.2 Human Apolipoproteins (Pownall, 1992; Li et al., 1988; Eisenberg, 1990; Jong et al., 1999).

<table>
<thead>
<tr>
<th>protein</th>
<th>chromosome</th>
<th>MW (kDa)</th>
<th>plasma concentration (mg/dl)</th>
<th>function</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoAI</td>
<td>11</td>
<td>28.4</td>
<td>60-160</td>
<td>activator of LCAT</td>
</tr>
<tr>
<td>apoAII</td>
<td>1</td>
<td>17.4 (dimer)</td>
<td>25-55</td>
<td>activator of LCAT</td>
</tr>
<tr>
<td>apoAIV</td>
<td>11</td>
<td>46.0</td>
<td>15-37</td>
<td>activator of LCAT</td>
</tr>
<tr>
<td>apoB100</td>
<td>2</td>
<td>512.0</td>
<td>60-160</td>
<td>ligand for the LDLR</td>
</tr>
<tr>
<td>apoB48</td>
<td>2</td>
<td>245.0</td>
<td>0-2</td>
<td>receptor ligand</td>
</tr>
<tr>
<td>apoCI</td>
<td>19</td>
<td>6.6</td>
<td>3-10</td>
<td>prevent receptor-mediated uptake, inhibitor of CETP, activator of LCAT</td>
</tr>
<tr>
<td>apoCII</td>
<td>19</td>
<td>9.0</td>
<td>1-6</td>
<td>activator of LPL, prevent receptor-mediated uptake, inhibitor of LCAT</td>
</tr>
<tr>
<td>apoCIII</td>
<td>11</td>
<td>8.8</td>
<td>4-20</td>
<td>inhibitor of LPL and HL, prevent receptor-mediated uptake, activator of CETP, inhibitor of LCAT</td>
</tr>
<tr>
<td>apoE</td>
<td>19</td>
<td>35.0</td>
<td>2.5-5</td>
<td>receptor ligand, inhibitor of LPL, activator of both LCAT and CETP</td>
</tr>
</tbody>
</table>
apolipoprotein. In addition to their structural role, apolipoproteins play a dynamic role in lipoprotein metabolism as enzyme activators and receptor ligands, and apolipoproteins themselves undergo exchange between lipoproteins. Some features of human apolipoproteins are listed in Table 1.2.

1.1.2 Apolipoprotein Family

1.1.2.1 Amphipathic Helices: the Key Structure of the Apolipoproteins

The α helix is a common secondary structural motif in proteins and polypeptide and is formed by repetitive H-bonds between backbone CO and NH groups located 4 residues apart. It normally tends to be right-handed (a result of the L amino acid residues) with a pitch of 3.6 residues per turn (varies between 3.5 and 3.7 on average) and a rise of 1.5 Å and a radial rotation of 100° between residues (Figure 1.1) (Voet D. and Voet JG., 1990).

Figure 1.1 Orientation of the main chain atoms of a peptide about the axis of a α helix.
The amphipathic α helix is a special α helix in which hydrophobic and hydrophilic residues switch about every 3 or 4 residues, therefore with opposing polar and non-polar faces oriented along the long axis of the helix. It can occur where α helixes interface with a nonpolar environment (Details will be discussed in Chapter 6). The amphipathic α helix is an often-encountered secondary structural motif in several classes of proteins, including lipid associating proteins and globular proteins, such as apolipoproteins, certain polypeptide hormones (Kaiser and Kezdy, 1983; Kaiser and Kezdy, 1984), Venoms (Bernheimer and Rudy, 1986; Argiolas and Pisano, 1984), antibiotics, human immunodeficiency virus glycoprotein and calmodulin-regulated protein kinases. Additional, amphipathic helices involved in both intra- and intermolecular protein-protein interactions have also been described in a number of proteins, including globular proteins, calmodulin-regulated protein kinases and coiled-coil-containing proteins (Segrest et al., 1992). These interactions have also been described between apolipoproteins, resulting in the self-association of apoE into tetramers (Aggerbeck et al., 1988), apoAI and apoAIY into dimer (Weinberg and Spector, 1985; Pownall, 1992). Recently, the study performed by MacPhee et al. showed that apoCII can also self associate in the solution or on the surface of lipid particles (egg yolk phosphatidylcholine EYPC). Additionally, their results revealed that the ability of apoCII fragment to self-associate on a lipid surface reflect a common property of lipid-bound amphipathic α-helices between homologous or heterologous apolipoproteins (MacPhee et al., 1999).

A model of the amphiphilic α helical structure of apolipoproteins has been proposed particularly for exchangeable apolipoproteins (Segrest et al., 1974). The essential feature of this model is that the α-helical regions of apolipoproteins contain two defined faces, a polar and a nonpolar face. The nonpolar face is associated with the fatty acyl chain of the hydrophobic region of the phospholipid monolayer that is half-buried at the surface of the phospholipid structure and surrounds the neutral lipid core. This therefore protects the hydrophobic parts from contact with water. The polar face compounding of Asp, Glu, Lys and Arg residues results in negatively charged residues at the centre, and positively charged residues at the periphery of this face. The polar face associated with the phospholipid polar head groups is oriented towards
the aqueous phase surrounding the lipoproteins. The lipid can thereby be dissolved in the aqueous environments as the lipid-apolipoprotein particles and is thus made soluble in aqueous plasma (Segrest et al., 1992).

Furthermore, the amphipathic helices of the apolipoproteins allow the rapidly reversible binding of the apolipoprotein to the lipid/water interface that enables their rapid movement between different lipoprotein particles. The mobility of the proteins at the surface of the lipoprotein particle facilitates their exchange between the lipoproteins and probably also makes possible adjustments necessary for interaction with receptors and enzymes easier.

1.1.2.2 Structural Features of the Apolipoproteins

Some apolipoproteins, such as apoB-100 and apoB-48, are not exchangeable between the lipoprotein particles and remain with one lipoprotein particle from biosynthesis to catabolism. However, most of the apolipoproteins are exchangeable between the lipoprotein particles. The structural features of the two groups of the apolipoproteins are different.

All the human exchangeable apolipoprotein genes have been cloned and sequenced. APOAI, APOA2, APOA4, APOC2, APOC3 and APOE belong to a multigene family since they have a similar gene arrangement (Li et al., 1988). In addition, several of these genes are located in clusters on the genome (Driscoll and Getz, 1986). Except for the apoAIV gene that lacks the first intron that consists of three exons and two introns, the genes of this apolipoprotein family consist of four exons and three introns. The introns differ in size but the organisation is similar. Briefly, intron 1 interrupt the 5' untranslated regions, intron 2 interrupt the 3' untranslated regions end of the sequence that codes for the signal peptide and intron 3 interrupts the sequence coding for the mature protein. Additionally most of the exons are of similar size apart from the last exon that differ in size. While exon 1 contains non-coding regions, exon 2 codes for a large part of the signal peptides, exon 3 for short amino terminus of the mature protein and exon 4 for the carboxy terminus of the proteins (Figure 1.1a).
Figure 1.1a Structural organisation of the human apoAI, AII, AIV, CII, CIII and E genes. Transcription is from left to right. The wide bars represent the exons and the thin lines represent the 5' flanking region, introns and 3' flanking region of the respective genes. The wide bars are divided into several regions: the open bars at the two ends represent the 5' and 3' untranslated regions, the hatched bars represent the signal peptide regions, and the solid bars represent the mature peptide regions of the respective genes. In apoAI and apoAII, the prosegment is represented by a narrow open bar between the signal peptide and mature peptide region. The number above the exons indicate the length (number of nucleotides) of the exons. The lengths of the exons are drawn to scale, except for the last exons in apoAI, E and AIV.
The most striking feature of the exchangeable apolipoproteins is the presence of similar internal 11 or 22 amino acid residue (tandem array of two 11 mers) repeats of functionally identical amino acids (Fitch, 1977) and most of them have the characteristics of amphiphatic α-helices. This is in contrast to apoB that does not show any similarity with these apolipoproteins and its structure is dominated by β-sheet (Schumaker et al., 1994). Based on their degree of homology and pattern of these internal repeats, Luo et al. calculated that the apolipoproteins share a common ancestral gene that occurred 680 million years ago (Luo et al., 1986).

All these soluble apolipoproteins are synthesised in the rough endoplasmic reticulum, and like all proteins that are destined for secretion, they contain hydrophobic leader sequences that are cleaved posttranslationally. The sequences are also found in many leader sequences, containing an amino terminal methionine residue, a middle region that is rich in hydrophobic amino acids and a carboxyl terminal region that contain many small neutral amino acids.

### 1.1.3 Lipoprotein Metabolism

#### 1.1.3.1 Exogenous Pathway

Dietary lipids provide for approximately 40% of the daily intake of energy (Doupont J, 1990). Together with the protein components (apoB-48, apoAI, apoAIV and apoCs) these lipids are packed in the intestinal epithelium into nascent chylomicrons (Table 1.1) (Bisgaier and Glickman, 1983). The nascent chylomicrons move through the lymph to the blood and acquire apoCs and apoE from HDL, in exchange for apoAI and apoAIV on the chylomicrons (Eisenberg, 1984). Lipoprotein lipase (LPL) on the endothelial surface of peripheral capillaries, which requires apoCII as an activator, hydrolyses the core TG in chylomicrons. The product of hydrolysis, free fatty acid (FFA) and glycerol are released and delivered to adipose tissue for storage, or to muscle and liver for their metabolic requirements. During hydrolysis, the particle diameter of the chylomicron is reduced and excess surface material (surface remnants, including of phospholipids, free cholesterol, apoAI and most of the apoCs) are released and transferred to HDL. The above transfer of the lipids is promoted by phospholipid transfer protein (PLTP) (Jiang et al., 1999; 2023).
Jauhianen and Ehnholm, 1999), and the cholesterol ester is transferred from mature HDL to the particles in exchange for TG by cholesteryl ester transfer protein (CETP). The resulting particles of chylomicron hydrolysis (chylomicron remnants) which contain apoB-48 and apoE, depleted of TG and enriched in cholesterol ester, are cleared through the LDL-receptor related protein (LRP) on hepatic parenchymal cells that bind apoE in the lipid particles with high affinity. CE, fat-soluble vitamins, some phospholipids and TG are thereby transferred to the liver (Eisenberg, 1990) (Figure 1.2).

1.1.3.2 Endogenous Pathway

Fatty acids surplus to oxidative requirements in the liver are esterified to form TG and packaged together with cholesterol, CE, phospholipids apoB-100, apoCs and apoE into nascent very low density lipoprotein (VLDL) and secreted into the blood. After taking up more apoCs from HDL, the TG in VLDL are hydrolysed by LPL, to form CE-rich intermediately density lipoprotein (IDL), but much more slowly than chylomicrons. The apoCs and the majority of phospholipids and free cholesterol are then released and transferred to HDL. The remnant particles of VLDL (IDL) are taken up by LDL receptors (LDLR) or LRP on the liver and irreversibly degraded, while the remaining IDL is further hydrolysed by hepatic lipase (HL), and following the loss of apoE to form LDL (Eisenberg and Levy, 1975). LDL is greatly enriched in cholesterol ester, and it is actually the lipoprotein that contains the majority of the plasma cholesterol. ApoB-100 is the only significant apolipoprotein present in LDL and acts as ligand for LDLR located on the liver and to a lesser extent, on extra-hepatic tissues. The cholesterol in these particles can be used for membrane and steroid hormone synthesis (Steinberg et al., 1989) (Figure 1.2).

1.1.3.3 Reverse Cholesterol Transport

HDL particles are formed in the circulation as a by-product of lipolysis of TRL, chylomicrons and VLDL. In reality, the concentration of HDL cholesterol is
Figure 1.2 Lipid metabolism schematic diagram.
dependent on the metabolism of TRL (Tall, 1990). Following hydrolysis of TRL, the surface remnant lipids are transferred to HDL by PLTP (Jiang et al., 1999; Jauhianen and Ehnholm, 1999) and PLTP may also play a role in the inter-conversion of HDL subclasses (Jauhianen et al., 1993). The nascent form of HDL is synthesised in the liver and intestine and secreted into plasma as discoidal particles containing apolipoproteins complexed with phospholipid and free cholesterol. These particles are good acceptors of cholesterol and can therefore take free cholesterol from the surface membranes of extra-hepatic cells as well as chylomicrons and VLDL, supplied by their hydrolysis by LPL. ATP-binding cassette transporter (ABC)A1, a cellular transmembrane transport protein, has been shown to regulate the apolipoprotein mediated lipid removal pathway from cells. The cholesterol is rapidly esterified by lecithin-cholesterol-acyl transferase (LCAT) into the centre of the particle and this resulted in the conversion of HDL from discs into spheres (Nichols et al., 1985). CE can be transported back to the liver by two pathways. Firstly, it can be transferred from HDL to IDL and LDL by CETP and is then taken up by hepatic receptors on the liver for disposal. Alternatively, the cholesterol in HDL can be selectively transferred to the liver without lipoprotein particle uptake and degradation through the HDL-receptor scavenger receptor B1 (SR-B1) on hver (Rigotti et al., 1997; Varban et al., 1998). These processes are called ‘reverse cholesterol transport’ by which excess cholesterol is returned from peripheral tissue to liver and therefore accounts for the protective role of HDL against cardiovascular disease (Figure 1.2).

### 1.1.4 Plasma Lipid Levels and Atherosclerosis

Atherosclerosis, the principal cause of coronary heart disease (CHD), heart attack and stroke, is responsible for 50% of all mortality in western society. Atherosclerosis develops over several years and tends to narrow the large and medium-sized arteries. Several factors are related to its progress including lipids, lipoproteins, platelets and clotting factors. Since the early stage of atherosclerosis involves retention of plasma lipoproteins, including the increased uptake of lipoproteins and the accumulation of lipids (mainly the cholesterol derived from circulation and not from local synthesis) in the vascular wall as well as the transport
of lipids from the arterial wall (Ross, 1993), much attention has been paid to lipoprotein metabolism in vascular biology. Additionally, this process is also related to age, gender, smoking, hypertension, family history and lack of exercise.

A strong positive relationship has been revealed between plasma cholesterol, specifically LDL-cholesterol, and CHD (Grundy, 1986) while low levels of HDL cholesterol are also believed to favour the appearance of CHD as this would decrease anti-atherogenic reverse cholesterol transport (Gordon et al., 1977). A ratio of total cholesterol/HDL-cholesterol is therefore considered to be a better indicator of CHD risk. In epidemiological studies the risk of CHD has been shown to markedly increase when the ratio is >4.5. In the absence of hypercholesterolaemia, hypertriglyceridaemia along with either a reduced level of HDL cholesterol or an elevated level of LDL cholesterol are associated with CHD (Reaven and Laws, 1990).

More recently, research has shown that TG levels are independent risk factors for CHD (Austin et al., 1998) and the strength of this effect lies in its ability to reflect the presence of atherogenic plasma TRL remnants, which highlight the accumulating evidence that TRL remnants are associated with the presence, severity and progression of atherosclerosis (Hodis, 1999) and have been shown to be an independent predictor of coronary events in patients with CHD (Kugiyama et al., 1999). The magnitude of postprandial hypertriglyceridaemia is therefore also a significant predictor of CHD (Davignon and Cohn, 1996) since it reflects the presence of the TRL remnant. In patients with type I hyperlipoproteinamia, very high TG levels are not at particular high risk of developing CHD but rather pancreatitis since chylomicrons or very large VLDL particles are increased in the circulation. Since these particles are unable to pass through the endothelial barrier, they are not directly atherogenic. In agreement with this, smaller VLDL and IDL are more atherogenic compared to larger VLDL and chylomicrons particles, since they have shown the increased capacity to enter the arterial wall (Kesaniemi, 1998). However, Benlian et al suggest that there is some evidence that type I patients are more likely to develop CHD (Benlian et al., 1996).

Taken together, the elevated or abnormal plasma lipid levels can increase the atherosclerotic process and the incidence of clinical vascular diseases, thus treatment of the lipid disorders can therefore reduce atherosclerosis. The World Health
Organisation system (WHO), based on the Fredrickson classification, provided a convenient means of describing the lipoprotein profile of the most commonly occurring patterns of hyperlipidaemia. The lipid elevations being determined by the composition of the elevated lipoprotein fractions (Table 1.3).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Lipoprotein abnormality</th>
<th>Major plasma lipid elevation</th>
<th>Minor plasma lipid elevation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>chylomicrons</td>
<td>TGs</td>
<td>cholesterol</td>
</tr>
<tr>
<td>IIa</td>
<td>LDL (β)</td>
<td>cholesterol</td>
<td></td>
</tr>
<tr>
<td>IIb</td>
<td>LDL (β) and VLDL (pre-β)</td>
<td>cholesterol and TGs</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>IDL (β)</td>
<td>cholesterol and TGs</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>VLDL (pre-β)</td>
<td>TGs</td>
<td>cholesterol</td>
</tr>
<tr>
<td>V</td>
<td>VLDL (pre-β) and chylomicrons</td>
<td>TGs</td>
<td>cholesterol</td>
</tr>
</tbody>
</table>

1.2 ApoCIII

1.2.1 ApoCIII: Gene

ApoCIII is the most abundant of the three C apolipoproteins (CI, CII and CIII). In humans, the APOC3 gene is linked to the APOA1 and APOA4 genes (Figure 1.3) (Karathanasis et al., 1983) and has been mapped to the long arm of chromosome 11 (11q23-qter) (Karathanasis et al., 1983) with approximately 3.1 kb in length. The APOC3 gene translates in the opposite direction to the other two and is divergently transcribed. The APOC3 gene encodes for a 99 amino acids peptide (Protter et al., 1984) and the removal of the signal sequence results in the mature 79 amino acids protein with a molecular weight (MW) of 8.8 kDa.
1.2.2 *ApoCIII: Protein*

ApoCIII protein is a glycoprotein and expressed predominantly in liver and in minor quantities by the intestine in mammalian and avian species (Haddad et al., 1986; Lenich et al., 1988). ApoCIII contains a single O-linked carbohydrate chain at Thr-74 with 1 mole each of galactose and galactosamine per mole of protein. Isoelectric focusing separates apoCIII into three isoforms that containing 0, 1, and 2 moles of sialic acid per mole of protein and designated as apoCIII-0 (no sialic acid), apoCIII-1 (one mole sialic acid) and apoCIII-2 (two moles sialic acid) (Brown et al., 1970). These three isoforms account for 14%, 59% and 27% of the total plasma apoCIII respectively (Kashyap et al., 1981). The glycosylation of apoCIII is not an absolute prerequisite for its secretion and ability to associate with plasma lipoproteins (Roghani and Zannis, 1988).

ApoCIII makes up 26% of the protein in VLDL and 2% of HDL protein (Herbert et al., 1999), but most of the circulating apoCIII is found on HDL, since total protein mass on VLDL is much lower than that on HDL. The normal plasma concentration of apoCIII is about 12 mg/dl (Nestel and Fidge, 1982). The postprandial state can change the apoCIII distribution. ApoCIII proteins are mainly associated with HDL in the fasting state while redistribute to the TRL, chylomicrons and VLDL in the fed state (Mahley et al., 1984). ApoCIII is transferred from HDL to VLDL during the early absorption phase (2 hours after meal) and then shuttles back to HDL after 6 to 8 hours (Barr et al., 1981). ApoCIII exchanges rapidly between the different lipoprotein fractions (Eisenberg, 1984) although there is a non-exchangeable apoCIII pool present on both VLDL and HDL that accounts for 30% to 60% of the total apoCIII mass in each lipoprotein fraction (Bukberg et al., 1985; Tomoci et al., 1993).

Enzymatic digestion with thrombin cleaves the protein between Arg40 and Gly41, yielding 2 fragments, 1-40 and 41-79 and the C-terminal of apoCIII carries the immunoreactivity of the apoCIII molecule (Mao et al., 1980).
Figure 1.3 The *APOA1-C3-A4* gene Region
1.3 In Vivo Functions of ApoCIII

1.3.1 Clinical Evidence: Can ApoCIII Be a New Tool for Assessing Cardiovascular Risk?

Plasma apoCIII levels are positively correlated with plasma TG and cholesterol levels (Shoulders et al., 1991; Le et al., 1988; Marz et al., 1987) and delayed postprandial clearance (Koren et al., 1996). Elevated apoCIII levels have been observed in hypertriglyceridemic individuals (Chivot et al., 1990) and in patients with CHD (Wiseman et al., 1991). These results strongly suggest its key role in TG metabolism and associations with hypertriglyceridemia and therefore a risk factor of CHD.

Moreover, the distribution of apoCIII between HDL particles and non-HDL particles is reported to be a strong predictor of coronary atherosclerosis and hyperlipoproteinaemias. The increased apoCIII levels can lead to an accumulation of apoB-rich particles and result in a shift in the apoCIII distribution between VLDL and HDL that result in decreased clearance and thus higher levels of circulating TRLs (Chivot et al., 1990). The apoCIII ratio in HDL:VLDL has been found to be negatively associated with the progression of atherosclerosis, the reduced levels of HDL-apoCIII (a reflection of increased levels of TRL and remnants) are a predominant risk factor for progression of coronary artery lesions (Aalto Setala et al., 1992). Each increase in HDL-apoCIII of 1.9 mg/dl can halve the risk of developing new lesions (Blankenhorn et al., 1990). In healthy individuals 2/3 of apoCIII is carried on HDL and 1/3 is on VLDL. Conversely, most of the apoCIII is associated with the VLDL fraction in Type IV patients, whereas in Type III patients the amount of apoCIII is balanced between VLDL, remnants and HDL (Fredenrich et al., 1997). Additionally, apoCIII/apoE ratio in different lipoprotein is another important predictor for hyperlipoproteinaemias. Compared to normolipidaemic individuals, all lipoprotein fractions in type III patients were characterized by a lower apoCIII/apoE ratio. In contrast, the apoCIII/apoE ratio in TRL of type IV patients was lower, whereas the IDL apoCIII/apoE ratio was significantly higher than that of the normolipidaemic individuals (Fredenrich et al., 1997).
Taken together, as a marker of TRL metabolism, the concentration of apoCIII in plasma and its distribution in lipoprotein fractions are a useful tool for determining the metabolism of TRL remnant fractions which are atherogenic, and therefore could be used as a predictor of CHD in hyperlipidaemic patients.

1.3.2 Transgenic Animal Studies

The major support for the role of apoCIII in the TRL metabolism has come from transgenic animal studies and these results are in agreement with the results obtained from the clinical studies.

The human APOC3 transgenic mice are hypertriglyceridemic, high levels of human apoCIII mRNA in the liver and intestine were observed in these animals, and the plasma TG levels were proportional to plasma apoCIII concentration, thus providing direct evidence for the causal involvement of apoCIII in hypertriglyceridemia (Ito et al., 1990; Aalto Setala et al., 1996; de Silva et al., 1994). The elevated TG in these animals is due to an increased number of large TG-rich VLDL in the circulation (Aalto Setala et al., 1992). The mouse apoC3 transgenic mice are also hypertriglyceridemic (Aalto Setala et al., 1996). The hypertriglyceridemia in the apoCIII overexpressing animals is likely to be a consequence of both the low fractional catabolic rate (FCR) of VLDL and the impaired clearance of TRL mediated by the cellular receptors (Aalto Setala et al., 1992). The latter is thought to be due to the increased plasma apoCIII and the decreased apoE levels observed in these animals, which results in the low apoE/CIII ratio in the lipid particles, leading to reduced apoE-mediated uptake of VLDL by the cellular receptors. ApoCIII is therefore suggested to interfere with the apoE-mediated clearance of triglyceride-rich lipoproteins through the displacement of apoE from these particles. This hypothesis is strengthened by the correction of plasma TG levels by cross breeding these mice with APOE overexpression mice (de Silva et al., 1994). However, overexpression of APOC3 can still result in hypertriglyceridemia in apoE knockout mice, which results from accumulation of the lowest density sub-fractions including chylomicrons and VLDL, as well as the significantly decreased clearance of VLDL-TG. The hypertriglyceridemia in these animals is mainly due to the decreased
lipolysis caused by higher plasma apoCIII levels (Ebara et al., 1997). ApoCIII is therefore suggested to be able to inhibit the TRL uptake alone, independent of interaction with apoE. Another explanation might be the interaction with apoB, which is in agreement with the previous results from the in vitro studies (Clavey et al., 1995; Agnani et al., 1991).

The disruption of the apoC3 gene results in the lower fasting plasma TG levels and the protection from the postprandial hypertriglyceridemia as well as an increased rate of chylomicron clearance in the apoC3 knockout mice (Maeda et al., 1994), thus confirming the involvement of apoCIII in the hydrolysis of the TRL.

Taken together these data suggests that apoCIII plays a key role in the in vivo clearance of TG through the impairment of both lipolysis and uptake of TRL.

1.4 In Vitro Functions of ApoCIII

In this section, the mechanism by which apoCIII affects TG metabolism will be presented according to both the inhibition of lipoprotein lipolysis and the uptake of lipid particles by cellular receptors.

1.4.1 Inhibition of Lipoprotein Lipolysis by ApoCIII

1.4.1.1 LPL

LPL was first discovered by Hahn in 1943 as the rate-limiting enzyme in plasma lipoprotein which plays a central role in lipoprotein metabolism and atherogenesis (Goldberg, 1996; Santamarina Fojo and Dugi, 1994).

The role of LPL is to hydrolyse TG and some phospholipids present in the circulating lipoproteins to generate mono-, di-glycerides and free fatty acids. However, the affinity of LPL for phospholipids is much lower than that of TG (Deckelbaum et al., 1992). LPL overexpression in mice appears to enhance the clearance of TRL (Liu et al., 1994), while LPL deficiency leads to severe hyperlipidemia (type 1 hyperlipoproteinaemia) and is present at a frequency of $10^{-4}$ in
the general population, the patients usually present with pancreatitis, abdominal pain and hepatosplenomegaly (Fojo, 1992).

LPL is an extracellular lipase that is synthesised by a number of tissues, primarily in adipose tissue, heart and muscle (Kirchgessner et al., 1989) thus providing these tissues with FFA as a source of energy and for storage. After secretion, LPL is transported to the endothelial cells where it is anchored to heparin sulphate proteoglycans (HSPG) (Olivecrona T. and Bengtsson-Olivecrona G., 1993), thus enabling LPL contact with the TRLs and hydrolysis of the TG in these lipid particles. ApoE, a strong heparin-binding protein in these lipid particles, allows lipoproteins to associate with cell surface HSPG and therefore can anchor the TRLs to the endothelial cells (Fielding et al., 1989; Ji et al., 1993). Moreover, LPL dissociated from the endothelial cell can still hydrolyse lipoprotein TG in the bloodstream, and in fact the LPL in solution is more effective than LPL on the surface of endothelial cells (Saxena et al., 1989).

Mature human LPL contains 448 amino acids with a molecular weight of 55 kDa (Wion et al., 1987), functions as a non-covalent dimer while the monomer proteins are inactive (Peterson et al., 1992). Its activity is greatly enhanced by the presence of its co-factor apoCII, however LPL still has some basal activity even in the absence of apoCII (Olivecrona and Bengtsson, 1983). The rate of LPL hydrolysis can increase as a function of apoCII concentration and is maximal at a molar ratio of LPL:apoCII of 1:1 (Shirai et al., 1982). However, *in vitro* studies have shown that apoCII can inhibit LPL at high concentrations (Shachter et al., 1994).

LPL has at least six binding areas for different ligands: heparan sulfate/heparin, apoCII, individual substrate molecules, lipid/water interface, fatty acids and LRP (Olivecrona and Bengtsson Olivecrona, 1985; Beisiegel et al., 1991). A consensus sequence shared by virtually all neutral lipases (G-X-S-X-G) is predicted to be part of a putative active site (Derewenda and Sharp, 1993). Binding of apoCII (Davis et al., 1992; Dichek et al., 1993) and the lipid substrate (Wion et al., 1987) has been suggested to occur in the N-terminal folding domain of LPL.

Apart from the enzymatic actions, LPL has been postulated to be a receptor ligand for lipoprotein removal, the so-called 'bridging function'. Similar to apoE, LPL
is a heparin-binding protein that can bind to cell-surface proteoglycans with high affinity, thereby facilitating internalisation through the receptor proteins (Beisiegel et al., 1994; Ji et al., 1994; Nykjaer et al., 1994). LPL was shown to act as a signal for hepatic removal of chylomicrons remnants (Felts et al., 1975) and also mediates the binding of VLDL (Chappell et al., 1992), chylomicrons (Beisiegel et al., 1991) and \( \beta \)-VLDL (Nykjaer et al., 1993) to the LRP. In addition, LPL was shown to enhance the binding of VLDL and LDL to the LDLR via HSPG (Mulder et al., 1992) and oxidised LDL to macrophages (Hendriks et al., 1996).

1.4.1.2 ApoCII

\( APOC2 \) gene is located in the gene cluster of \( APOE/C1/C2/C4 \) on chromosome 19 (19q13.2) (Smit et al., 1988) and spans a region of 3.4 kb. Genetic deficiency of \( APOC2 \) results in the high circulating levels of TG and the patients are phenotypically similar to the patients with LPL deficiency, indicating that apoCII is the specific activator of LPL (LaRosa et al., 1970; Fojo and Brewer, 1992).

ApoCII is a 79 amino acids glycoprotein with a calculated molecular weight of 9.0 kDa (Jackson et al., 1977; Myklebost et al., 1984). It is primarily expressed in liver and intestine (Myklebost et al., 1984) and the normal plasma apoCII concentration in human is approximately 4 mg/dL (Table 1.2), which is in excess of the amount necessary to achieve maximal activation of LPL (Saheki et al., 1991). Both in vitro and in vivo studies have estimated that a concentration of apoCII corresponding to about 10% of the normal level would be sufficient for maximal stimulation of LPL (Breckenridge et al., 1978). Therefore most heterozygotes for apoCII deficiency with half the normal amount of apoC2, have normal plasma TG levels (Beil et al., 1992). Early studies suggested that LPL was not able to efficiently hydrolyse TG in vitro without apoCII (Korn, 1955). However LPL was then shown to have some basal activity even in the absence of apoCII (Olivecrona and Bengtsson Olivecrona, 1985). The rate of LPL hydrolysis can increase as a function of apoCII concentration and is maximal at a molar ratio of LPL: apoCII of 1:1 (Shirai et al., 1982).
*In vitro* studies have shown that apoCII inhibits both apoE and apoB-mediated binding of TRL to the LDLR (Sehayek and Eisenberg, 1991; Clavey et al., 1995) and to the LRP (Kowal et al., 1990). However, the inhibitory capacity is less than that of apoCI (on apoE mediated binding) and apoCIII (on apoB mediated binding). ApoCII can also inhibit LCAT (Nishida et al., 1986). Transgenic mice overexpressing human APOC2 are hypertriglyceridaemic, caused by an accumulation of TG-rich VLDL particles in the circulation, and mild elevation of plasma cholesterol levels (Shachter et al., 1994).

The structure of apoCH is predicted to contain 3 helical regions between 13-22, 29-40 and 43-52, which are thought to be involved in phospholipid binding and self-association in solution or on the lipid particles (MacPhee et al., 1999). Tryptic cleavage of apoCII results in two fragments, peptide 1-50 and peptide 51-79 and the C-terminal fragment was shown to be able to activate LPL but the N-terminal fragment was not (Musliner et al., 1977). This is in agreement with the results from the studies using synthetic peptides of apoCII, which suggested that LPL interacts with the C-terminal of apoCII from 56-79, and the smallest fragment that was able to activate LPL *in vitro* was peptide 61-79 (Kinnunen et al., 1977).

1.4.1.3 *In Vitro* and *In Vivo* Inhibition of LPL Activity by ApoCIII

ApoCIII has been shown to inhibit LPL activity both *in vivo* and *in vitro* and is the most specific and efficient inhibitor of LPL activity among the apolipoproteins.

The LPL inhibition by apoCIII had been suggested to be due to its interaction with apoCII, the natural activator of LPL. This hypothesis has been supported by several studies. The apoCIII/apoCII ratio on the TRL was shown to be an important factor for their clearance from plasma (Carlson and Ballantyne, 1976; Erkelens et al., 1979). Normally this ratio is about 2:1 or 3:1 and elevation of VLDL apoCIII, or a higher apoCIII/apoCII ratio (around 5:1) has been observed in hypertriglyceridemia (Le et al., 1988; Wang et al., 1985; Carlson and Ballantyne, 1976; Matsuoka et al., 1981). *In vitro* studies indicated that apoCIII/apoCII ratio of 20 is essential for LPL inhibition (Wang et al., 1985; Jackson et al., 1986). The LPL inhibition by apoCIII was therefore thought to be due to the displacement of apoCII by apoCIII from the
enzyme and/or the lipid substrate (Smith and Pownall, 1984). However, in vitro studies showed that apoCIII acted as a direct non-competitive inhibitor of LPL and can therefore directly inhibit LPL rather than through the interaction with apoCII (Wang et al., 1985; Smith and Pownall, 1984), this would require the presence of an apoCIII binding site on LPL, although this site has not been reported yet. Further in vitro studies performed by Catapano (1987) showed that apoCIII inhibited the LPL activity induced by a fragment of apoCII lacking the lipid binding domain, suggested that apoCIII may inhibit lipolysis by inhibiting the apoCII-LPL interaction.

In the study of APOC3 overexpression in apoE knockout mouse, on the basis of in vitro binding studies using the heparin-conjugated agarose column, decreased association of VLDL with cell-surface glycosaminoglycans, and consequent decreased in vivo lipolysis on cell surface were proposed as the predominant mechanism of hypertriglyceridemia in human APOC3 transgenic mice (Ebara et al., 1997).

1.4.1.4 | Hepatic Lipase (HL)

HL belongs to the same super gene family as LPL (Kirchgessner et al., 1987; Carriere et al., 1995). It is a glycoprotein of approximately 66 kDa that is synthesised primarily by the liver, secreted and bound to the HSPG at the endothelial cell surface of hepatic sinusoids (Santamarina Fojo et al., 1998). In vitro studies have clearly demonstrated that HL catalysed the hydrolysis of TGs, diglycerides and phospholipids in native lipoproteins without the requirement of an activator (Deckelbaum et al., 1992; Coffill et al., 1997; Shirai et al., 1981). It can therefore act sequentially to LPL in remodelling of the lipid particles. Similar to LPL, HL can also enhance TRL binding to receptors (Kounnas et al., 1995).

HL plays a key role in the remodelling and catabolism of HDL. Post-heparin plasma activity of HL has consistently been found inversely related to plasma HDL-cholesterol concentration (Blades et al., 1993; Applebaum Bowden et al., 1985; Kuusi et al., 1989; Jackson et al., 1990), particularly in the HDL₂ (Applebaum Bowden et al., 1985). The increase in HL activity appears to precede the decrease in plasma HDL-cholesterol concentrations (Applebaum Bowden et al., 1987). Clinical studies
have suggested a role for HL activity in the formation, remodelling and metabolism of LDL. LDL size is inversely associated with HL activity (Zambon et al., 1993; Watson et al., 1994).

ApoCIII has a strong inhibitory effect on HL (Kinnunen and Ehnolm, 1976) and the mechanism is suggested to be the impaired apoE-mediated binding of TRL remnants to HSPG (Landis et al., 1987). Additionally, \textit{in vitro} studies suggested that apoCIII stimulates CETP activity and inhibits LCAT activity (Jong et al., 1999).

\textbf{1.4.2 Inhibition of Cellular Uptake of Lipid Particles by ApoCIII in Vitro}

TRLs are cleared from plasma predominantly in the liver by receptor- and non-receptor-mediated pathways (Cooper, 1997), and are mainly carried out by the former. The LRP is the major receptor involved in this process. The non-receptor-mediated uptake can be performed without requiring the participation of the receptors, but rather through HSPG-mediated endocytosis, where apoE, LPL and/or HL function as bridges between the remnants and the HSPG. I will only focus on the cellular receptors shown to be affected by apoCIII. In addition, apoE will be introduced in this section since it plays the key roles as a ligand in the receptor uptake and is normally located on the same lipid particles as apoCIII.

\textbf{1.4.2.1 ApoE: the Key Ligand of the Cell Surface Receptors}

ApoE is an important apolipoprotein that plays key roles in the TG metabolism and is located on several classes of plasma lipoproteins including chylomicrons, VLDL, IDL and HDL (Table 1.2) (Mahley and Innerarity, 1983). As mentioned earlier in this section, it binds with high affinity to cell surface receptors, therefore acts as a ligand for several receptors including LRP and LDLR, and thus can mediate the uptake of IDL, LDL and chylomicrons remnants through these receptors. Noticeably, apoE is necessary for the clearance of apoB-48-containing TRLs from plasma. Furthermore, apoE plays an important role in the liver for the secretion-capture process of TRL particles in the space of Disse (Ji et al., 1994) and is
also reported to enhance both LCAT (De Pauw et al., 1995) and CETP (Kinoshita et al., 1993) activity. The *in vivo* importance of apoE for cholesterol homeostasis, and thereby for the development of atherosclerosis was demonstrated in transgenic animal studies. Reduced VLDL and LDL cholesterol concentrations thought to reduce susceptibility to atherosclerosis were found in mice overexpressing human *APOE* (Shimano et al., 1995; Shimano et al., 1992), while spontaneous hypercholesterolaemia and severe atherosclerosis were found in *apoE* knockout mice (Zhang et al., 1992; Plump et al., 1992).

The human *APOE* gene is located in the gene cluster of *APOE-C1-C2* on chromosome 19 (19q13.2) and spans a region of 3.7 kb (Wei et al., 1985; Allan et al., 1995). Similar to apoCIII and other water-soluble apolipoproteins, the *APOE* gene has four exons and three introns, can also move rapidly between lipoproteins. The mature apoE is a 299 amino acids protein with a molecular weight of 34.2 kDa (Rall, Jr. et al., 1982) and is primarily synthesised in the liver and also by a number of tissues such as spleen, kidney, adrenals, gonads, brain and macrophages (Mahley, 1988). The plasma concentration of apoE in humans is in the range of 2.5-5 mg/dl (Eisenberg, 1990) (Table 1.2).

Results from the study using synthetic peptide of human apoE have shown that only multimer of apoE are biologically active (Dyer et al., 1991). Thrombin digestion of the protein produces two fragments that are consistence with its functional domains. The 22 kDa N-terminal fragment (residues 1-191) is the receptor-binding domain and is therefore responsible for the binding of apoE to LDLR (Innerarity et al., 1983), and only binds to lipids weakly (Gianturco et al., 1983). The 12 kDa C-terminal fragment (residues 192-299) is the lipid and HSPG binding domain of apoE (Gianturco et al., 1983) and does not bind to the LDLR (Innerarity et al., 1983). The X-ray crystallography studies of the N-terminal apoE fragment indicate the presence of a 4 helix bundle globular structure, that are located at residues 24-42, 54-81, 87-122 and 130-164, with the latter containing the LDLR-binding domain (Wilson et al., 1991).

Three common apoE isoforms of apoE are established according to the amino acid at residues 112 and 158 (Weisgraber et al., 1981) with different charge (Table 1.4) and result in six possible genotypes (Table 1.5). The frequencies of these apoE
variants differ between ethnic groups and the overall high frequency of the apoE3 form make it the wild type while the apoE2 allele is the most rare. These variants can be detected by isoelectric focusing (IEF) and reflect a different affinity for their binding to the cellular receptors. ApoE2 has only 1-2% of the LDLR-binding activity compared to the apoE3 (Schneider et al., 1981). In the general population, the apoE2 homozygotes exhibit lower plasma cholesterol concentration, while the apoE4 homozygotes have higher plasma cholesterol concentration than the apoE3 homozygotes (Dallongeville et al., 1992).

**Table 1.4 ApoE isoforms**

<table>
<thead>
<tr>
<th></th>
<th>residue 112</th>
<th>residue 158</th>
<th>relative charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoE2</td>
<td>Cys</td>
<td>Cys</td>
<td>0</td>
</tr>
<tr>
<td>apoE3</td>
<td>Cys</td>
<td>Arg</td>
<td>+1</td>
</tr>
<tr>
<td>apoE4</td>
<td>Arg</td>
<td>Arg</td>
<td>+2</td>
</tr>
</tbody>
</table>

**Table 1.5 ApoE genotypes and their frequencies (Davignon et al., 1988)**

<table>
<thead>
<tr>
<th>genotype (homozygous)</th>
<th>frequency (%)</th>
<th>genotype (homozygous)</th>
<th>frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4/E4</td>
<td>3</td>
<td>E4/E3</td>
<td>22</td>
</tr>
<tr>
<td>E3/E3</td>
<td>60</td>
<td>E3/E2</td>
<td>12</td>
</tr>
<tr>
<td>E2/E2</td>
<td>1</td>
<td>E4/E2</td>
<td>2</td>
</tr>
</tbody>
</table>

Apart from acting as the mediator of TRL binding to cellular receptors, apoE is also involved in the LPL mediated lipolysis. Yamada et al. (Yamada and Murase, 1980) observed an activation of LPL by apoE toward triolein emulsions. Additionally, as described in section 1.3.1.1, apoE in the TRLs can favour the
association between the lipid particles and the LPL bound on the endothelial cells and therefore stimulate the lipolysis, although it is not related to the enzymatic role of LPL. In agreement with this, Clark and Quarfordt (Clark et al., 1984) showed an enhancing effect of apoE on emulsion triolein hydrolysis by LPL that was immobilised to heparin-Sepharose. However, an increasing body of evidence points to an inhibitory role of apoE in LPL-mediated lipolysis both in vitro and in vivo, although the mechanism is still controversial. Connelly et al. (Connelly et al., 1994) have suggested that it is because apoE acts as a non-competitive regulator of lipolysis, and mediates the binding of LPL to TG-poor lipoproteins to form the enzyme complexes thus preventing LPL to complex with TRL. However, the studies reported by Saxena et al. and Rensen et al. showed that apoE inhibited LPL lipolysis by substrate dissociation from LPL rather than inactivation of substrate-associated LPL (Saxena et al., 1992; Rensen and van Berkel, 1996; Saxena et al., 1993; Saxena et al., 1995). In the in vitro system described in these studies, LPL appeared to increase LDL retention to glycosaminoglycans of the sub-endothelial matrix, while purified apoE and apoE-rich HDL were able to affect these associations by displacement of LDL from LPL. Physiologically, it can be envisioned that enrichment of lipoproteins with apoE during LPL-mediated lipolysis results in dissociation of the lipoprotein remnants from endothelium-bound LPL, leading to the accessibility of the remnants for uptake by hepatic apoE-specific receptors. The inhibition of LPL by apoE might also be concluded from the characterisation of lipoproteins in apoE-knock-out mice, since the VLDL isolated from these animals appears to contain less TGs compared to VLDL from control mice (van Ree et al., 1994).

1.4.2.2 LDLR

LDLR was first discovered by Brown and Goldstein (Brown and Goldstein, 1986) in the studies on familial hypercholesterolaemia, a genetic disease caused by defects in the LDLR. Understanding of the structure-functional relations of LDLR has provided the portrait of the other multifunctional receptors, such as LRP that will be mentioned later, since they share the similar structural features.
LDLR binds lipoproteins containing apoB and/or apoE (hence is also called the B, E receptor) (Mahley and Innerarity, 1983; Brown and Goldstein, 1986) and has a far higher affinity for lipid particles containing several molecules of apoE compared to those lipid particles containing a single molecule of apoB (Choi et al., 1991). It binds LDL, VLDL remnants (IDL), chylomicrons (Cooper, 1997) and VLDL (Chappell et al., 1993). LDLR is involved in the LDL catabolism that occurs primarily in the liver (Pittman et al., 1979; Pittman et al., 1982) and extra hepatic tissues via two routes. Firstly, LDLR limits LDL production by the removal of IDL, the precursor of LDL from the circulation. Secondly, it enhances LDL degradation by mediating cellular uptake of LDL. A deficiency of LDLR results in LDL to accumulate as a result of both over-production and of delayed removal. The contribution of the removal of chylomicrons by LDLR was studied using anti-LDLR antibodies or mice with a deficiency of the apoE gene, and suggested that LDLR accounted for the removal of half the chylomicrons (Ishibashi et al., 1996).

1. Cysteine-rich - ligand binding domain
2. EGF precursor homology
3. O-linked sugar
4. Membrane spanning region
5. Cytoplasmic domain

Figure 1.4 Cartoon of the domains of the LDLR

LDLR is a 160 kDa glycoprotein that presents in the plasma membrane of all cells (Brown and Goldstein, 1986). The LDLR gene is located on the short arm of chromosome 19 (p13.1-p13.3), spans 45 kb and comprises 18 exons and 17 introns
The LDLR mRNA is 5.3 kb long and about half of it codes for a 860 amino acids protein including a 21 amino acid signal peptide (Yamamoto et al., 1984). LDLRs are synthesised in response to a fall in cellular free cholesterol concentration. The 839 amino acids mature protein consists of five domains: the 292 amino acids ligand-binding domain contains seven imperfect cysteine rich repeats of approximately 40 amino acids each. The 400 amino acids epidermal growth factor (EGF) precursor homology domain that includes three cysteine rich repeats of 40 amino acids each that differ from the ligand binding repeats. The 58 amino acid O-linked sugar domain that is rich in serine and threonine residues, many of which are the attachment sites for carbohydrate chains. The 22 hydrophobic amino acids of the membrane-spanning domain and the 50 amino acids cytoplasmic domain (Brown and Goldstein, 1986), the latter contains a tetrameric signal sequence (NPXY) that mediates the association of the LDLR with coated pits (Chen et al., 1990).

1.4.2.3 Lipoprotein Receptor-Related Protein (LRP)

Apart from the cholesterol clearance related to the LDL catabolism, the LDLR is also involved in clearance of TRLs (Ishibashi et al., 1996; Brown et al., 1981). However, LDLR deficiency, such as the LDLR knockout mouse (Ishibashi et al., 1993) and individuals with familial hypercholesterolaemia (Rubinsztein et al., 1990) does not lead to chylomicron or VLDL remnant accumulation. This suggests an alternative route for the clearance of these lipoproteins. In this context, interest has been directed towards LRP. In addition, TRL remnants were found to accumulate in the LDLR knockout mice that overexpresses the receptor-associated protein (RAP) (a high-affinity ligand to the LRP), strongly suggesting that LRP is involved in the clearance of TRL remnants in vivo (Willnow and Herz, 1994).

LRP was first reported by Herz and colleagues (Herz et al., 1988). It is a large integral membrane glycoprotein with MW of 600 kDa and highly homologous to the LDLR. LRP has multifunctional roles (Herz J, 1993). In lipid metabolism, LRP contributed to the clearance of chylomicron remnants that occurred primarily in the liver (Kowal et al., 1989; Beisiegel et al., 1989). LRP was first identified as an apoE specific receptor, however, LPL and HL have also shown the directly interact with LRP (section 1.3.1.1 and 1.3.1.3). LRP binds all three apoE isoforms (Kowal et al.,
1989; Beisiegel et al., 1989; Mahley, 1988) while its binding capacity for apoE2 is lower than that for the other two isoforms (Weisgraber et al., 1990).

LRP is highly expressed in the liver, where most of the uptake activity is detected, as well as many other tissues including the brain (Herz et al., 1988; Wolf et al., 1992) and a wide variety of cells (Lund et al., 1989). LRP is synthesised as a single chain precursor of 4525 amino acids. It is composed of two types of cysteine-rich repeat sequences and both of them are similar to that in the LDLR. One series of repeats are designated as ligand binding repeats and known to be able to bind apoE. The other series of repeats are designated epidermal growth factor repeats and found in many other proteins and appears to play a variety of roles. LDLR contains seven ligand-binding repeats and three EGF-repeats, LRP contains five and seventeen, respectively. The cytoplasmic portion of LRP also contains two NPXY sequences.

1.4.2.4 Inhibition of Receptor Mediated Uptake of Lipid Particles by ApoCIII

In experimental liver perfusions, excess apoCIII was shown to inhibit hepatic uptake of TG-rich emulsions and TRL (Quarfordt et al., 1982; Windler et al., 1980). Consistent with these results, apoCIII was shown to effectively inhibit the apoE-mediated uptake of TG-rich emulsions by HepG2 cells and rat hepatocytes in culture (Oswald and Quarfordt, 1987). Further in vitro studies have shown that apoCIII decreases the binding of human VLDL and IDL to the LDLR expressed in human fibroblasts (Sehayek and Eisenberg, 1991), as well as LRP (Kowal et al., 1990).

The mechanism by which apoCIII inhibits lipoprotein uptake has been the subject of debate. One possibility is that apoCIII can displace apoE from the lipid particles and thus diminish their binding to receptors. Another possibility is that apoCIII simply masks or alters the conformation of apoE proteins on the lipid particle surface and interferes with their interaction with receptors (Weisgraber, 1990). The recent results from the transgenic mice studies using crossbreeding of APOC3 transgenic mice with apoE knock out mice suggest that apoCIII can inhibit the hepatic uptake independent of apoE, and maybe through the interaction with apoB on the surface of the lipid particles (Ebara et al., 1997). Due to the lack of the common
structure features between the two proteins (section 1.1.2.2), apoCIII is unlikely to displace apoB from the lipid particles. The above interaction is therefore probably due to the conformational change of apoB induced by apoCIII or the masking of the apoB functional domain by apoCIII (Clavey et al., 1995).

1.5 Regulation of ApoCIII Expression

Expression of apoCIII can be affected by dietary, hormonal, environmental and developmental factors. In this section, I will focus on the regulation of apoCIII expression at the transcriptional levels through the functions of peroxisome proliferator-activated receptors (PPARs), the members of nuclear receptor superfamily and by insulin.

1.5.1 Inhibition of ApoCIII Expression through PPARs

1.5.1.1 PPARs

PPARs are ligand-activated transcription factors belonging to the nuclear receptor subfamily, they are activated by FFAs and derivatives. Additionally, the diverse group of peroxisome proliferators is also known to activate PPARs. The name 'PPARs' derives from the fact that they are all activated by peroxisome proliferators in a transient transfection assay (Issemann and Green, 1990). Three distinct PPARs termed α, δ(β) and γ have been identified. They are encoded by separate genes on different chromosomes and shown a distinct tissue distribution pattern (Beamer et al., 1997).

Activated PPARs can heterodimerise with another nuclear receptor, the retinoid X receptor (RXR) and alter the transcription of target genes after binding to specific peroxisome proliferator-activated elements (PPREs), a direct repeat of the nuclear receptor hexameric AGGTCA DNA core recognition motif separated by one or two nucleotides (DR1 and DR2) (Schoonjans et al., 1996) (Figure 1.4). My description will focus on PPARα since the expression of apoCIII is inhibited by PPARα (Fruchart et al., 1999).
Figure 1.5 The PPARα signaling pathways. After activation, PPARα heterodimerise with RXR and alter the transcription of target genes after binding to specific PPREs.

1.5.1.2 Inhibition of ApoCIII Expression by Hypolipidaemic Drugs

Since apoCIII plays a key role in the TG metabolism, and the overexpression of human apoCIII results in hypertriglyceridaemia with a positive linear relation between apoCIII levels and TG concentration, compounds that suppress apoCIII expression can therefore be used as hypertriglyceridemic drugs. Fibrates are widely used hypolipidaemic drugs in clinical practice for over 30 years, however, the molecular mechanism by which fibrates function has only been revealed after the identification of PPARs.

The fibrates drugs are derivatives of peroxisome proliferators, which also contain fatty acid, certain herbicides and phtalate ester plasticizers. They were shown to activate the nuclear PPARα and reduce apoCIII and induce LPL gene expression.

Administration of fibrates decreased the synthesis of apoCIII mRNA to 90% in vivo in rat liver as well as in vitro in isolated human and rats hepatocytes, this change occurred rapidly and was dose- and time-dependent and reversible (Staels et al., 1995). In hypertriglyceridaemic patients treated with combined diet restriction
and one of the fibrates, fenofibrate, the plasma apoCIII level was reduced by 48% (Malmendier et al., 1989).

The regulation of APOC3 gene transcription is complex, being governed by an ensemble of transcription factor binding sites within 1kb upstream of the transcription initiation site. Among these sites is the C3P (also called CIIIB) site, to which a number of nuclear receptors such as hepatic nuclear factor (HNF-4), apoAI regulatory protein-1 (ARP-1), RXR and PPARα bind. HNF-4, RXR and PPARα can activate APOC3 gene transcription, while ARP-1 acts as repressors. Different mechanisms can be invoked to explain the transcriptional suppression of APOC3 gene expression by fibrates.

Firstly, the suppression of apoCIII by PPARα activators is due to a displacement of the strong transcriptional activator HNF-4 by the less active PPARα/RXR complex, resulting in lower apoCIII promoter activity (Auwerx et al., 1996).

Secondly, PPARα activators such as MEDICA 16 (β,β'-tetramethylhexadecanedioic acid) may decrease the expression of HNF-4 but this has not been observed with clinically used fibrates such as fenofibrate or ciprofibrate (Vu Dac et al., 1998).

Thirdly, PPARα-activators may induce the expression of repressor proteins, such as ARP-1 or Rev-erbα, fibrates induce Rev-erbα expression both in rat and human liver cells through PPARα interacting with a PPRE in the Rev-erbα gene promoter (Vu Dac et al., 1998; Gervois et al., 1999). Furthermore, Rev-erbα deficient mice exhibit increased plasma TG and apoCIII concentrations and liver apoCIII mRNA levels are elevated. Finally, activated PPARα has been shown to interfere with certain transcription factor pathways, which are all operative in liver cells and the participation of such a mechanism in the repression of apoCIII expression can not be excluded.
1.5.2 Inhibition of ApoCIII Expression by Insulin

Accumulated evidence has shown that insulin can regulate the expression of a wide range of genes, followed by the binding of insulin via a nuclear receptor to its response element on the target gene, insulin response element (IRE). Insulin was shown to transcriptionally downregulate apoCIII expression through the IRE on APOC3 gene. In diabetes, the lack of insulin may therefore lead to enhanced apoCIII expression and contribute to the hypertriglyceridemia observed in addition to the failing antilipolytic effect (Chen et al., 1994). Hypothetically, the hypertriglyceridaemic effect of the insulin-resistant metabolic syndrome could be partially dependent on over-secretion of apoCIII.

1.6 Molecular Variants in the Human APOC3 Gene

1.6.1 Restriction Fragment Length Polymorphisms (RFLPs) of the APOA1-C3-A4 Gene Cluster and Their Association

The studies of genetic variation around the APOC3 gene have identified several polymorphic restriction enzyme recognition sites (Figure 1.3). Most of these polymorphisms are caused by sequence changes outside the coding region of the APOC3 gene, and therefore do not alter the amino acid sequence of apoCIII protein. These sequence changes might therefore have some effects on the expression of the apoCIII in the plasma.

The first reported polymorphism located in the 3' untranslated region (UTR) is a C to G change at position 3238 detected by the presence of an additional SstI site and is found associated with hypertriglyceridaemia in several distinct populations. The absence of the cutting site, G3238, is associated with raised apoCIII and plasma TG concentrations and therefore increased risk of CHD (Rees et al., 1983).

Five polymorphic sites in the proximal promoter of the apoCIII gene, T-455C, C-482T, T-625C, T-630C and T-641C were identified (Naganawa et al., 1997) and showed complete or strong allelic associated with each other and with the SstI polymorphism. Variation at either the -455 or -482 sites, both within the IRE led to
the abolition of insulin responsiveness of the apoCIII promoter. The T-482 or C-455 variant promoter is active at all concentration of insulin while the wild type promoter is transcriptionally downregulated by insulin (Li et al., 1995). Bandshift assays showed that the base change at -455 alone reduced the affinity of the IRE for a specific nuclear factor. Without changing an amino acid residue, two additional common variants within the apoCIII gene were identified: a C1100T variant in exon 3, which is in the wobble position of the codon 13, and a T3206G variant in the 3'UTR of exon 4. The individuals with the T1100 allele have higher levels of plasma TGs due to the increased number of VLDL and IDL in the circulation of familiar combined hyperlipidaemia (FCH) patients. Both variants showed strong allelic association with each other and with the SstI polymorphism too (Xu et al., 1994).

The PstI RFLP is created by a sequence change approximately 300 bp 3' of the APOAI gene (Kessling et al., 1985; Ordovas et al., 1986) which abolishes a restriction enzyme site. The XmnI RFLP occurs about 4 kb upstream of the apoAI transcriptional start site (Coleman et al., 1986). The rare allele was created by the presence of a new restriction site and the allele frequency varies among different racial groups (Cole et al., 1989). Both RFLPs of PstI and XmnI have also been reported to be associated with hypertriglyceridemia (Kessling et al., 1985) or CHD (Ordovas et al., 1986).

The MspI polymorphic site was identified in the promoter region of the APOAI gene. This RFLP is created by a G to A substitution at position -75 abolishing a MspI cutting site. It was reported that XmnI polymorphism together with MspI and SstI aggravated hypercholesterolaemia and hypertriglyceridaemia in FCH probands (Jeenah et al., 1990).

Waterworth et al. investigated the postprandial response to glucose and fat tolerance tests in individuals with different apoCIII genotypes, including C3238G (SstI), C1100T, C-482T and T-2854G. The results showed that the postprandial response was regulated by variation at the T-2854G and C3238G sites. After the oral fat load test (OFTT), carriers of the rare alleles had delayed clearance of TG; G-2854 carriers showed a largest effect on TG, and G3238 carriers showed a smaller response. However, after adjustment for fasting levels of TG, only the effect with the T-2854G remained significant. Variation at the C-482T (IRE) determined the
response to the oral glucose test (OGTT), with carriers of the rare T-482 having significantly elevated glucose and insulin concentrations, while none of the other 3 apoCIII polymorphisms showed any significant association with insulin or glucose levels. The results obtained from this study suggested that specific genetic variants at the apoCIII gene locus differently affected postprandial response to glucose and fat tolerance tests and also suggested a novel mechanism for the effects of variation at this locus on risk for atherosclerosis (Waterworth et al., 1999).

1.6.2 APOC3 Deficiency

The deficiency of apoCIII is rare. A deletion of the APOAI-CIII gene complex was found in the kindred resulted in apoAI and apoCIII deficiency (Norum et al., 1982). Additionally, a complete deletion of the APOAI-CIII-AIV gene cluster has also been reported to cause familial deficiency of these three proteins (Ordovas et al., 1989). In both cases, plasma HDL levels were undetectable, TG levels were low, conversion of VLDL to IDL and LDL was increased which suggested an inhibitory role for apoCIII in vivo and homozygous individuals had premature atherosclerosis. However, since the APOC3 deficiency was concomitant with APOAI and APOA4 deficiency, the exact impact of APOC3 deficiency alone on lipid metabolism is not estimated yet.

1.6.3 Rare ApoCIII Variants

1.6.3.1 Previously Identified ApoCIII Variants

The first apoCIII variant was found in a German family by the presence of an abnormal retention time in reversed-phase high-performance liquid chromatography (HPLC) and a higher molecular mass which was shown to be due to the oversialylation of the protein. The carrier was hypertriglycerideremic also family studies failed to establish a causal role of this variant in the development of hypertriglycerideremia (Jabs and Assmann, 1987). However the other studies (Holdsworth et al., 1982) indicated that hypertriglycerideremia was associated with the sialylation modification of apoCIII. Additionally, the abnormal lipoprotein was
shown to be a less efficient substrate for purified bovine milk LPL than control lipoproteins.

ApoCIII-Thr74Ala was found in a Japanese family with a significant reduced concentration of the sialylated isoforms apoCIII1 and apoCIII2, due to the prevented O-glycosylation of apoCIII, and was not associated with any dyslipoproteinemia. Functional study indicated that this variant did not interfere with the synthesis and lipid binding of apoCIII and the missed O-glycosylation was not found to interfere the secretion of apoCIII (Maeda et al., 1987; Roghani and Zannis, 1988), suggested that the degree of apoCIII sialylation have little or no impact on lipoprotein metabolism.

ApoCIII-Lys58Glu was identified in a small German family and was associated with 30-40% lower plasma apoCIII levels which presented as both lower VLDL-apoCIII levels (15% of normal) and lower HDL-apoCIII levels (25% of normal), and also large, apoE-enrich HDL. The substitution of residue Lys58 by Glu was suggested to abolish the only positive charge at the α-helical hydrophilic site. The hyperalphalipoproteinemia was suggested to be caused by both the decreased apoCIII levels and the structural defect in apoCIII that might result in the lack of inhibition of lipolysis, since remnants of TG-rich particles are precursors of HDL. However, the number of carriers for this mutation was too small to indicate a direct relationship between the mutation and lipid metabolism (von Eckardstein et al., 1991).

ApoCIII-Gln38Lys was identified in a Mexican-American and associated with increased apoCIII and TG levels. Family studies in 16 heterozygous individuals revealed mildly elevated plasma TG levels. This amino acid substitution was predicted to result in an additional charge on the protein, which might enhance lipid binding and/or alter the effect on LPL, therefore resulted in the raised plasma apoCIII and TG levels. However no in vitro studies were carried out to confirm this (Pullinger et al., 1997).

ApoCIII-Asp45Asn was identified in a Turkish patient who previously had undergone coronary bypass surgery and in two of his three children. The family was too small to demonstrate a significant effect of the variant on lipid metabolism and
therefore failed to show a clear association between the mutation and the abnormal lipoprotein metabolism (Luttmann et al., 1994).

1.6.3.2 Background to This Study: Identification of the Novel ApoCIII Variant: Ala23Thr

ApoCIII-Ala23Thr was identified in three Mayan Indians from the Yucatan peninsula by Dr CF Xu, a postdoctoral worked in our group several years ago in collaboration with Dr R Ferrell. This mutation was associated with apoCIII deficiency and lower plasma TG levels. The identification of this novel apoCIII variant led us to investigate the functional basis of this naturally occurring mutation and to study the structure-function relationships of apoCIII.

The Mayan Indians inhabit in the Yucatan peninsula and other parts of Central America. The samples used in this study were collected in 1985 and 1987 from a series of local villages, as part of a study of chronic disease risk factors in Amerindians. All known first and second degree relatives were excluded (Ferrell et al., 1990). Sixteen individuals from one remote village of the original sample were included in our study.

The nucleotide sequences in the 5' and 3' flanking regions and all exons of the \textit{APOC3} gene were first compared between two individuals with decreased plasma apoCIII levels (1.6-1.9 mg/dl) and two individuals with normal (9.0 mg/dl) or elevated (18.4 mg/dl) apoCIII levels. A G to A transition in exon 3 of the \textit{APOC3} gene was identified in two of the apoCIII deficient subjects, causing an Ala23Thr substitution and abolishing an \textit{AcI}\textsubscript{I} (CCGC) site. Using PCR and \textit{AcI}\textsubscript{I} digestion, all the Mayan Indians samples were screened for this mutation, as well as the random sample of 192 male Caucasians aged 51-60 years and free from CHD from south London. None of the Caucasians carried this mutation. Other Mayan Indians, who also had moderately decreased plasma apoCIII levels (5.5 mg/dl), was identified with the mutant allele. The third carrier who was older than the other carriers had slightly higher plasma TG and cholesterol levels than the non-carriers. All three individuals with this mutation were heterozygous and the available lipid and lipoprotein data of
Table 1.6 Plasma apoCIII and lipid levels (mg/dl) and APOA1-C3-A4 genotype in Mayan Indians

<table>
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<th>ApoCIII (mg/dl)</th>
<th>Tg (mg/dl)</th>
<th>Chol (mg/dl)</th>
<th>HDL (mg/dl)</th>
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<th>SstI</th>
<th>C1100/T</th>
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</table>

Mean(±SD) from the other thirteen Mayans (5 males and 8 females) included in the study.

57
them are presented in Table 1.6 and compared to that of the 13 other Mayan Indians who do not carry the mutation.

To identify the haplotype on which the apoCIII-Thr23 occurred, DNA polymorphisms of the APOAI-C3-A4 gene cluster (the G-75/A substitution of the APOAI gene, the SstI polymorphism, the C1100/T transition, and the PvuII polymorphism of the APOC3 gene) were detected. The data are compatible with the mutation for Thr23 being present on the haplotype A-75, S-, T1100, and V- in all three individuals (Table 1.6).

My role in the project was to express the wild type and three variant proteins and perform structure-function studies on these proteins.

1.6.4 Structure-Functional Relationships of ApoCIII

Earlier studies have suggested that the amphipathic helical region of apoCIII is located between residues 40 to 67 (Segrest et al., 1974). However, the more recent sequence analysis and secondary predictions applied to apoCIII indicate the existence of two helical domains that are rather amphipathic and therefore involved in lipid binding of the protein, which are amino acids 11-28 and 35-52 respectively (Brasseur, 1990). However, by detecting the fluorescence and circular dichroism (CD) changes in the presence of lipid, the lipid binding domain of apoCIII was shown to be located between amino acid 41-79 (Sparrow et al., 1973). In other supporting experiments using the thrombin cleaved apoCIII fragment, apoCIII (41-79) but not apoCIII (1-40), was found to interact with phospholipid (Sparrow et al., 1977).

Catapano (1987) showed that the LPL inhibitory effect of apoCIII could be localised to the C-terminal domain between residues 41-79, which also bound phospholipids (Sparrow et al., 1977). However, results from the study using synthetic apoCIII peptide have concluded that the N-terminal domain of apoCIII, particular the segments containing amino acids 1-7 and amino acids 17-25 is the primary domain modulating LPL activity (McConathy et al., 1992).
Additionally, sequencing analysis results showed that the N-terminal domain and particularly residues 12-40 of the protein is well conserved among species compare to the C-terminal domain of apoCIII, indicating the functional importance (Bengtsson Olivecrona and Sletten, 1990). Apart from the above results, the structure-functional relationship of apoCIII was not well understood yet.

1.7 Aims of the Thesis

As mentioned in section 1.6.3 there are six rare apoCIII variants identified today, however, the current studies on these variants have not shown the functional effects of these mutations and the altered lipoprotein metabolism. In addition, the current knowledge about the structure-functional relationship of apoCIII is not well defined. In order to have a better understanding of apoCIII it would be necessary to perform structure-functional relationship studies of this important apolipoprotein.

The aim of this thesis is to use molecular biology technique to express recombinant wild type and mutant apoCIII-A23T to perform the structure-functional analysis by comparing the difference of their lipid binding properties and LPL inhibition, and to extend this project by analysis site directed mutations in apoCIII, which were generated according to molecular modelling results that would disrupt putative LPL or lipid binding domains, thus obtaining better structure-function predictions.
Chapter 2

Materials and Methods

2.1 Materials

Unless otherwise stated, the majority of chemicals were Analar grade obtained from BDH Ltd (Dorset, UK), Fisher Scientific (Leicestershire, UK) or Sigma (Irvine, UK). Suppliers of kits are given in the text.

The insect cell line sf9, sf21 and Hi5 were obtained from Invitrogen (San Diego, CA, USA) and all the medium as well as the supplements used for insect cells culture were provided by Gibco BRL, Paisley, UK. The flasks, Petri dishes and pipette used for the cell culture were provided by Falcon (Becton Dickinson Labware, New Jersey, USA).

Restriction enzymes and their appropriate buffers were obtained from either New England Biolabs (Herts, UK), Gibco BRL, or Boeringer Mannhein (East Sussex, UK). The DNA molecular weight size standards and oligonucleotides were obtained from Gibco BRL. Agarose was from Helena Biosciences (Sunderland, Tyne & Wear, UK). Western Blotting membranes were from either Schleicher & Schuell (Dassel, Germany). Protein molecular weight markers were obtained either from Amersham (Surrey, UK) or Sigma.

2.2 Methods

2.2.1 Plasmid DNA Preparation

Qiagen kits were used for plasmid deoxyribonucleic acid (DNA) preparation. These methods are based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to anion-exchange resin under appropriate low salt and pH
conditions. Ribonucleic acid (RNA), proteins, dyes and low MW impurities were removed by a medium salt wash. DNA was eluted under high salt conditions and desalted by isopropanol precipitation.

DNA was dissolved in TE buffer and detected with 0.5 mg/ml EtBr by adding it to the melted agarose containing 1xTAE buffer, prior to casting and the gels were viewed on an ultraviolet (UV) (260nm) transilluminator.

2.2.2 Cloning of DNA

2.2.2.1 Restriction Enzyme Digestion

DNA sample was digested with restriction enzymes according to the manufacturer's instructions in the appropriate buffer with the addition of 100 ng/μl bovine serum albumin (BSA). Digestions were carried out in water baths at the recommended temperature for the enzyme. Prior to cloning, 1 to 5 μg of DNA sample was digested with 1 to 5 units of enzyme for two hours.

2.2.2.2 DNA Clean up from Agarose Gels

DNA fragments were separated by electrophoresis on low melting point (LMP) agarose gel and excised from the gel under UV illumination. The DNA fragment was purified using Wizard PCR preps kit (Promega, Southampton, UK). The gel slice was placed in an eppendorf tube with 1 ml of DNA binding resin and incubated at 65 °C for 5 minutes. A 2ml syringe was then used to apply the resin-gel mixture to a spin-column that was subsequently washed using 80% isopropanol. The spin column was then placed in an eppendorf tube and spun for 20 seconds in a micro centrifuge to remove the excess isopropanol. The column was air-dried for a few minutes before adding 50 μl distilled water. The DNA was eluted by spinning the column into a fresh eppendorf tube for 20 seconds.
2.2.2.3 Ligation

Insert DNA and vector DNA were digested with appropriate restriction enzymes and purified from agarose gels using Wizard PCR preps kit (Promega, Southampton, UK) as described in 2.2.2.2. An approximately 3:1 molar ratio of insert to vector DNA (use 100 ng of vector DNA) was ligated using 10 units of T4 ligase (Boeringer Mannhein) in total volume of 20 μl containing the manufacturer’s supplied buffer at 14 °C for 12 hours.

2.2.2.4 Transformation

10 μl of ligation reaction or 10 ng of plasmid DNA was mixed with 100 μl of competent cells and was then incubated on ice for 30 minutes. The mixture was heat shocked at 42 °C for 45 seconds and then allowed recovering in 1ml of LB (see section 2.3) at 37 °C for 1 hour. The cell mixture was then spreaded between two of the LB agar plates containing appropriate antibiotics depending on the resistance conferred by the different vectors. Plates were incubated at 37 °C for 16 hours.

2.2.3 Polymerase Chain Reaction (PCR)

Unless otherwise stated the apoCIII gene was amplified by the following method of PCR condition. Reactions consisted of approximately 50 ng DNA as template, 250 ng each primer and 1 unit DNA polymerase (Gibco-BRL) in a buffer consisting of: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.001% Gelatin, 0.1% W-1 detergent, 0.2 mM each 2’-deoxy-N-5’ triphosphate (dNTP) and 1.5 mM MgCl₂ in final volume of 50 μl. After initial denaturing of the DNA for 5 mins at 94 °C, annealing of the primers (66 °C, 1 min) and extension (72 °C, 2 mins), the program used was followed by 35 cycles of: 94 °C, 30 seconds; 66 °C, 1 min and 72 °C, 1 mins. Reactions were performed in automated Omigene PCR machine (Hybaid Ltd, Middlesex, UK).
2.2.4 Mutagenesis

All the apoCIII variants were generated using QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). Plasmid DNA of wild type (WT)-apoCIII was used as template. Two synthetic oligonucleotide (oligo) primers that containing the desired mutation and complementary to opposite strands of the vector, are extended by using PfuTurbo DNA polymerase, according to the conditions provided by the manufacturer’s instructions of the kit. PCR was performed by initial denaturation at 95 °C for 30 sec, followed by 16 cycles of the following programme: 95 °C for 30 sec, 55 °C for 1 min and 68 °C for 12 min. After DpnI digestion of the above PCR product to remove the parental dam-methylated template, the synthesised mutated DNA was transformed into *E. coli* XL1Blue super-competent cells. Candidate clones were screened by sequencing the entire apoCIII insert on an ABI 377 prism DNA sequencer, both to confirm the presence of the mutation and to ensure no alterations at other sites.

All the primers used in the mutagenesis reactions were list in Table 2.2.

2.2.5 Sequencing by Fluorescence Dye Terminator Technology

2.2.5.1 Sequencing Reaction

The dRhodamine terminator cycle sequencing chemistry kit (Perkin Elmer Applied Biosystems, UK) was used to perform the sequencing reactions. Normally, the 10 μl sequencing reactions were carried out. To each 0.5 ml tube, 0.5 μg of plasmid DNA from mini prepare was added, 4 μl of dye terminator premix and 1 μl of 1.6 pmol/μl primer was added and the final volume was made up to 10 μl with sterile water. The mixture was vortexed, centrifuged, overlaid with 20 μl of mineral oil and placed on a preheated (96 °C) PCR machine. The PE-ABI DNA thermal cycler model 480 and the cycle conditions were employed as recommended by the manufacturers.
2.2.5.2 Purification of Sequencing Products

The sequencing products were taken from the reaction tube after being diluted with 10 μl of dH₂O, and incubated with 300 μl of 95% ethanol and 10 μl of 3 M NaOAc (pH 5.2) at RT for an additional 15 minutes. The mixture was then spun at 13,000 rpm at desktop centrifuge for 20 minutes. The supernatant was removed and the resulting invisible pellets were washed twice with 200 μl of 70% ethanol by briefly vortexing and spinning. The final pellets were placed on a PCR block for 5 minutes at 80 °C to remove all traces of ethanol and stored either at room temperature for a day or -20 °C for longer periods.

2.2.5.3 Preparation of Sequencing Gel and Loading of Samples

3 μl of formamide loading buffer was added to the samples (dextran sulphate at 30 mg/ml in a ratio of 5:1 (v/v) 25 mM EDTA (pH 8.0) to deionised 98% formamide, 10 mmol/L EDTA), denatured for 2 minutes at 96 °C and immediately placed on ice, 2 μl of the product was loaded onto a sequencing gel.

2.2.5.4. Sequencing Analysis

Sequencing analysis was carried out using Sequencing analysis 2.0 (PE-ABI, UK) programme.

2.2.6 Protein Analysis

2.2.6.1 Protein Analysis by Sodium Dodecyl Sulphate (SDS) -Polyacrylamide Gel Electrophoresis (PAGE)

The recombinant apoCIII proteins with MW ranging from 12-43 kDa, including glutathione S-transferase (GST)-apoCIII fusion protein, S-protein fusion apoCIII (see detail in Chapter 5) and apoE were separated on 15% SDS-PAGE
according to described by Maniatis et al (Maniatis T et al., 1989), using Pharmacia
(Uppsala, Sweden) system according to manufacturer's instruction.

The 6xHis-tag fused apoCIII proteins (both C-terminal and N-terminal fusion
proteins) were separated on 17.5 % precast Tricine-SDS-PAGE (separation range
MW 26.6-1.4 kDa) provided by Bio-Rad (Hertfordshire, UK).

2.2.6.2 Coomassie Blue Staining of Polyacrylamide Gels

Coomassie blue staining was performed according to described by Maniatis et
al. (Maniatis T et al., 1989). Gels were immersed in at least 5 volumes of a
concentrated solution of the dye (0.5% Coomassie Brilliant Blue in 10% HAC, 30%
isopropanol) and placed on a slowly rotating platform at room temperature for at least
30 minutes. The gels were then destained in 30% methanol, 10% HAC solution as
described above. The excess dye was then allowed to diffuse from the gel during a
prolonged period of destaining as long as required for the appearance of the bands.
The gels were rinsed with distilled water and fixed in distilled water containing 20%
glycerol prior to dry.

2.2.6.3 Silver Staining of Polyacrylamide Gels

Visualisation of protein on polyacrylamide gels was achieved by the method of
Merril (1981). All solutions except developer could be kept at room temperature for
months. Gels were fixed by soak in 50% methanol, 10% HAC for 20 minutes followed
by soak in 5% MeOH and HAC for 20 minutes. After washed in distilled water for 10
minutes, gels were soaked in 10 μg/ml DTT for 20 minutes then immersed in 0.1%
AgNO₃ for 20 minutes and washed with distilled water briefly. Precipitation of silver
was achieved by first rinsing gels in freshly made developer (30 g Na₂CO₃, 0.5 ml
formaldehyde per litre) until the solution turned yellow-brown (30-40 seconds) and
then soaking in the developer as long as required for the appearance of the bands.
Finally the reaction was stopped by adding HAC. The gels were then rinsed well with
distilled water and fixed in distilled water containing 20% glycerol prior to dry.
2.2.6.4 Western-blotting

**Protocol 1**

The separated proteins were transferred onto a nitrocellulose membrane using a Pharmacia transblotting device and then the membrane was put in TBST buffer containing 3% BSA for 30 minutes at room temperature. After 2 hours incubation with a rabbit-anti apoCIII polyclonal antibody (1:100 dilution in BSA/TBST), the membrane was washed for 3x15 minutes in TBST. Then the membrane was incubated with anti-rabbit IgG alkaline phosphatase conjugate (1:20000 in BSA/TBST) for 1 hour. Finally the membrane was washed three times for 5 minutes in TBST. ApoCIII protein was visible in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl\textsubscript{2} containing 0.4 mM nitroblue tetrazolium (NBT) and 0.4 mM 5-bromo-4-chloro-3-indolylphosphosphate (BCIP). The reaction was stopped by rinsing the membrane in 20 mM Tris-HCl (pH 8.0) containing 5 mM EDTA.

**Protocol 2**

The separated proteins were transferred onto a Immobilon membrane (Bio-Rad) using a semi-dry blotting apparatus (Bio-Rad) in a Tris (5mM)-Glycine (192 mM) buffer at pH 8.3 containing 20% methanol. Proteins were visualised with a 1:100 dilution of polyclonal antibodies of rabbit-anti apoCIII (for apoCIII detection), or a rabbit-anti apoE (for apoE detection), and a secondary peroxidase labelled goat anti-rabbit antibody (Sigma). The process was performed similarly to described in Protocol 2, apart from using 1% dry milk powder in phosphate buffer saline (PBS, pH 7.5) containing 0.02% Tween-20 instead of the TBST buffer containing 3% BSA. The bound peroxidase was visualised using the BM Chemiluminescence luminal substrate provided by CN-DAB kit (Pierce).
2.2.6.5 Detection of Free Protein Concentration

The protein concentration of all the free apoCIII-6xHis fusion proteins and some of the apoCIII:phospholipid particles mentioned in this thesis was determined by OD measurement at 280 nm using a molar extinction coefficient 19,630 (M⁻¹.cm⁻¹). For free GST-apoCIII proteins, the protein concentration was determined in the same way apart from using a molar extinction coefficient 45,630 (M⁻¹.cm⁻¹).

2.2.6.6 Enzyme-linked Immunosorbent Assay (ELISA)

In addition to the description in section 2.2.6.5, the protein concentration of both apoCIII variants and apoE in lipid particles obtained from gel filtration on a Superose 6HR (see section 2.2.9.4) in this thesis was measured by a sandwich ELISA (enzyme-linked immunosorbent assay).

100 μl of the antibodies (5 mg/ml in PBS) (polyclonal antibodies of rabbit-anti apoCIII (for apoCIII detection), or a rabbit-anti apoE (for apoE detection) were first coated on the 96-ELISA plates prior to the experiment. Residual binding sites were blocked and 100 μl of samples or standards are incubated on the plates for 24 hours at 37 °C. After washing the plates four times with 10 mM PBS containing 0.05% Tween-20 to remove any unbound substances with, a peroxidase labelled polyclonal antibody was incubated on the plates for 2 hours at 37 °C. Following a wash to remove any unbound antibody-enzyme reagent by four washes with 10 mM PBS containing 0.05% Tween-20, the amount of bound peroxidase was revealed with a chromogenic substrate. The plates were read on the plate reader (Biotek Reader adapted with KC4 software), the standard curve and samples were calculated with the software provided with the reader. The sensitivity of the assay is approximately 2 ng.
2.2.7 Protein Expression in Insect Cells

2.2.7.1 Insect Cell Culture and Infection with Baculovirus

The insect cells used in this study were sf9, sf21, Hi5 cells (*Spodoptera frugiperda* cells), which were derived from pupal ovarian tissue of fall armyworm. Sf9 and sf21 cells were cultured in the 500 ml spinning bottle at 27 °C with constant stirring at 120 rpm in suspension, in TC-100 medium containing 10% fetal calf serum (FCS), 100 U/500 ml penicillin and 100 U/500 ml streptomycin (the resulting medium was called complete TC-100 medium), up to a density of 1x10⁶ cells/ml. Cells were then seeded at the required density onto petri dishes and allowed to adhere for 30 minutes at room temperature before infected with virus. Media was removed from cells by aspiration, prior to infection by adding virus stock drop-wise onto the cells and incubating at room temperature with gentle rocking for 1 hour. After remove the viral solution from the infected cells by aspiration, plates (90 mm) were then supplemented with 4-10 ml more TC-100 completed medium, and incubated at 27 °C with humidity for 4 days, unless otherwise stated. Hi5 cells were always cultured in monolayer in flasks using the same media under the same conditions, and were infected by the baculovirus using the same method. Alternatively, the cells were cultured in the serum free conditions by using SFM-II medium containing the same amount of the antibiotics. The medium was changed every 3-4 days.

2.2.7.2 Cationic Liposome Mediated Transfection of Insect Cells

1 ml of TC-100 medium (containing no supplements) was incubated with 1 μg of linear *Autographa Californica* nuclear polyhedrosis virus (AcMNPV) DNA, 3 μg of plasmid DNA (apoCIII in transfer vector) and 20 μl of the Cationic liposomes solution (Invitrogen, San Diego, CA, USA) at room temperature for 15 minutes. This mixture was then added to 2x10⁶ of log phase sf9 cells in 60 mm plates and slowly rocked at room temperature for 4 hours, following by adding 1 ml of TC-100 complete medium into the plates and then incubated at 27 °C in a humidified
incubator. The medium in the plates was harvested in 48 hours and stored at 4 °C as virus stock.

2.2.13 Plaque Purification of Recombinant Virus and the Determination of the Virus Titer

1.5x10⁶ of log phase sf9 cells in 35-mm plates were infected with 100 µl of the virus stock by incubating at room temperature for 1 hour. The virus stock was then removed completely by aspiration, followed by adding 1.5 ml of 1% Sea Plaque Agarose solution (pre-made and incubated at 37 °C, by mixing 2% aqueous Sea Plaque Agarose solution with equal volume of Grace medium containing 150 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)) into the dishes. These dishes were then left at room temperature for approximately 30 minutes to allow the agarose to set before being incubated in a humidified incubator at 27 °C. Blue plaques appeared after 5-6 days incubation.

For the pure baculovirus, this method can also be used to detect the viral titer (pfu/ml) according to the number of the plaque obtained.

2.2.7.4 Amplification of the Virus from the Plaque

Viral occlusion bodies are formed in the nucleus and comprise enveloped nucleocapsid embedded in a crystalline protein matrix. They have a bright shiny appearance that is readily visualised under the light microscope. Blue plaques without occlusion bodies in the insect cells (occ-) were picked using a sterile Pasteur pipette and bulb. The agarose plugs were soaked in 1 ml of complete TC-100 medium overnight and then vortexed. The resulting mixture was stored at 4 °C as plaque-pick. In order to amplify the virus in the plaque-pick, 100 µl of the plaque-pick solution was used to infect 5x10⁷ of log phase sf9 cells at room temperature for 1 hour. 2 ml of the complete TC-100 medium was then added into the plates in prior to incubate at 27 °C in a humidified incubator. The medium covering the cells was harvested after 4 days.
and stored as Pass-1 virus stock after centrifugation at 1500 rpm to pellet the cell debris. Pass-2 virus stock was made in the same way except that $5 \times 10^6$ of log phase sf9 cells were inoculated with the 0.5 ml of the Pass-1 stock.

The amplification of the large scale of the pure virus was performed as same as described above except using pure virus instead of the plaque-pick solution.

2.2.7.5 Extraction of Viral DNA

0.75 ml of virus stock (normally Pass-2) was mixed with an equal volume of 20% PEG solution in 1 M NaCl in a fresh 1.5 ml eppendorf tube by inverting twice and the resulting mixture was allowed to stand at room temperature for 30 minutes. After the mixture was spun at 14000 rpm for 10 minutes at room temperature, all the supernatant was removed. The remaining pellets were then dissolved in 100 $\mu$l of sterile H$_2$O followed by addition of 10 $\mu$l of Proteinase K (5-10 mg/ml). The resulting solution was then incubated at 50 °C. After 1 hour, the solution was extracted with an equal volume of phenol/chloroform (1:1) to remove all the proteins. 10 $\mu$l of 3M NaOAc (pH5.2), 5 $\mu$l of glycogen and 220 $\mu$l of ethanol were added and the resulting mixture was incubated at -20 °C. After at least 20 minutes, the above mixture was spun at 14000 rpm for 15 minutes at 4 °C and all the supernatant was removed. The remaining pellets were washed with 0.5 ml of 80% ethanol and spun at 14000 rpm for 5 minutes to remove all trace of ethanol. The final viral DNA pellets were either stored at 4 °C for a day or at -20 °C for longer periods.

2.2.7.6 Time Course of Recombinant ApoCIII Expression

$5 \times 10^6$ of log phase insect cells were grown in the 6-well plates and infected with pure baculovirus at multiplicity of infection (MOI) (plaque forming units/cell) of 5, 7.5 and 10 for 1 hour. Amount of titered virus (detected as described in section 2.2.7.3) needed to infect the cells was calculated as the equation:
\[ \text{ml of virus} = \text{MOI} \times 5 \times 10^6 \text{ (number of cells)} \]

\[ \text{titer (pfu/ml)} \]

Cells were then washed 3 times with TC-100 medium (no serum) to remove all FCS contained in the TC-100 complete medium used previously and finally covered with 3 ml of SFM-II serum free medium. At 12-hour time points from 0-96 hours after infection, 500 \( \mu \)l of medium were removed and centrifuged at 1500 rpm for 5 minutes. Additionally, the cells scratched from one well were lysed by sonication in 200 \( \mu \)l of PBS containing 1 mM of PMSF for 3x15 seconds bursts at a medium intensity setting while holding the suspension at ice bath. Above samples of both the medium and the cell lysate were stored at -20 °C until all the samples of each time point were obtained and then used for the detection of the expression level. Finally, 25 \( \mu \)l of the medium and 5 \( \mu \)l of the cell lysate on 17.5 % precast Tricine-SDS-PAGE as described in section 2.2.6.1.

### 2.2.7.7 Large Scale Expression of Recombinant ApoCIII Proteins in Insect Cells

500 ml of insect cells (sf9 or sf21) grown in 1 L spinning bottle at density of 1x10^6 were infected with the pure baculovirus stock at MOI of 5 by mixing the cells and the virus in the spinning bottle at room temperature for 1 hour. The medium was then removed after the cells were spinning down at 1000 rpm for 10 minutes at 4 °C in a Sorvall RC5 or the equivalent equipped with a SORVALL® SLA-1500 rotor. The cell pellet was then resuspended in complete TC-100 medium and grown at 27 °C with constant stirring at 120 rpm in suspension. These cells were harvested at 72 hour after infection.

### 2.2.7.8 Isolation of 6xHis-apoCIII Fusion Proteins from Insect Cells

(A) Native Condition:

The infected sf9 and sf21 cells grown in the spinning bottle were harvested by spinning down at 3000 rpm for 15 minutes at 4 °C. Cell pellets obtained from 1L of
cell culture were resuspended in 200 ml native binding buffer (see section 2.3.1) containing 1 mM of PMSF and lysed by sonication with 3x20 seconds bursts at a high intensity setting while holding the suspension at ice bath. The cell lysate was centrifuged at 18000 rpm for 30 minutes at 4 °C to remove the insoluble debris and the clear supernatant was incubated with 1 ml of nickel chelating resin (Invitrogen) pre-equilibrated with the native binding buffer at 4 °C for 2 hours. The resin was pelleted by centrifugation at 3000 rpm for 5 minutes prior to twice batch-wash with 4 ml of the native binding buffer (pH 7.8) and twice batch-wash with 4 ml of the native washing buffer (pH 6.0). The washed resin was transferred to a fresh column (Bio-Rad) and proteins were eluted from the column with the native elution buffer (pH 4.0).

For the infected Hi5 cells grown in monolayer, the cells were scratched from the flasks/dishes and resuspended in the media. The cells were then harvested by spinning down at 3000 rpm for 15 minutes at 4 °C, the further purification procedure was carried out similarly as that of sf9 and sf21 cells.

(B) Denaturing Conditions:

The infected sf9, sf21 and Hi5 cells were harvested as described in 2.2.7.7 (A). The cell pellet was resuspended in 200 ml of Guanidinium lysis buffer (see section 2.3.1) containing 1 mM of PMSF and slowly rocked at room temperature for 1 hour to assure thorough cell lysis. Resulting mixture was sonicated with 3x20 seconds bursts at a high intensity setting while holding the suspension at ice bath. The cell lysate was centrifuged at 18000 rpm for 30 minutes at 4 °C to remove insoluble debris and the clear supernatant was incubated with 1 ml of nickel chelating resin (Invitrogen) pre-equilibrated with the Guanidinium lysis buffer at 4 °C for 2 hours. The resin was pelleted by centrifugation at 3000 rpm for 5 minutes prior to twice batch-wash with 4 ml of the denaturing binding buffer and twice batch-wash with 4 ml of the denaturing washing buffer (pH 6.0) as well as 4 ml of the denaturing washing buffer (pH 5.3). The washed resin was transferred to a fresh column (Bio-Rad) and proteins were eluted from the column with the denaturing elution buffer (pH 4.0).
2.2.8 Expression and Isolation of Recombinant ApoCIII Protein from E. coli Cells

2.2.8.1 Detection of Small Scale Expression of Recombinant ApoCIII Protein

The plasmid DNA containing the apoCIII in the expression vector was transformed into the appropriate E. coli cells as described in section 2.2.2.4. The single clone was put in 15 ml of medium (2xYT for GST-apoCIII fusion proteins and SOC medium for apoCIII-6xHis fusion proteins) with 75 µg/ml of ampicillin (shaken at 220 rpm) at 37 °C. Cells were induced with isopropyl β-D-thiogalactoside (IPTG) at final concentration of 0.5 mM when the optical density (O.D) of the cell culture reached 0.6. After 2 hours induction the cells were harvested by spinning at 3000 rpm for 10 minutes at 4 °C in a Sorvall RC5 or the equivalent equipped with a SORVALL® SA-600 rotor. Cell pellets were lysed in 0.75 ml of lysis buffer containing 1 mM of phenylmethylsulphonyl fluoride (PMSF) by sonication with 3x20 seconds bursts at a high intensity setting while holding the suspension at ice bath. 10 µl of the SDS sample-loading buffer (section 2.3.3) was added in 10 µl of cell lysate and heated at 95 °C for 3 minutes prior to analysis the cell lysate samples on the SDS-PAGE.

Sometimes the recombinanted apoCIII proteins (such as the apoCIII-6xHis fusion protein) were not expressed at high level therefore they were not easily detectable from the cell lysate samples on Coomassie blue stained gels. In these cases, the cell lysate was first purified with Talon™ affinity resin (Clontech) in prior to detect on Coomassie blue stained gels. Briefly, all the cell lysate (0.75 ml) were mixed with 20 µl of Talon™ affinity resin (bed volume) pre-equilibrated with the lysis buffer (section 2.3.2) at room temperature for 10 minutes. After removing the cell lysate and washing the above resin by 1.5 ml of the lysis buffer, 50 µl of 0.1 M EDTA was added to the Talon™ affinity resin in order to elute all the bound proteins. 20 µl of the eluate was separated on 17.5 % precast Tricine-SDS-PAGE followed by Coomassie blue staining.
2.2.8.2 Large Scale Expression of Recombinant ApoCIII Protein

The plasmid DNA containing the apoCIII in the expression vector was transformed into the appropriate *E. coli* cells (B834 for WT-apoCIII and apoCIII-A64A65 while BL21 for apoCIII-T23,apoCIII-T9L20,apoCIII-A21 and all the GST-apoCIII proteins) as described in 2.2.2.4. The single clone was cultured in 100 ml of LB medium that containing 75 μg/ml of ampicillin (shaken at 220 rpm/min) at 37 °C for overnight. 30 ml of the overnight culture was used to infect 1L of medium (2xYT for GST-apoCIII fusion proteins and SOC medium for apoCIII-6xHis fusion proteins) that containing 75 μg/ml of ampicillin. The resulting culture was shaken at 220 rpm/min at 37 °C. Cells were then induced with IPTG at final concentration of 0.5 mM when the O.D._{600} of the cell culture reached 0.6. After certain time (1 hour for recombinant apoCIII-T23-6xHis and 2 hours for all the other apoCIII variants), the cells were harvested by spinning down at 3000 rpm for 15 minutes at 4 °C in a Sorvall RC5 or the equivalent equipped with a SORVALL® SLA-1500 rotor.

2.2.8.3 Isolation of Recombinant GST-ApoCIII Fusion Protein from the *E. Coli* Cells

The cell pellets contained the GST-apoCIII fusion protein from 1L of culture were sonicated in 200 ml ice-cold PBS containing 1 mM of PMSF and 1% (v/v) Triton X-100 for 3x20 seconds bursts at a high intensity setting while holding the suspension at ice bath. The cell lysate was centrifuged at 18000 rpm for 30 minutes at 4 °C to remove insoluble debris. The clear supernatant was incubated with 1 ml of glutathione-agarose (bed volume) (Pharmacia) pre-equilibrated with PBS at 4 °C for 2 hours. The glutathione-agarose was then collected by centrifugation at 3000 rpm for 3 minutes. After batch washed with 5 ml of ice-cold PBS, the GST-apoCIII fusion protein was eluted in 5 ml of ice-cold PBS (pH 7.5) containing 10 mM reduced glutathione from the glutathione-agarose.
2.2.8.4 Isolation of Recombinant ApoCIII-6xHis Fusion Protein

The cell pellets contained the apoCIII-6xHis fusion protein obtained from 1L of cell culture were resuspended in 200 ml lysis buffer (pH 8.0) (see section 2.3.2) containing 1 mM of PMSF and incubated at ice for 1 hour. Resulting mixture was then lysed by sonication for 3x20 seconds bursts at a high intensity setting while holding the suspension at ice bath. The cell lysate was centrifuged at 18000 rpm for 30 minutes at 4 °C to remove insoluble debris. The clear supernatant was incubated with 1 ml of Talon™ affinity resin (Clontech) pre-equilibrated with lysis buffer (pH 8.0) at 4 °C for 2 hours. The resin was then pelleted by centrifugation at 3000 rpm for 5 minutes and then batch-washed 4 times with total of 5 ml of the bed-volume of the resin and then transferred to a fresh column (Bio-Rad). Recombinant apoCIII-6xHis fusion proteins were eluted from the column with the elution buffer, pH 6.0-6.3.

2.2.8.5 Purification of ApoCIII-6xHis Fusion Protein using FPLC

The eluate containing apoCIII-6xHis was dialysed against 4 M urea, 5 mM NH₄HCO₃ (pH 8.0) at 4 °C for overnight. The resulting sample was spinning down at 14000 rpm for 15 minutes by desktop centrifuge at 4 °C to removed any possible debris for chromatography on an anion exchange column (MonoQ) using fast protein liquid chromatography (FPLC) (Pharmacia). The column was eluted with a linear gradient solution of NaCl (from 0.1 M to 0.25 M) over 20 ml in 4 M urea 5 mM NH₄HCO₃ (pH 8.0) buffer, at 0.5 ml/minute. The protein eluate profile was collected every minute and the protein samples were monitored by OD₂₈₀.

2.2.8.6 Cleavage of GST from the GST-ApoCIII Fusion Proteins Using Proteases

Thrombin cleavage was completed in PBS (pH 7.5) using 50 μg of GST-apoCIII fusion protein in final volume of 10 μL. The eluted GST-apoCIII fusion proteins were dialysed against PBS (pH 7.5) prior to the cleavage reaction. 2 μL of the mixture was then analysed on 17.5% precast Tricine-SDS-PAGE.
Enterokinase cleavage was completed in 30 μl of the enterokinase reaction buffer (EKmax™ buffer: 50 mM Tris-HCl, pH 8.0, 1 mM CaCl₂, 0.1% Tween-20) (Invitrogen) using 20 μg of fusion protein and 0.001 unit of EKmax™ (Invitrogen, San Diego, USA). The eluted GST-apoCIII fusion proteins were dialysed against EKmax™ buffer prior to the cleavage reaction. 15 μl of the resulting reaction mixture was then analysed on 17.5% precast Tricine-SDS-PAGE.

Factor Xa was used at an enzyme:substrate (w/w) ratio of about 1:100 in 10 μl of 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1 mM CaCl₂ to perform the cleavage reaction using 10 μg of GST-apoCIII protein. The resulting mixture was then analysed on 17.5% precast Tricine-SDS-PAGE. The eluted GST-apoCIII fusion proteins were dialysed against above enzyme reaction buffer prior to the cleavage reaction. All the mixture was then analysed on 17.5% precast Tricine-SDS-PAGE.

Cleavage of the mixture of GST-apoCIII fusion protein and plasma apoCIII using enterokinase/factor Xa was performed as described above, except using a mixture of the GST fusion apoCIII (20 μg) and plasma apoCIII (5 μg) instead of GST-apoCIII fusion protein alone. The resulting mixture containing 1 μg of total protein was then analysed on 17.5% precast Tricine-SDS-PAGE followed by silver staining as described in 2.2.6.3.

2.2.9 Lipid Binding Properties

2.2.9.1 Prepare the 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) Multilamellar Vesicles (MLV)

DMPC (Sigma) was first dissolved in chloroform and dried under nitrogen to a film during 2 hours and then re-dissolved in 5 mM NH₄HCO₃ (pH 8.0) at concentration of 2 mg/ml by vortex at room temperature until the emulsion was homogeneous.
2.2.9.2 Lipid Binding Properties of Recombinant ApoCIII

Lipid binding properties of recombinant apoCIII variants were followed by monitoring the turbidity decrease of DMPC MLV at 325 nm after mixed with the recombinant proteins (obtained as described in section 2.2.9.1) as a function of the temperature from 16 °C to 36 °C. Normally, 40 μg of apoCIII protein was added to 80 μg of DMPC MLV in 5 mM NH₄HCO₃ buffer (pH 8.0) to perform the experiment. The turbidity of DMPC was measured in an Uvikon 931 spectrophotometer.

2.2.9.3 Preparation of ApoCIII:DMPC Complexes

Dependent on the amount of the apoCIII:DMPC complexes required, the complexes were prepared by incubation of the recombinant apoCIII with DMPC MLV at DMPC/protein (w/w) ratios of 2:1 in 5 mM NH₄HCO₃ (pH 8.0) in the shaking water bath at 25 °C for 16 hours. The concentrations of the complexes were expressed by their protein contents.

2.2.9.4 Complex Isolation and Characterisation

All the protein:phospholipid complexes were isolated by gel filtration on a Superose 6HR column (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK) in 0.01 M Tris-HCl buffer, pH 7.6 and 0.2 g/L NaN₃ in FPLC system (Waters). The eluate was collected every minute and the complexes were detected by measuring both the absorbency at 280 nm and the tryptophan (Trp) fluorescence emission at 330 nm (excitation at 295 nm).

The Superose 6HR column was calibrated with a set of protein standards ranging from thyroglobulin (MW 668 kDa) to cytochrome c (MW 65 kDa) (Pharmacia). The resulting calibration curve was used for the determination of the MW or Stokes radii of the particles eluting from the column. The composition and the size of the complexes were determined on the fractions corresponding to the maximal of the elution peaks.
2.2.10 Physicochemical characteristics of the native and lipid bound apoCIII

2.2.10.1 Circular Dichroism (CD) Measurements

CD spectra of each apoCIII protein with 50% 2,2,2-Trifluoro-Ethanol (Sigma) (TFE) and without TFE were measured on a Jasco 600 spectropolarimeter between 184 and 260 nm at room temperature. All the samples are placed in a 0.1 cm path length quartz cell. Measurements were carried out at a protein concentration of 0.2 mg/ml in 5 mM NH₄HCO₃ buffer (pH 8.0). Spectra data from 260 to 184 nm are collected at 2 nm intervals with a scanning speed of 20 nm/minutes and a time constant of 2 seconds. Nine spectra were collected and averaged for each sample. The secondary structures of each samples were obtained by curve-fitting on the entire ellipticity curve between 184 and 260 nm using the software available on the Internet http://bioinformatik.biochemtech.ini-halle.de/cdnn/.

For the denaturation experiments, the signal measured at 222 nm and expressed as molar ellipticity is plotted as a function of the GdnHCl concentration (M) in the sample.

2.2.10.2 Denaturation

Denaturation was performed by incubation both lipid free apoCIII proteins and the apoCIII:DMPC complexes at protein concentration of 100 µg/ml with increasing GdnHCl concentrations in 5 mM NH₄HCO₃ buffer (pH 8.0) between 0 and 6 M, at 4 °C for 12 hours.

2.2.10.3 Fluorescence Measurements

The maximal emission wavelength of the Trp fluorescence emission spectra of lipid-free apoCIII at protein concentration of 25 µg/ml and lipid bound apoCIII variations (apoCIII:DMPC complexes) at protein concentration of 60 µg/ml were
recorded at wavelengths between 300 and 450 nm, with the excitation wavelength set at 290 nm. Measurements were performed on an Aminco Bowman Series 2 Spectrofluorimeter (Vanloo et al., 1991) at room temperature.

2.2.11 Displacement of ApoE by ApoCIII Variants from Discoidal ApoE: Dipalmitoylphosphatidylcholine (DPPC) Complexes

2.2.11.1 Preparation of Discoidal ApoE:DPPC Complexes

The apoE:DPPC complexes were prepared by the sodium cholate dialysis method described by Labeur et al (Labeur et al., 1998) and the recombinant apoE3 was used for the preparation of the DPPC complexes.

The DPPC (Sigma)/protein mixtures at a ratio (w:w) of 2:1 in 5 mM NH₄HCO₃ (pH 8.0) were incubated overnight at 43 °C and then extensively dialysed against 10 mM Tris-HCl buffer (pH 8.0), containing 150 mM NaCl and 0.1 g/L of disodium-EDTA and 1mM azide, each time during 24 hours at 43 °C, at room temperature and at 4 °C. The homogeneity of the apoE:DPPC complexes was verified by gel-filtration on a Superose 6 PG column where the complexes eluted in one homogeneous peak. The protein concentration in the complexes was determined by optical density measurement at 280 nm.

2.2.11.2 Displacement of ApoE by ApoCIII Variants from Discoidal ApoE:DPPC Complexes

This was performed by mixing the apoE:DPPC complex with the apoCIII proteins at a ratio of 1:1 (w:w) and incubating the mixture at room temperature for 2 hours.
2.2.12 In Vitro LPL Inhibition by ApoCIII Variants

The following protocol of LPL assay was drawn from Nilsson-Ehle and Schotz (Nilsson Ehle and Schotz, 1976) and outlined below.

2.2.12.1 Substrate Emulsion

50 µl of glycerol tri[9,10-\textsuperscript{3}H]oleate (with specific activity of 23.7 mCi/mg and radioactive concentration of 5.0 mCi/ml) previously blow-dried by N\textsubscript{2} was mixed with 2 ml of Intralipid\textsuperscript{TM} (100 mg/ml) (Pharmacia Laboratories, Milton Keynes, UK). The mixture was then pause sonicated in ice for 20 minutes for every 45 seconds. The emulsion was then kept at 4 °C for several weeks. 6 µl of the resulting \textsuperscript{3}H-labeled lipid emulsion was then mixed with 100 µl of incubation mixture (see section 2.3.2) and each apoCIII variants which final concentration ranging from 0 to 15 µM, with or without apoCII*. Sterile H\textsubscript{2}O was also added into above mixture in order to adjust final volume of above mixture to 190 µl. The final mixture, substrate emulsion was then incubated for at least 15 minutes at 25 °C and used for one LPL assay sample.

* The lyophilised human plasma apoCII (a kind gift from Dr. Gunilla Olivecrona, Umeå, Sweden) was dissolved in 5 M urea, 10 mM Tris-Cl, (pH 8.5) at 2 mg/ml, 2 µl of the 1/100 dilution of above apoCII sample was used if required.

2.2.12.2 LPL Assay

Bovine LPL (EC 3.1.1.34) (Sigma) was used in an \textit{in vitro} LPL assay to investigate LPL inhibition by apoCIII variants. 10 µl of bovine LPL (1.5 µg/ml) was added into the substrate emulsion (obtained as described in section 2.2.12.1) to a total volume of 200 µl. The mixture was shaken on a water bath at 25 °C for 30 minutes. The reaction was terminated by adding 2 ml of Borgstrom solution (see section 2.3.2) and 0.5 ml of H\textsubscript{2}O followed by vortex for 30 seconds. Finally 0.8 ml of the top aqueous layer was separated and taken after centrifugation at 3000 rpm for 10 minutes and mixed with 1 ml of alkaline ethanol (see section 2.3.2), and extracted twice by 3 ml heptan.
In order to measure the radioactivity of each sample, 0.8 ml of the resulting top layer was mixed with 4 ml of biodegradable scintillation solution, Ecoscint™A (National Diagnostics, Atlanta, Georgia, USA), followed by standard liquid scintillation counting procedures on a Packard TRI-CARB®, 2000 CA liquid scintillation counter. Enzyme activity in the sample was expressed in mU/ml, 1 mU of enzyme activity is defined as 1 nmol of fatty acid released per minute at 25 °C, and was calculated based on the formula found in Nilsson-Ehle and Schotz (1976):

\[
\text{Activity (mU/ml)} = \frac{\text{net cpm} \times \frac{1}{30 \text{ minutes}} \times \frac{1}{\text{sp. act}} \times \frac{1}{\text{LPL sample (\mu l)}}}{2.4 \times 1000}
\]

Specific activity (sp.act) is expressed in cpm per nmole triglyceride

Samples were assayed in triplicates.

2.3 Solutions

The constituents of standard solutions and culture media referred to in this thesis (10xPBS, 10xTAE, 10xTBE, 10xTBST, 10xSDS-PAGE running buffer, LB medium, LB agar plate, 2xTY medium, TB medium and SOC medium) are given in Maniatis et al. (Maniatis T et al., 1989).

2.3.1 Solutions for Purification of 6xHis-ApoCIII Protein from Insect Cells

2.3.1.1 Native Buffers

Native Binding Buffer

20 mM \( \text{NaH}_2\text{PO}_4 \),

500 mM \( \text{NaCl (pH 7.8)} \)
Native Wash Buffer, pH 6.0-5.0
(Used for Imidizole Elution and pH Elution protocols)
20 mM  NaH$_2$PO$_4$
500 mM  NaCl

Native-pH Elution Buffer
20 mM  NaH$_2$PO$_4$
500 mM  NaCl (pH 4.0)

2.3.1.2 Denaturing Buffers

Guanidinium Lysis Buffer:
6 M  GdnHCl
20 mM  NaH$_2$PO$_4$
500 mM  NaCl (pH 7.8)

Denaturing Binding Buffer:
8 M  urea
20 mM  NaH$_2$PO$_4$
500 mM  NaCl (pH 7.8)

Denaturing Wash Buffer:
8 M  urea
20 mM  NaH$_2$PO$_4$
500 mM  NaCl (pH 6.0)

Denaturing Elution Buffer:
8 M  urea
20 mM  NaH$_2$PO$_4$
500 mM  NaCl (pH 4.0)
2.3.2 Solutions for Purification and Detection of ApoCIII-6xHis Protein from E. coli Cells

Lysis Buffer
50 mM \( \text{NaH}_2\text{PO}_4 \)
10 mM Tris-HCl (pH 8.0)
8 M UREA
500 mM NaCl

Elution Buffer
50 mM \( \text{NaH}_2\text{PO}_4 \)
8 M UREA
20 mM PIPES
500 mM NaCl (pH 6.0-6.3)

2.3.3 Solutions for Detection of Recombinant ApoCIII Protein

5 x SDS PAGE Sample Buffer
15% \( \beta \)-mercaptoethanol
15% SDS
50% Glycerol
1.5% Bromophenol blue

Tricine SDS-page running Buffer (bottom)
0.2 M Tris-HCl (pH 8.9)

Tricine SDS-page Running Buffer (top)
1 M Tris (pH 8.45)
0.1 M Tricine
0.1% SDS
TBST Buffer
10 mM Tris-HCl (pH 8.0)
150 mM NaCl
0.1% Tween-20

2.3.4 Solutions for LPL Assay

Incubation Mixture
12 % BSA
0.2 mg/ml Heparin
0.3 M Tris-HCl (pH 8.5)

Borgstrom Solution: heptane/isopropanol/1M H₂SO₄ = 483:400:10 (v:v:v.)

Alkaline Ethanol: 50 mM NaOH, 47.5%
### 2.4 Primers

**Table 2.1 Primers used to generate the apoCIII construct for the apoCIII expression**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHII</td>
<td>5'-CGAGGATCCCGAGGCCGAGGATGCC-3'</td>
</tr>
<tr>
<td>HindIII</td>
<td>5'-CAGCCCTGAAGCTTGCAAGGAC-3'</td>
</tr>
<tr>
<td>BEI</td>
<td>5'-CGAGGATCCCGAGGCCGAGGATGCC-3'</td>
</tr>
<tr>
<td>viral forward</td>
<td>5'-TTTACTGTTTTTCGTTACAGTTGT-3'</td>
</tr>
<tr>
<td>viral reverse</td>
<td>5'-CAACAACGCAACAGAATCTAG-3'</td>
</tr>
<tr>
<td>apoCIII</td>
<td>5'-TCCTTGTGTGGCCCTCCTC-3'</td>
</tr>
<tr>
<td>apoCIII forward</td>
<td>5'-GTGCTCCAGTACTTTCAGG-3'</td>
</tr>
<tr>
<td>apoCIII reverse</td>
<td>5'-TCCTTGTGTGGCCCTCCTC-3'</td>
</tr>
<tr>
<td>PhedrinFP</td>
<td>5'-AAATGATAACCATCTCGC-3'</td>
</tr>
<tr>
<td>PhedrinRP</td>
<td>5'-GTCCAAGTTTCCCTG-3'</td>
</tr>
<tr>
<td>ECOR I</td>
<td>5'-GGACCTTGGGAATTCCAGG-3'</td>
</tr>
<tr>
<td>BKMAX</td>
<td>5'-GCAGCATGGATCCCGACGATGATGATAAATCAGAGG-3'</td>
</tr>
<tr>
<td>GSTEN 3'</td>
<td>5'-CAGTCACAGTAATCAGTTACTCAGGCGAGCCA-3'</td>
</tr>
<tr>
<td>FAC</td>
<td>5'-CCGCGTGATCCATCGAAGTCGTTACAGAGGCC-3'</td>
</tr>
<tr>
<td>5' pGEX sequencing primer</td>
<td>5'-GGGGCTGGCAAGCCACGTTTGTTG-3'</td>
</tr>
<tr>
<td>3' pGEX sequencing primer</td>
<td>5'-CCGAGAGGCATGTGAGTGTCAGAGG-3'</td>
</tr>
<tr>
<td>5'NDEI23B</td>
<td>5'-GGGAATCCCATATATCGAGGAGGCCGAGGAT-3'</td>
</tr>
<tr>
<td>3'XHOI23B</td>
<td>5'-GTGTCATCGAGGAGGCACGCGCTGCTGAAAG-3'</td>
</tr>
<tr>
<td>T7 promoter primer</td>
<td>5'-TTAATACGACTCAGTATAGGG-3'</td>
</tr>
<tr>
<td>T7 terminator primer</td>
<td>5'-GTATTGCTAGCGGTG-3'</td>
</tr>
</tbody>
</table>
Table 2.2 Primers used to generate apoCIII variants by the *in vitro* site directed mutagenesis reactions

| 5’K21A | 5’-CATGAAGCACGCCACCCGCGACCAGGGATGCAC-3’ |
| 3’K21A | 5’-GTGCATCTCTGGGTGCGGTGCTTCATG-3’ |
| 5’F64AW65A | 5’-AAGTTCTCTGAGGCCGCGTGACCTTTGGGACC-3’ |
| 3’F64AW65A | 5’-GGGTCCAATACTCGGCGCTCAGAGAAGCTT-3’ |
| 5’L9T | 5’-AGGATGCTCCCTCCCTACCAGCTTCATGCAGGG-3’ |
| 3’L9T | 5’-CCCTGCATGAAAGCTGTAAGGGAGGCATCT-3’ |
| 5’T20L | 5’-GCTACATGAAGCAGCCTCTCAAGAGCAGGCAAGGATG-3’ |
| 3’T20L | 5’-CATCCTTGGCCTTTGGGCTTTGATGTCATGTAGC-3’ |
| 5’K21N | 5’-GAAGCACGCACCCACCCCGCGCCAAGGATG-3’ |
| 3’K21N | 5’-CATCCTTGGCCTTTGGGCTTTGATGTCATGTAGC-3’ |
| 5’F61A | 5’-GCACCGTAAAGGACAAGGCGCTCTGATTTGGGATTT-3’ |
| 3’F61A | 5’-AAATCCCAAGACTAGAGACCTTTGTCCTTAAACGGTGC-3’ |
| 5’A23T | 5’-CCACCAAGACCAACAGGATGCA-3’ |
| 3’A23T | 5’-TGATCCTTGGGTCTTGGGAT-3’ |
Chapter 3
Protein Modelling

3.1 Introduction

Protein modelling is commonly used to understand the function of the new protein according to the prediction of its structure, it has been aimed for designing new proteins and predicting the effect of point mutations.

The prediction of the structure of the new proteins is initially carried out by detection of the significant similarities of sequence to proteins of known structure. If the sequence of the protein with unknown structure has a significant sequence identical to a protein of known three-dimensional (3D) structure, the two proteins will probably share the same structures within the similar sequence. However, only a very small portion of new sequences has sequence similar to known structures. Among the human apolipoproteins, until now, only the structure of N-terminal apoE has been characterised by X-ray crystallographic studies (Wilson et al., 1991). Therefore, the most realistic prediction of functions of proteins with unknown structure is normally performed by understanding its structure according to the extracted properties from the amino acid sequence of functional proteins. It is well accepted that structure is more conserved than sequence, thus proteins with no significant sequence similarity can adopt the same 3D structure.

In the current studies, protein modelling was carried out to identify differences in functional properties of apoCIII variants, and also used to predict the potential functional changes caused by the amino acid substitution of the protein. In order to improve the accuracy of the protein modelling results, several methods were used to predict the structure-functional relationships of apoCIII. All the protein modelling work involved in this thesis was carried out by Dr. Laurence Lins and Prof. Robert Brasseur, in Centre de Biologique Moléculaire Numérique, Univeristy of Gembloux, Belgium. I thus describe background, methods and results of this part of work in this chapter, distinct from the other work carried out for this thesis.
3.2 Methods

3.2.1 Secondary Structure Prediction

The secondary structure prediction was carried out at the NPS@ (Network Protein Sequence Analysis) web site (http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=./NPSA/npsa_seccons.html). Different predictive methods were used to obtain the consensus: SOPMA (Geourjon and Deleage, 1995), PHD (Rost and Sander, 1993; Rost and Sander, 1994), Predator (Frishman and Argos, 1996), GORIV (Garnier et al., 1996), DPM (Deleage and Roux, 1987), DSC (King and Sternberg, 1996), SIMPA96 (Levin, 1997; Levin et al., 1986), and HNNC (Guermeur et al., 1999).

3.2.2 Molecular Hydrophobicity Potential (MHP) Calculations - 3D Construction of the Peptide

Construction of the peptides was carried out as previously described (Brasseur et al., 1992). The method accounted for the contribution of the lipid-water interface, the concomitant variation of the dielectric constant, and the transfer energy of atoms from a hydrophobic to a hydrophilic environment (Brasseur, 1990). The computational methodology presented here is more elaborated than the conventional search for internal homology based upon sequence similarities. Its major advantage is to provide a better insight in the conformation and hydrophobic properties of the apolipoprotein helical segments, such as the hydrophobic portion of the helix, and therefore provides the valuable information about the lipid binding ability of the amphipathic α-helical structure. The MHP results applied not only to the helical segments of apolipoproteins but can also account for differentiation between the helix response for the different functions.

The calculation of the MHP along the peptide was carried out assuming that the hydrophobic interaction between an atom and a point M decreased exponentially with the distance according to the equation (Brasseur, 1991):

\[ \text{MHP} = E_{\text{tri}} \exp (r_i - d_i) \]
where $E_{tri}$ is the energy of transfer of atom $i$, $r_i$ is the radius of the atom $i$, $d_i$ is the distance between atom $i$ and a point $M$.

Calculations were made in a plane perpendicular to the mean orientation of the long axis of the helix, which was moved every $2\AA$ along this axis, and the MHP was computed for all atoms contained in this plane.

3.3 Protein Modelling Results of the ApoCIII Variants

Consensus secondary structure prediction suggests that apoCIII is made of two helical domains, one in the N-terminal part, from approximately residues 5 to 40 and a second C-terminal helical domain localised at residues 45 to 60 (Figure 3.1). The region from residue 15 to 33 was shown to correspond to the core of the predicted N-terminal helical domain. Hydrophobic cluster analysis (HCA) of the entire apoCIII sequence identified two hydrophobic clusters at residues 6 to 20 and 42 to 66 localised within the two helical domains as detected from the secondary structure predictions analysis (Figure 3.2). The shape and hydrophobic repartition in these clusters indicate that they are amphipathic helices, suggesting a potential role in lipid binding. Both peptides were constructed in a 3D model and their favoured orientation at the lipid/water interface was calculated according to the method described (Brasseur, 1990) (Figure 3.4 A, C).

To investigate the respective role of the N- and C-terminal domains of apoCIII, we designed mutants, by modifying the amphipathicity and the aromatic content. The protein modelling results of these apoCIII variants are described as below.

3.3.1 ApoCIII-Ala23Thr (apoCIII-T23)

Protein modelling results suggested residue 23 lies on the N-terminal helical domains of the protein, from approximately residues 5 to 40. Additionally, it locates on the core of this helical domain region from residue 15 to 33. The distribution of the hydrophobic and hydrophilic envelopes suggests that this domain is amphipathic,
Figure 3.1 Secondary structure prediction of apoCIII. Different predictive methods were used (see methods) and a consensus was obtained.

h: α helix; e: β sheet; c: coil; t: turn
Figure 3.2 Hydrophobic cluster analysis (HCA) plot of the apoCIII sequence. Hydrophobic residues (F,Y,W,M,L,I,V) are circled and hatched; red stars are Pro; black diamond-shaped are Gly, white boxes are Thr and pointed boxes are Ser. Negatively charged residues are red and positively charged ones are blue. The two boxes indicate the main hydrophobic clusters of apoCIII.

since there is a rather good segregation between the hydrophobic and hydrophilic faces of the helix (Figure 3.3). This domain is therefore important in lipid binding but not in LPL inhibition. The Ala23Thr substitution induces a hydrophilic domain into the hydrophobic potential and therefore disturbs the amphipathicity of this α-helix (Figure 3.3 b, c) and therefore suggested to influence the affinity of the apoCIII-Thr23 to a lipid surface.

3.3.2 ApoCIII-Leu9Thr/Thr20Leu (apoCIII-T9L20)

The permutation of the Leu9-Thr20 residues avoided changes in hydrophobicity of the protein (that could have an effect by itself on the lipid-binding properties) and restored amphipathicity, since the mean axis of this peptide was oriented mostly parallel to the interface. Based on the protein modelling results, hydrophobic cluster 6-20 of apoCIII is a tilted peptide, which can associate with
Figure 3.3 Molecular hydrophobicity potential (MHP) surfaces around the 15-33 segment. CPK representation of the 15-33 peptide of A) WT-apoCII and B) apoCIII-T23. N terminus is left and C terminus is right. MHP surfaces around the 15-33 segment in the same orientation. Green surfaces represent hydrophilic domains while orange surfaces represent hydrophobic domains. The surfaces are cut with a plane to visualise the mutated residue identified with an arrow.
phospholipid at an angle of 45° (Figure 3.4, A), the permutation of the Leu9-Thr20 residues could alter this angle to 0° and affect the lipid binding properties of apoCIII (Figure 3.4, B).

3.3.3 ApoCIII-Lys21Ala (apoCIII-A21)

This mutation was designed to alter the first protein-protein binding-domain in the N-terminus of apoCIII and therefore affect LPL inhibition of apoCIII.

3.3.4 ApoCIII- Phe64Ala/Trp65Ala (apoCIII-A64A65)

Protein modelling results has shown that the 42-66 peptide is amphipathic and with its main axis mostly parallel to the interface (Figure 3.4, C). The N-terminal peptide was tilted towards the interface, due to an uneven segregation of the hydrophilic and hydrophobic residues along the long axis of this particular α-helix (Figure 3.4, C). Another interesting feature of the 42-66 helix was the unusually high content of aromatic residues (Phe, Trp and to a lesser extent Tyr), especially at the C-terminus of the peptide (Figure 3.2).

The double mutation apoCIII-A64A65 was designed to perturb the lipid binding properties of apoCIII by replacing two adjacent C-terminal aromatic amino acids with Alanine. These mutations changed both the amphipathicity (Figure 3.4, D) and the aromatic content as compared to the 42-66 WT-apoCIII fragment (Figure 3.4, C). The mutation of those two bulky hydrophobic residues located at the C-terminal extremity of the predicted second amphipathic helix was shown to drastically improve the propensity of this segment to fold as a α-helix, and therefore favour α-helix formation. However, the aromatic content change caused by the double mutation largely affects the hydrophobic interactions between the protein and phospholipid. The lipid binding results from this variant can thus help us to compare the contribution of two effects in the lipid binding of apoCIII: 1) the interaction between the amphipathic α-helical structures and the fatty acid chain of phospholipid. 2) the initial interaction between the polypeptide and the fatty acid chain of
phospholipid during the initial phases of the lipid binding. Our results showed that the latter is more important for the interaction between the protein and phospholipid.

Figure 3.4 Orientation of the different apoCIII peptides (represented in CPK) at the lipid/water interface (orange plane). Lipid phase is up and water phase is below. A. 6-20 peptide; B. 6-20 peptide of apoCIII-T9L20 mutant; C. 42-66 peptide; D. 42-66 peptide of apoCIII-A64A65 mutant.
Chapter 4

Expression of ApoCIII Protein in Insect Cells Using
Baculoviral Vector

4.1 Introduction

In order to obtain large quantities of apoCIII proteins to perform the structure-functional studies, it was necessary to develop a suitable apoCIII expression system. We first started with the baculoviral expression system since this is an eukaryotic expression system and thus uses many of the protein modification processing and transport system present in higher eukaryotic cells, such as glycosylation, a modification relevant to apoCIII.

Baculovirus belongs to a diverse group of large double-stranded DNA viruses that infect many different species of insects as their natural hosts. The baculovirus genome is replicated and transcribed in the nuclei of infected host cells where the large baculovirus DNA is packaged into rod-shaped nucleocapsids. Since the size of these nucleocapsids is flexible, recombinant baculovirus particles can accommodate large amounts of foreign DNA. The baculoviral expression system uses a helper-independent virus that can be propagated to high titres in insect cells adapted for growth in suspension cultures, making it possible to obtain large amounts of recombinant protein. Additionally, compared to mammalian cells, insect cells are relatively easier to culture and can grow in both monolayer and suspension.

There are over 500 baculoviruses reported in the literature. However, *Autographa Californica* nuclear polyhedrosis virus (AcMNPV) is the most extensively studied virus of the baculovirus family and was chosen to use as the parental viral DNA in this study, since AcMNPV-based systems have a number of advantages for most common applications. Cell lines supporting AcMNPV replication are superior in growth characteristics than cells lines supporting other commercially provided baculoviruses. The insect cell sf9 and sf21 used for the propagation of AcMNPV typically double every 18-24 hours and are less time consuming compared to the cells
used for the other baculoviruses. Additionally, expression levels of the cell lines supporting AcMNPV are higher than that of other baculoviruses. Furthermore, the range of transfer vectors and parent viruses available for the AcMNPV-based system is much greater than that of the other parental baculoviruses (O'Reilly D.R. et al., 1993a).

A very strong promoter, polyhedrin (polh) promoter was used for the apoCIII expression in the current study. This promoter functions very late in infection and is normally responsible for the synthesis of polyhedra, the occlusion body matrix protein that is involved in a visible plaque characteristic, although it is nonessential for the life cycle of the baculovirus under the tissue culture conditions. Non-recombinant virus still contains an intact polyhedron gene and so cells infected with the wild-type virus are seen to contain bright granules of polyhedron protein in the occlusion body in these cells (occ'). Insertion of the APOC3 gene downstream of the polh promoter displaces polyhedra gene and the insect cells infected with the recombinant virus do not contain the bright granules, therefore favouring the screen of the recombinant baculovirus by selection of the occ' infected cells.

In order to establish whether this expression system could provide sufficient amount of recombinant apoCIII proteins for the structure-functional study, an initial expression study was carried out with WT-apoCIII and apoCIII-T23 (the brief procedures are described in Figure 4.1). However, due to their low expression levels and the difficulty in purifying the recombinant proteins, we did not carry on the expression of other apoCIII variants using this system. The results involved in this chapter are therefore only limited to the studies of WT-apoCIII and apoCIII-T23.

4.2 Expression of ApoCIII Protein by using pBlueBacIII as Transfer Vector

The pBlueBacIII is a non-fusion vector that provides the AcMNPV polh promoter sequences to drive high expression and a multiple cloning site for single subcloning. This vector also co-expresses β-galactosidase from the AcMNPV early to late (ETL) promoter (a late promoter which is much weaker than the polyhedrin promoter) as a reporter for visual identification of blue recombinant plaques in the presence of X-gal.
Figure 4.1 Procedure of Wild Type ApoCIII Expression Using pBlueBacIII as Transfer Vector in Insect Cells

amplify the WT-apoCIII insert from pBSSK/apoCIII by PCR

↓

close the apoCIII insert into pBlueBac III (pBBIII/WT-apoCIII)

↓

cotransfect pBBIII/apoCIII and AcMNPV into insect cells using a cationic liposome mediated transfection procedure

↓

identify pure recombinant virus by plaque (picking the blue plaques)

↓

amplify the virus from the identified plaques

↓

extract viral DNA from the amplified virus

↓

check the purity of the virus by PCR using the viral DNA as template

↓

further amplify the identified pure virus

↓

infect the insect cells to express apoCIII proteins
4.2.1 Plasmid Construction of pBBIII/WT-apoCIII

The human apoCIII cDNA in the pBSSK vector (Stratagene) was used as a template for the amplification of the apoCIII gene by PCR as described in section 2.2.3. Two primers, the 5' forward primer (BamHI) with an internal BamHI site and a 3' reverse primer (HindIII) (Table 2.1) with an internal HindIII site were used for the PCR. The resulting 390 bp fragment of wild type human apoCIII was digested with BamHI/HindIII and subcloned into a similarly digested pBlueBacIII vector as described in section 2.2.2 (Invitrogen) (Figure 4.2). The ligation reactions were transformed into *E. coli* strain Top10 as described in section 2.2.2.4 and the transformed cells were selected with ampicillin (75 µg/ml) on LB-agar plates. The entire apoCIII cDNA sequence was confirmed in both directions using the primer apoCIII cDNA forward and apoCIII cDNA reverse (Table 2.1) respectively, on an ABI 377 prism DNA sequencer (Perkin-Elmer) as described in section 2.2.5. The isolated plasmid DNA (pBBIII/WT-apoCIII) of the selected clone was stored at -20 °C.

![Figure 4.2 pBlueBac III vector](image-url)
4.2.2 Generation of pBBIII/A23T-apoCIII Mutation

The mutation pBBIII/A23T-apoCIII was generated using the QuikChange™ Site-Directed Mutagenesis Kit as described in section 2.2.4 with pET23b/WT-apoCIII as template and two synthetic oligo primers 5’A23T and 3’A23T that containing the desired mutation (see Table 2.2) and complementary to opposite strands of the vector. Candidate clones were screened by sequencing the apoCIII insert on an ABI 377 prism DNA sequencer as described in section 2.2.5, both to confirm the presence of the mutation and to ensure no alterations at other sites.

4.2.3 Identification of Recombinant AcMNPV/pBBIII/apoCIII Virus

Insertion of the apoCIII insert into the transfer vector allowed the apoCIII gene to be cloned downstream of the viral polh promoter and flanked on both sides by viral sequences that would target the gene and promoter to a particular region in the viral genome. Recombinants of the plasmid pBBIII/apoCIII and parental viral DNA virus AcMNPV had been achieved by the homologous recombination occurred between the regions of viral DNA flanking the foreign gene in the plasmid and their homologous counterparts in the viral genome. When the purified plasmid DNA of pBBIII/apoCIII was co-transfected into the insect cells (sf9) with parental viral DNA (AcMNPV) using a cationic liposome mediated transfection procedure (see section 2.2.7.2), the enzymes in the cells recombine these DNAs. The medium of the co-transfected cells was then used to perform the plaque purification to obtain the pure recombinant virus as described in section 2.2.13. In our case, the recombinant virus was identified by a white-blue screening of the plaques by picking the blue occ- plaques. The pure virus was further confirmed by PCR using the viral primers: viral forward primer and viral reverse primer (Table 2.2) as described in section 2.2.3 using the viral DNA (see section 2.2.7.5) isolated from the amplified viral stock (see section 2.2.7.4) as template. By using these primers, the PCR product of the pure parental virus AcMNPV is 838 bp (Lane 7 of Figure 4.3). The PCR product of a recombinant virus depends on the size of both the inserted gene and the transfer plasmid used (620 bp for pBlueBac III), therefore, the PCR product of pure AcMNPV/pBBIII/apoCIII virus was 1010 bp (390 + 620) (Lane 1 of Figure 4.3). The PCR products of impure recombinant
AcMNPV/pBBIII/apoCIII virus contaminated with the parental virus AcMNPV were detected as two bands with different sizes (838 bp and 1010 bp) (Lane 2 to Lane 6 of Figure 4.3). Additionally, PCR products obtained by using the combination of the viral primer and the internal apoCIII primer (apoCIII cDNA forward and apoCIII cDNA reverse), for example apoCIII cDNA forward primer + viral reverse primer or apoCIII cDNA reverse + viral forward primer (data not shown) can confirm that apoCIII had been inserted into the recombinant virus at the correct location and in the correct orientation.

![Image of gel with bands](image)

**Figure 4.3 PCR detection of apoCIII insert in isolated recombinant virus.**

PCR was performed using viral primers: viral forward primer and viral reverse primer and the amplified fragments were detected on 1% agarose gel.

M: DNA marker  
Lane 1 is the PCR product of pure recombinant AcMNPV/pBBIII/apoCIII virus.  
Lane 2 - Lane 6 are the PCR products of recombinant AcMNPV/pBBIII/apoCIII virus contaminated with the parental virus AcMNPV.  
Lane 7 is the PCR product of the parental virus AcMNPV

### 4.2.4 Expression of Recombinant ApoCIII Protein

Protein expression of both wild type apoCIII and apoCIII-T23 were first performed in sf9 insect cells using SFM-II medium (see section 2.2.7.6) and the expression levels were initially determined from time course studies (see section...
2.2.7.6), although both proteins were expressed, expression levels were low (Figure 4.4).

![MW (kDa)](image)

**Figure 4.4 Expression of recombinant WT-apoCIII protein using pBlueBac III in insect cells.** Insect cells sf9, sf21 and Hi5 cells were infected with wild type apoCIII-AcMNPV recombinant viruses at MOI of 10 (pfu)/cell and harvested at 72 hours post infection. Both the cell lysate and medium samples were separated on 17.5% precast Tricine-SDS-PAGE and detected by Western blotting using the polyclonal anti-apoCIII antibody.

M: molecular weight marker
Lane 1: 200 ng of human plasma apoCIII (SIGMA)
Lane 2: media from sf9 cells
Lane 3: cell lysate from sf9 cells
Lane 4: media from sf21 cells
Lane 5: cell lysate from sf21 cells
Lane 6: cell lysate from Hi5 cells
Lane 7: media from Hi5 cells

When the human apoCIII inserts were cloned into the pBlueBac III vector, their original signal peptide was kept. This was based on the consideration that the mammalian signal peptide could be processed normally in the insect cells (O'Reilly D.R. et al., 1993b) and that this signal peptide would direct the recombinant human apoCIII protein into the endoplasmic reticulum (ER) and be cleaved from the recombinant protein. The mature recombinant apoCIII protein would then be secreted into the cell culture media. If the expression and secretion levels of recombinant apoCIII are high enough, the media could be used in functional studies directly without further purification. Even if the expression and secretion levels of apoCIII are not high...
enough to allow the direct use of the media, the purification of the recombinant protein from the media will still be much easier than that from the insect cells. From our results, however, the recombinant proteins remained in the sf9 cells rather than being secreted into the medium, since the recombinant apoCIII was only detectable from the cell lysate (obtained as described in section 2.2.7.6) but not from the medium (Figure 4.4, Lane 2 & 3), indicating the signal peptide of human apoCIII was not used efficiently. Additionally, the recombinant apoCIII proteins were detected as two bands with different sizes, suggesting two different glycosylation levels or absent cleavage of the signal peptide. This result is consistent with the above hypothesis that the signal peptide of human apoCIII was not processed normally by the sf9 cells. However, the answer to these questions was not discovered in our studies.

Furthermore, the recombinant apoCIII proteins remained in the cells at a low level of only about 1-2 mg/L culture. The recombinant protein was therefore not detectable on a Coomassie Blue stained gel as described in section 2.2.6.2, and was only detectable by Western-blotting using a polyclonal antibody (Figure 4). In order to overcome these problems, we have tried to use the wider range of MOI (1, 2, 12 and 15) and the other insect cells sf21 and High 5 (Hi5). Both sf9 and sf21 cells originated from IPLB-SF-21 cells, which were derived from *S. frugiperda* pupal ovarian tissue at the USDA Insect Pathology Laboratory at Beltsville, Maryland. The Hi5 cell line was established originally from minced adult ovaries of *T. ni* (cabbage loop) and do not grow in suspension, making it inconvenient for large-scale cell culture (O'Reilly D.R. et al., 1993a). We tried this cell line since it is reported to exhibit up to one log higher levels of protein expression compared to sf9 (Invitrogen Corporation advertisements). However, use of the two additional cell lines and the different MOI improved neither the apoCIII expression levels nor its secretion levels.

Taken together, both the lack of secretion of the recombinant apoCIII into medium from the insect cells and the low level of the recombinant apoCIII remained in the insect cells (less than 5% of the host cell proteins), made purification difficult. We therefore decided to use pBlueBacHis 2A as the transfer vector to perform the apoCIII expression.
4.3 Expression of ApoCIII Using pBlueBacHis 2A as Transfer Vector

Using pBlueBacHis 2A this vector, recombinant apoCIII proteins are fused to a tag of six tandem Histidine residues followed by an enterokinase cleavage site at its N-terminus. The 6xHis tag creates a high-affinity metal binding site therefore allowing purification of recombinant fusion proteins on a nickel chelating resin and the His tag can be cleaved away from the fusion protein by enterokinase without destroying the proper folding and functional properties of the protein.

Another change combined with using of pBlueBacHis 2A as the transfer vector is the use of Bac-N-Blue™ AcMNPV DNA as the parental viral DNA instead of wild type AcMNPV. Bac-N-Blue™ AcMNPV DNA is linearized to remove some sequence domains essential for efficient propagation of the virus. When recombination occurs between pBlueBacHis2 and Bac-N-Blue™ AcMNPV DNA, these essential sequences in the viral DNA are restored, since the pBlueBacHis2 contain these sequence domains homologous to those found in Bac-N-Blue™ AcMNPV DNA. The virus is therefore viable, and recombinant virus is then produced. Theoretically, the entire viable virus should be recombinant. Obviously, these processes should increase the efficiency of the recombination between the DNAs.
4.3.1 Plasmid Construction

Plasmid DNA of pBBIII/WT-apoCIII and pBBIII/apoCIII-T23 were used as templates for PCR respectively as described in section 2.2.3. Two primers, the forward primer (BE1) with an internal *Bam*HI site and a reverse primer (PhedrinRP) with an internal *Hind*III site to remove the original apoCIII signal peptide sequence were used (Table 2.1). The resulting 350 bp apoCIII fragments were digested with *Bam*HI/*Hind*III and subcloned into a similarly digested pBlueBacHis 2A vector (see section 2.2.2) (Invitrogen) (Figure 4.5). The ligation reactions were transformed into *E. coli* strain JM109 cells as described in section 2.2.2.4 and the transformed cells were selected with ampicillin (75 µg/ml) on LB-agar plates. The entire apoCIII cDNA sequence was sequenced in both directions, using an ABI 377 prism DNA sequencer (Perkin-Elmer) as described in section 2.2.5. The isolated plasmid DNA (pBBH2A/WT-apoCIII and pBBH2A/T23) of selected clone was stored at -20 °C.

4.3.2 Expression and Purification of Recombinant 6xHis-apoCIII Protein

Co-transfection and identification of the two recombinant viruses of AcMNPV/pBBH2A/apoCIII (wild type and the T23 mutation) were performed as described above using Bac-N-Blue™ rather than AcMNPV DNA. Both apoCIII variants were expressed and remained in the insect cells and were separated on 17.5 % precast Tricine-SDS-PAGE. However, as previously described in section 4.2.4, the results obtained from the time course study of recombinant apoCIII expression (see section 2.2.7.6), showed the recombinant 6xHis-apoCIII proteins were expressed at a low level even at the wide range of the infection conditions (MOI of 5-10). Figure 4.6 shows the results obtained by using sf9 cells, the recombinant apoCIII protein in the cell lysate samples were only detectable by Western-blotting using a polyclonal antibody but not on a Coomassie Blue stained gel (data not shown). Use of sf21 and Hi5 cells showed the same results (data not shown).
Figure 4.6 Detection of recombinant 6xHis-apoCIII fusion proteins expressed in sf9 cells infected by different MOI. All the cells were infected with wild type AcMNPV/pBBH2A/apoCIII recombinant virus and harvested at 72 hours post infection. The cell lysate samples were separated on 17.5 % precast Tricine-SDS-PAGE and detected by Western blotting using the polyclonal anti-apoCIII antibody.

M: molecular weight marker  
Lane 1: 200 ng of human plasma apoCIII (from SIGMA)  
Lane 2: cell lysate from AcMNPV infected cells  
Lane 3: cell lysate from sf9 cells infected at MOI = 5  
Lane 4: cell lysate from sf9 cells infected at MOI = 7.5  
Lane 5: cell lysate from sf9 cells infected at MOI = 10

Despite the low expression level of the recombinant 6xHis-apoCIII protein (1-5 mg/L), the large amount of sf21 cells infected by AcMNPV/pBBH2A/WT-apoCIII recombinant virus (see section 2.2.7.7) were used to purify these fusion proteins with nickel chelating resin (Invitrogen) as described in section 2.2.7.8. This purification system is based on the strong interaction between electropositive metals, such as Ni²⁺, and histidine residues (Figure 4.7). Metals such as nickel have six sites available, arranged in an octahedral configuration around each metal atom. Each co-ordination site is capable of interacting with an electron-rich ligand such as histidine, tryptophan, or cysteine. Therefore the 6xHis tag creates a binding site with high affinity to the metal within proteins to allow the purification of recombinant fusion proteins with a nickel-chelating resin. The binding affinity of a 6xHis tag to a nickel chelating resin is approximately $K_d = 10^{-13}$, which was higher than most antibody/antigen or enzyme/substrate interactions (pET System Manual, 1997a). This interaction between 6xHis tag and nickel chelating resin can thus tolerate strong washing conditions that can remove the contaminating host protein. However, we did not get any pure apoCIII
fusion protein under both the native and the denaturing conditions (see section 2.2.7.8). This was probably due to the fact that expression level of 6xHis-apoCIII was too low, about 1-5 mg/L culture, less than 10% of the total cell protein, which made it impossible to allow the fusion protein to be purified by the nickel-chelating resin from the contaminated cell proteins.

Figure 4.7 Schematic diagram of 6xHis-tag affinity purification system

Part A. Nickel-chelating resin; P: polymer (Agarose). TC: tetridentate chelator, 2+: metal ion
Part B. H: 6xHis tag fused recombinant protein

4.4 Summary

Both the wild type apoCIII and the apoCIII-T23 proteins were expressed at low level in the baculovirus system using either pBlueBacIII or pBlueBacHis 2A as transfer vectors. Additionally, when using pBlueBacIII, the recombinant apoCIII proteins (wild type and mutated) were not secreted into the medium as we had expected, suggesting the signal peptide of human apoCIII has not been used efficiently by the insect cells. However, these results do not mean that the baculovirus expression system is not a suitable system for the apolipoprotein expression. The same system has been used to express human apoB-17 successfully in this laboratory, and provided a high level of the secreted apoB-17 proteins (unpublished data). The signal peptide of the human apoB-17 was processed very well in the insect cells and directed the
recombinant apoB-17 into the ER and was cleaved when the protein was secreted. The previous study by Gretch (Gretch et al., 1991) has shown the successful expression of the recombinant baculovirus encoding human apoE3 that retains its native properties. The low expression levels of apoCIII could partly due to its small size, since the recombinant protein might be degraded by the proteases in the cells more rapidly, or maybe because that its DNA structures do not favour the expression of the recombinant protein. However, it is still unknown why apoCIII was not expressed well in this system.

Having failed with the baculoviral expression system, we therefore turned our attention to an *E. coli* expression system.
Chapter 5
Expression of ApoCIII in E. coli

5.1 Introduction

Due to the unsatisfactory results with the baculoviral expression system, we therefore turned our attention to an E. coli expression system that has the advantage of being low cost and the less time consuming. As a procaryotic system, the E. coli expression system is unable to use many of the protein modification processing and transport system present in higher eukaryotic cells such as glycosylation, which is part of process for native apoCIII expression. However, it is generally believed that the glycosylation of apoCIII has little impact on its lipid binding properties. Formation of the amphipathic α-helix, the key motif responsible for the lipid binding properties of apoCIII does not require this modification. This point was confirmed by the study reported by Roghani and Zannis, which introduced the *in vitro* site-directed mutation into human apoCIII gene to alter Thr74 to Ala74 and therefore abolish the glycosylation site of the protein. Their results showed that cell clones expressing the APOC3 gene exclusively secreted the un-sialylated apoCIII, suggesting intracellular O-glycosylation of apoCIII was not required for its intracellular transport and secretion. Additionally, lack of the glycosylation had no effect on its relative affinities for plasma VLDLs and HDLs (Roghani and Zannis, 1988). Furthermore, the results from the study of synthetic apoCIII (McConathy et al., 1992) indicated that the glycosylation of apoCIII was not necessary for its LPL inhibition. The recombinant apoCIII proteins obtained from the E. coli expression system would therefore be suitable to perform the structure-functional studies. Further evidences to support this point came from the functional studies of Guinea pig apoCII using the recombinant apoCII proteins obtained from the E. coli expression system, that showed the efficient lipid binding ability and the stimulation of the LPL activity (Andersson et al., 1997).
Several bacterial expression vectors were employed to express apoCIII variants. But apart from pGEX2T and pET23b vectors, they gave either a very low expression level or no detectable expression.

5.2 Expression and Purification of Recombinant GST-ApoCIII Fusion Protein Using the pGEX2T Vector

The pGEX2T vector is designed for inducible, intracellular expression of genes or gene fragments fused with GST, a 26 kDa protein. Fusion proteins (GST-apoCIII) can be easily purified from bacterial lysate by affinity chromatography using Glutathione Sepharose 4B. Cleavage of the desired protein from GST can be achieved using a site-specific protease, thrombin, whose recognition sequence is located immediately upstream from the multiple cloning sites on the pGEX2T plasmid.

5.2.1 Plasmid Construction

![Figure 5.1 pGEX-2T vector](image)

Figure 5.1 pGEX-2T vector
Plasmid DNA pBBH2A/WT-apoCIII (See section 4.2.1) and pBBH2A/apoCIII-T23 (See section 4.2.2) were used as templates for PCR, respectively, as described in section 2.2.3. The 5' forward primer with an internal BamHI site (BEI) and a 3' reverse primer with an internal EcoRI site (ECOR I) were used. The resulting 278 bp fragment were digested by BamHI/EcoRI and subcloned into the similar digested pGEX-2T vector (Figure 5.1) as described in section 2.2.2. The candidate clones were selected with ampicillin (75 μg/ml) on LB-agar plates. The entire apoCIII cDNA sequence was sequenced in both directions, using 5’ pGEX sequencing primer and 3’ pGEX sequencing primer (Table 2.1) with an ABI 377 prism DNA sequencer (Perkin-Elmer) as described in section 2.2.5. The isolated plasmid DNA (pGEX-2T/WT-apoCIII and pGEX-2T/apoCIII-T23) of selected clone was stored at -20 °C. The resulting construct had a tac promoter followed by the sequence of GST, the thrombin cutting site and the sequence of apoCIII.

5.2.2 Expression and Isolation of Recombinant GST-apoCIII

The plasmid DNA of pGEX-2T/WT-apoCIII and pGEX-2T/apoCIII-T23 were transformed into the E. coli strain BL21 (DE3) as described in section 2.2.2.4. Both of the GST-apoCIII fusion proteins were expressed in BL21 (DE3) cells as described in section 2.2.8.2 at high levels and were purified successfully by one-step purification using Glutathione Sepharose 4B as described in 2.2.8.3. About 30-50 mg of the pure GST-apoCIII fusion proteins was obtained from 1L of E. coli culture. The expression and purification of the GST-apoCIII fusion proteins were analysed by 15% SDS-PAGE as described in section 2.2.6.1 followed by Coomassie Blue staining as described in section 2.2.6.2 (Figure 5.2), the eluted GST-apoCIII fusion proteins with molecular weight of 35 kDa were at least 95% pure. Western blotting results using rabbit-anti apoCIII polyclonal antibody (performed as described in section 2.2.6.4, protocol 1) have confirmed the presence of apoCIII in the fusion protein (data not shown).
Figure 5.2 Purification of GST-apoCIII fusion proteins. The samples were separated on 15% SDS-PAGE and were detected by Coomassie Blue staining.

M: molecular weight marker
Lane 1, 2, 3: E. coli lysate. From left to right: GST-WT-apoCIII fusion protein, GST-apoCIII-Thr23 fusion protein and GST protein only
Lane 4, 5, 6: Elute from Glutathione Sepharose 4B. From left to right: GST-WT-apoCIII fusion protein, GST-apoCIII-T23 fusion protein and GST protein

5.2.3 Cleavage of GST from the GST-ApoCIII Fusion Proteins Using Thrombin

Both GST-apoCIII fusion proteins were sent to our collaborator Dr. Gunilla Olivecrona at Umeå in Sweden and used in the LPL assay to detect their LPL inhibitory activity. However, neither GST-WT-apoCIII nor GST-apoCIII-T23 showed any effect on LPL activity similar to the GST protein control (data not shown). It was therefore necessary to remove the GST from the GST-apoCIII fusion protein prior to performing the structure-functional studies of apoCIII. Although there is an internal thrombin cleavage site in the apoCIII protein between Arg40 and Gly41, thrombin was first used to perform the cleavage of the GST since the internal thrombin cleavage site is the less optimal recognition sequence compared to the thrombin cleavage site in the GST-apoCIII fusion protein. It was thought that it might be possible to manipulate the
thrombin cleavage sites by titration, so that thrombin only cleaved at the better site and therefore the GST-apoCIII fusion protein could be cleaved under certain conditions without the internal cleavage.

![Digestion of GST-apoCIII fusion protein by thrombin](image)

**Figure 5.3 Cleavage of GST-apoCIII fusion protein by thrombin.** GST-apoCIII fusion protein was digested by thrombin in glutathione elution buffer at 22 °C for 10 hours. The samples were separated on 17.5 % precast Tricine-SDS-PAGE and detected by Coomassie Blue staining.

M: molecular weight marker  
Lane 1: GST-apoCIII fusion protein, incubated in glutathione elution buffer only  
Lane 2 to 11: GST-apoCIII fusion protein, incubated in glutathione elution buffer with 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5 units of thrombin.  
Lane 12: GST protein only

Thrombin cleavage was completed using 50 µg of GST-apoCIII fusion protein as described in section 2.2.8.6. In order to determine the optimal cleavage conditions, different temperatures (4 °C, 16 °C, 22 °C and 37 °C), different amounts of thrombin (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 unit of thrombin) and different incubation times (2, 4, 6, 8, 10, 12, 14 and 16 hours) were performed as described in section 2.2.8.5. The cleavage mixture was analysed on 17.5 % precast Tricine-SDS-PAGE (Bio-Rad) as described in section 2.2.6.1 and first detected by Coomassie Blue staining as described in section 2.2.6.2. Since the unfused apoCIII fragments were not identified, the cleavage mixture was further detected by silver staining as described in
section 2.2.6.3 and Western blotting using rabbit-anti apoCIII polyclonal antibody as described in section 2.2.6.4 (protocol 1). However the desired unfused apoCIII was never detected (data not shown) probably due to the fact that the thrombin cleavage occurred on the apoCIII proteins cleaved from the GST-apoCIII fusion protein. To overcome this problem, more specific protease cleavage sites were introduced into the above construct between GST and apoCIII after the thrombin site by PCR to replace the thrombin site (Figure 5.4).

5.2.4 Introducing the Enterokinase/Factor Xa Cleavage Site into the pGEX-2T /apoCIII Construct

Having failed to obtain the unfused recombinant apoCIII protein through the thrombin cleavage of the GST-apoCIII fusion protein, we therefore turned our attention to factor Xa and enterokinase cleavage instead, since there are no factor Xa and enterokinase internal cleavage sites in the apoCIII protein. Both enzymes, particularly enterokinase, are supposed to be highly specific and only tolerate very few changes to their recognition sites. Enterokinase recognises the sequence -(Asp)$_4$Lys and cleaves after the lysine residue, the four-aspartyl residues act as a signal for enterokinase cleavage. It has been reported that the rate of hydrolysis is reduced with only three aspartyl residues and the two aspartyl residues preceding the lysyl residues are the minimum number of amino acid residues needed to maintain its function (Light and Janska, 1989). Both of the cleavage sites were inserted into the construct of GST-thrombin site-apoCIII (pGEX-2T/WT-apoCIII and pGEX-2T/apoCIII-T23) by PCR as below.

Plasmid DNA pBBH2A/WT-apoCIII (See section 4.2.1) and pBBH2A/apoCIII-T23 (See section 4.2.2) were used as templates for PCR respectively as described in section 2.2.3. The 5' forward primer with an internal BamHI site (FAC) and the 3' reverse primer with an internal EcoRI site (GSTEN 3') were used. The resulting 290 bp fragments were subcloned into pGEX-2T vector as described in section 5.1.1. The new constructs (pGEX-2T/GST-FX site-apoCIII) had a tac promoter followed by the sequence of GST, a thrombin cutting site, a factor Xa cleavage site and
Figure 5.4 Insertion of enterokinase and factor Xa cleavage sites in GST-apoCIII construct by PCR

```
[ Thrombin ] [ Enterokinase ] [ ApoCIII →
Leu Val Pro Arg↓ Gly Ser Asp Asp Asp Asp Lys↓ Ser Glu Ala Glu Asp
CTG GTT CCG CGT GGA TCC GAT GAT GAT GAT AAA TCA GAG GCC GAG GAT
\[BamHI\]  
introduced sequence

[ Thrombin ] [ Factor Xa ] [ ApoCIII →
Leu Val Pro Arg↓ Gly Ser Ile Glu Gly Arg↓ Ser Glu Ala Glu Asp
CTG GTT CCG CGT GGA TCC ATC GAA GGT CGT TCA GAG GCC GAG GAT
\[BamHI\]  
introduced sequence
```
apoCIII. An enterokinase cleavage site was inserted in the same way except using oligonucleotides EKMAX instead of FAC (see Table 2.1) for PCR and obtained a fragment of 293 bp.

All the new constructs were transformed into the *E. coli* strain BL21 (DE3) for protein expression and all the recombinant GST fusion proteins from the above four constructs were expressed at high levels (30-50 mg/L culture) and isolated successfully.

### 5.2.5 Cleavage of the GST-apoCIII Fusion Protein Using Enterokinase and Factor Xa

The cleavage of GST from the fusion proteins using both enterokinase and factor Xa (performed as described in section 2.2.8.6) were still problematic.

Both the cleavage reactions were studied extensively under different conditions as described in section 2.2.8.6. Different time points, different amounts of proteases and different temperatures were employed. In order to determine the optimal cleavage conditions, enterokinase cleavage was completed in the enterokinase reaction buffer (EKmax™ buffer) at different temperatures (4 °C, 16 °C, 22 °C and 37 °C) and detected at different time points (2, 4, 6, 8, 10, 12, 14 and 16 hours). Factor Xa cleavage was also performed under the same conditions. The GST-apoCIII fusion protein could always be cleaved under certain conditions, since the products with the smaller size than GST-apoCIII fusion protein were observed on 17.5 % precast Tricine-SDS-PAGE as shown in Figure 5.3. However the desired unfused apoCIII was never detected.

Furthermore, time course studies of cleavage reactions using both enterokinase and factor Xa were performed at 22 °C respectively as described in section 2.2.8.6. The mixture of equal molar of plasma apoCIII (5 μg) and the GST-apoCIII fusion proteins (20 μg) were used in these studies and samples were taken at 0, 20, 40, 80, 120 and 180 minutes after the digestion and analyzed on Tricine-SDS-PAGE followed by silver staining. Figure 5.5 shows the results of the time course study using
enterokinase. Instead of seeing the accumulation of the cleaved apoCIII from the GST-apoCIII fusion protein, the free apoCIII was found to disappear rapidly and the plasma apoCIII was cleaved even quicker than the GST fusion apoCIII. Similar results were obtained in factor Xa digestion. These results confirmed that the cleavage had occurred internal of the apoCIII protein and also suggested that the incorrect or unspecific cleavage occurred in GST-apoCIII fusion protein. This may be because that GST (26 kD) is four times bigger than apoCIII (8.8 kD) and presumably the inserted protease

MW (kDa)

Figure 5.5 Co-digestion of human plasma apoCIII protein and GST-WT-apoCIII fusion protein by enterokinase. 5 µg of human plasma apoCIII protein and 20 µg of recombinant GST-WT-apoCIII fusion protein were mixed and digested with enterokinase in EKmax™ buffer at 25 °C in the final volume of 30 µl for the time course study. 1.5 µl of each sample was separated on 17.5 % precast Tricine-SDS-PAGE and followed by silver staining.

M: molecular weight marker
Lane 0: 200 ng of human plasma apoCIII
Lane 1 to 6: the reaction mixture taken at 0, 20, 40, 80, 120, 180 minutes after the digestion
Lane 7: 200 ng of human plasma apoCIII was incubated in EKmax™ buffer at 25 °C for 180 minutes
recognition site or even the N-terminal of apoCIII were masked by GST secondary structure and were not exposed properly. In order to establish if this was the real reason for the inability to produce cleaved intact apoCIII, the cleavage reaction was also studied under denaturing conditions in which the protease recognition site in the fusion proteins would be better exposed to the protease. Different levels of urea (0, 0.25, 0.5, 0.75, 1 and 2M) and Tween-20 (0.2, 0.3, 0.4, 0.5 and 1%) (final concentrations) were employed to denature the fusion protein prior to the cleavage by both enterokinase and factor Xa, but there was little improvement. When the GST fusion protein was denatured by heat, there was no significant improvement with regard to the cleavage reaction although a tiny band corresponding to the apoCIII on the gel appeared (data not shown). However, it was not possible to isolate and purify it, because the small amount of apoCIII was contaminated with the large amount of other proteins caused by the non-specific cleavage.

These results suggested that the GST-apoCIII construct was not suitable for the expression of apoCIII due to the difficulties to cleave GST from the fusion protein to obtain the pure recombinant apoCIII protein.

5.3 Expression and Purification of Recombinant ApoCIII Protein Using pET23b Vector

Having failed to express apoCIII using pGEX-2T vector, I have tried several other bacterial expression vectors to express apoCIII protein, including pET29a (Novagen), pET15b (Novagen), pRSETA (Invitrogen), pQE9 (Qiagen) and pET23b (Novagen).

The proteins expressed by pRSETA and pET15b are fused to a N-terminal 6xHis tag and the proteins expressed by pET29a are fused to a N-terminal S-tag. S-tag is a 15 amino acid peptide which has high binding affinity with the 104 amino acid S-protein ($K_d = 10^{-4}$) portion of ribonuclease A to form a tightly bound complex (pET System Manual, 1997b). The two tags allow for easy affinity purification using affinity resins and could also be cleaved from the fusion proteins. Since these two tags are
smaller than GST, the cleavage of these tags from the fusion proteins was thought to be easier than the cleavage of GST. However, vectors pRSETA and pET15b resulted in very low expression levels while pET29a although it resulted in the reasonable expression level the cleavage of the S-tag from the resulting apoCIII fusion protein was prone to be difficult, since the intact apoCIII cleaved from the GST-apoCIII fusion protein was not detected. We therefore converted to using the vectors that provide the un-cleavagable 6xHis tag, pQE9 and pET23b. pQE9 is designed for expression of N-terminal 6xHis tag fusion proteins but only offered very low expression levels of apoCIII. Finally apoCIII was expressed using pET23b vector as a C-terminal 6xHis tag fusion protein. The details of the expression and purification of apoCIII-6xHis protein are described below.

5.3.1 Plasmid Construction

Vector pET23 (Novagen) was provided as pET23a, b and c with different reading framing. pET23b was used for apoCIII expression as the most appropriator to give the correct reading frame.

Plasmid DNA of pBBIII/WT-apoCIII (See section 4.2.1) and pBBIII/apoCIII-T23 (See section 4.2.2) were used as templates for PCR respectively as described in section 2.2.3. Two primers, the 5’ forward primer 5’NDEI23B with an internal NdeI site and a 3’ reverse primer 3’XHOI23B with an internal XhoI site were used (See Table 2.1). The resulting 263 bp fragments were digested with NdeI/XhoI and subcloned into a similarly digested pET23b vector (Figure 5.6) as described in section 2.2.2. The candidate clones were selected as described in section 5.1.1. The entire apoCIII cDNA sequence was sequenced in both directions, using both T7 promoter primer and T7 terminator primer (Table 2.1) with an ABI 377 prism DNA sequencer (Perkin-Elmer) as described in section 2.2.5. The isolated plasmid DNA (pET23b/WT-apoCIII and pET23b/apoCIII-T23) of selected clone was stored at -20 °C. All the new constructs (pET23b/apoCIII) had a T7 RNA polymerase promoter followed by the sequences of apoCIII and 6xHis-Tag.
5.3.2 Generation of ApoCIII Variants

ApoCIII variants were generated by *in vitro* Site-directed Mutagenesis reaction as described in section 2.2.4 using pET23b/WT-apoCIII as template. pET23b/apoCIII-A21 was generated using primer 5'-K21A and 3'-K21A while pET23b/apoCIII-A64A65 was generated using primers 5'-F64AW65A and 3'-F64AW65A. pET23b/apoCIII-T9L20 was generated by two-steps mutagenesis reaction by first change L9 to T9 using primers 5'-L9T and 3'-L9T, the plasmid DNA from the resulting construct was used as a template to performed the second mutagenesis reaction using 5'-T20L and 3'-T20L and this resulted in the pET23b/apoCIII-T9L20 construct. All the primers used in the *in vitro* site-directed mutagenesis reactions are listed in Table 2.2. The new constructs were screened as described in section 5.3.1. The apoCIII insert was detected by sequencing on an ABI 377 prism DNA sequencer using both T7 promoter primer and T7 terminator primer as described in section 2.2.5, both to confirm the presence of the mutation and to ensure no alterations at other sites.
5.3.3 Optimisation of the Expression Conditions of ApoCIII-6xHis Fusion Proteins

Due to the fact that these experiments were very time consuming, in studies of the optimisation of the expression conditions of apoCIII-6xHis fusion proteins, all the apoCIII-6xHis proteins were first expressed in small scale in different expression conditions and detected as described in section 2.2.8.1.

Initially the recombinant apoCIII-6xHis fusion proteins were not expressed at high levels, and all the fusion proteins were insoluble inside the cells. This might have been due to the fact that the proteins were bound to the cell membrane or did not fold properly, rather than the fusion proteins were expressed as insoluble aggregates (inclusion bodies) because the expression level (1-5 mg/L culture) was quite low. In order to overcome this obstacle, different growth conditions to optimise expression levels were studied to encourage cells to express at higher levels of the soluble recombinant proteins. The media, E. coli strain, length of induction and induction conditions were the factors that could affect the cellular environment of E. coli. The effect of these factors on the yield of both total recombinant fusion protein and soluble recombinant protein were systematically examined, but none of them improved the solubility of apoCIII fusion protein when cells were broken by sonication in the presence of 1% Triton X-100. However the yield of the total recombinant fusion protein was largely improved to up to 5% of the total cell protein in certain conditions although these proteins still remained insoluble.

The following results describe the optimisation of expression conditions for every apoCIII variants.

5.3.3.1 Media

Plasmid DNA of pET23b/WT-apoCIII was transformed into BL21 (DE3) cells and expressed in the different media with varying glucose levels (0, 5, 10, 20 and 40 mM). Several different media were used for the expression studies, including 2xTY, LB, Super Broth, TB and SOC media. Figure 5.7 shows the different expression levels of WT-apoCIII-6xHis fusion protein in these media. All the samples were purified
from the cell lysate from 15 ml cell cultures by Talon™ cobalt metal affinity resin as
described in section 2.2.8.1. The resulting samples were separated on 17.5% precast
Tricine-SDS-PAGE followed by the visualisation of Coomassie Blue staining. LB,
Super Broth and 2xTY media provided very low expression levels. Using TB medium
improved the expression levels but SOC (containing 20 mM of glucose) appeared to be
the optimal medium for the expression of apoCIII-6xHis fusion protein.

MW (kDa)

Figure 5.7 Expression of recombinant wild type apoCIII-6xHis in the different
media. The samples were purified from the E. coli cell lysate by Talon™ cobalt metal
affinity resin and separated on 17.5% precast Tricine-SDS-PAGE followed by the
visualisation of Coomassie Blue staining.

M: molecular weight marker
Lane 1-5: TB (terrific broth) medium containing 0, 5 mM, 10 mM, 20 mM and 40 mM
of glucose.
Lane 6-10: 2xTY medium containing 0, 5 mM, 10 mM, 20 mM and 40 mM of glucose.
Lane 11-14: SOB medium containing 0, 5 mM, 10 mM and 20 mM of glucose.

5.3.3.2 E. coli Strains

The different E. coli strains including BLR (DE3), HMS174 (DE3), BL21
(DE3), B834 (DE3), JM109 (DE3) and NOVABlue (DE3) were used to detect the
expression levels of the different apoCIII variants respectively. Plasmid DNA of each
pET23b/apoCIII variant was transformed in these cells respectively and expressed in SOC medium contained 20 mM of glucose.

**Figure 5.8** Expression of recombinant wild type apoCIII fusion protein in different *E. coli* strains. Wild type apoCIII was expressed in different cells using SOC (with 20 mM glucose) medium and induced by 0.2 mM IPTG at 37 °C for 2 hours. The samples were purified from the *E. coli* cell lysate by Talon™ cobalt metal affinity resin and separated on 17.5% precast Tricine-SDS-PAGE followed by the visualisation of Coomassie Blue staining.

M: molecular weight marker
Lane 1: NOVABlue (DE3) cells
Lane 2: HMS174 (DE3) cells
Lane 3: BLR (DE3) cells
Lane 4: B834 (DE3) cells
Lane 5: JM109 (DE3) cells
Lane 6: BL21 (DE3) cells

Figure 5.8 shows the different expression levels of WT-apoCIII in the different *E. coli* strains. The cell lysate from 15 ml of *E. coli* cultures were purified by Talon™ cobalt metal affinity resin as described in section 2.2.8.1. All the resulting samples were separated on 17.5% precast Tricine-SDS-PAGE and detected by Coomassie Blue staining. The different cell strains provided the significantly different expression levels
for each apoCIII variant. According to these results, WT-apoCIII and apoCIII-A64A65 were therefore expressed in B834 (DE3) cells while apoCIII-T23, apoCIII-T9L20 and apoCIII-A21 were expressed in BL21 (DE3) cells for the best expression.

5.3.3.3 Different Induction Conditions

The optimal induction time of the different apoCIII variants in the different E. coli strains, including 30 minutes, 1 hour, 2 hours, 3 hours and overnight were compared. Apart from apoCIII-T23, with the optimal induction time of 30 minutes, all the other apoCIII variants required optimal induction times of 2 hours. Figure 5.9 shows the time course studies of WT-apoCIII and apoCIII-T23 expression. The WT-apoCIII in B834 (DE3) cells and apoCIII-T23 in BL21 (DE3) cells were cultured in SOC medium (containing 20 mM of glucose) and 15 ml of cell cultures were collected at different time. The cell lysate from these E. coli culture were purified by Talon™ cobalt metal affinity resin as described in section 2.2.8.1 and separated on 17.5% precast Tricine-SDS-PAGE followed by the visualisation of Coomassie Blue staining as described in section 2.2.6.1 and 2.2.6.2. This figure shows that recombinant apoCIII with a reasonable expression level could be first detected as early as 30 minutes after induction. Expression level of apoCIII-T23 protein starts falling very early, indicating its instability in the E. coli cells and therefore required short induction time (1 hour). Compared with apoCIII-T23, recombinant proteins of all the other apoCIII variants were more stable and therefore required longer induction time (2 hours) (data not shown). As shown in figure 5.9, the expression level of WT-apoCIII continued to increase until 2 hour after induction before the protein levels finally started falling. However, after overnight induction, almost no recombinant protein remained in the cells, possibly due to intracellular degradation, indicating that recombinant apoCIII proteins were not stable for a long time in the E. coli cells at 37 °C.
Figure 5.9 Time course of apoCIII-6xHis expression. Recombinant WT-apoCIII and apoCIII-Thr23 fusion proteins were expressed in SOC (with 20 mM glucose) medium and induced by 0.2 mM IPTG at 37 °C for different induction times. The samples were purified from the *E. coli* cell lysate by Talon™ cobalt metal affinity resin and separated on 17.5% precast Tricine-SDS-PAGE followed by the visualisation of Coomassie Blue staining.

Lane 1-6: The samples that were collected at 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours and overnight respectively.
M: molecular weight marker
Lane 7-12: The samples that were collected at 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours and overnight respectively.

The different induction temperatures were therefore compared. Plasmid DNA of WT-apoCIII was transformed into B834 (DE3) cells and cultured in SOC medium (containing 20 mM glucose) at 37 °C before being induced by 0.5 mM IPTG for 2 hours at different temperatures (37 °C, 30 °C, 25 °C and 18 °C). 15 ml of the cell culture was used to detect the expression levels as described above. Figure 5.10 shows the different expression levels of WT-apoCIII-6xHis at different induction temperatures. 37 °C was shown to be the optimal temperature for WT-apoCIII expression, and hopefully indicating the common induction temperature for all the tested apoCIII variants.

<table>
<thead>
<tr>
<th>Host cells</th>
<th>WT-apoCIII</th>
<th>apoCIII-T23</th>
</tr>
</thead>
<tbody>
<tr>
<td>B834 (DE3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.10 Induction of recombinant WT-apoCIII at different temperatures. WT-apoCIII was expressed in SOC (with 20 mM glucose) medium and induced by 0.5 mM IPTG at different temperatures (37 °C, 30 °C, 25 °C and 18 °C) for 2 hours. The samples were purified from the *E. coli* cell lysate by Talon™ cobalt metal affinity resin and separated on 17.5% precast Tricine-SDS-PAGE followed by the visualisation of Coomassie Blue staining.

M: molecular weight marker
Lane 1-4: The samples induced at different temperatures (37 °C, 30 °C, 25 °C and 18 °C respectively).

5.3.4 Purification of Recombinant ApoCIII-6xHis Protein

Since all the recombinant apoCIII proteins were insoluble inside the *E. coli* cells, apoCIII proteins had to be purified under denaturing condition in the presence of 8 M urea. Because proteins expressed by pET23b vector were fused at the C-terminus to a tag of six tandem Histidine residues, the fusion proteins were thus firstly purified using Talon™ cobalt metal affinity resin. Compared to nickel-chelating resin (mentioned in section 4.3.2, used in purification of recombinant 6xHis-apoCIII from insect cells), this resin binds 6xHis tag less tightly and exhibits a significantly reduced affinity for non-6xHis fused proteins. After the first purification using Talon™ cobalt metal affinity resin performed as described in section 2.2.8.4, the recombinant apoCIII proteins were the major component of the eluate although contaminated with some proteins of the host cells. In order to remove the contamination protein, more stringent purification conditions were employed using a lower washing buffer pH (pH 7.5, 7.0...
and 6.5), however, little improvement was obtained and one of the contaminating host proteins with molecular weight of approximately 26 kDa bound to the Talon™ cobalt metal affinity resin even stronger than the recombinant apoCIII fusion protein (data not shown). It was therefore impossible to obtain the pure apoCIII from the cell lysate through the one-step purification only using Talon™ cobalt metal affinity resin.

![MW (kDa)](image)

**Figure 5.11 Expression and purification of the recombinant WT-apoCIII fusion protein.** The samples were separated on 17.5% precast Tricine-SDS-PAGE followed by the visualisation of Coomassie Blue staining.

Lane 1: molecular weight makers  
Lane 2: bacterial extracts of the un-induced cells  
Lane 3: bacterial extracts of the cells induced by 0.2 mM IPTG for 2 hours at 37 °C  
Lane 4: elute from Talon™ cobalt metal affinity resin  
Lane 5: pure apoCIII fraction after MonoQ column chromatography

Further purification by anion exchange chromatography was performed in FPLC system at 4 °C as described in section 2.2.8.5. The fractions containing the pure apoCIII were eluted at NaCl concentration of approximately 0.15 M. All the apoCIII variants were at least 95% pure. The 26 kDa host protein was shown to behave very similarly to apoCIII-6xHis fusion protein during the anion exchange chromatography, since the two proteins shared the same peak and fortunately apoCIII-6xHis was eluted...
at the earlier fractions. After the two step purification, approximately 5 mg of the pure recombinant apoCIII fusion protein was obtained from 1 L of the culture. Figure 5.11 showed the samples during the purification of the recombinant WT-apoCIII protein from the cell lysate. The pure apoCIII fractions were then dialysed against 5 mM NH₄HCO₃ at 4 °C to remove urea and used for the functional studies.

5.4 Discussion

Many eukaryotic proteins are poorly expressed in *E. coli*. In order to obtain the high expression levels, human apoCIII variants were initially expressed as their GST fusion proteins since the expression of the fusion protein is primarily dependent on the expression level of the N-terminal target protein, GST protein. The relatively larger size of GST protein compared to apoCIII (26 kD and 8.8 kD) would be likely to reduce the potential negative effect on the expression levels of the GST fusion proteins caused by apoCIII. In agreement with this, none of the other N-terminal fusion apoCIII proteins fused to either S-protein (15 amino acids) or 6xHis tag gave higher expression levels than the GST-apoCIII fusion proteins. However, the recombinant apoCIII was finally expressed as a C-terminal 6xHis tag fused protein without any N-terminal fusion partners, though the expression levels were much lower than that of the GST-apoCIII fusion proteins.

Due to the low solubility of the apoCIII-6xhis fusion protein in the cells, the proteins were purified under the presence of a high concentration of urea and followed by dialysis prior to the functional studies, thus allowing the restructure of recombinant proteins into their native structures. All the proteins remained soluble after the dialysis and were used in the functional studies directly. However, it was unclear if the structure of the apoCIII refolded properly until the secondary structures of the proteins were detected by CD showed about 20% α-helix in the free proteins, which was similar to the previous results obtained from plasma apoCIII. The dialysed recombinant apoCIII proteins therefore was properly folded (Chapter 6).
Although all the recombinant proteins were C-terminal 6xHis tag fusion proteins, all the physicochemical data point to the similar behaviour of the fusion and natural apoCIII, confirming that function was not affected by its presence. Functional characterisation of un-cleaved N-terminal 6xHis-tagged recombinant apoAI (Rogers et al., 1998; Bergeron et al., 1997) and C-terminal 6xHis-tagged recombinant LCAT (Chisholm et al., 1999) have been reported to be no different when compared to plasma apoAI or LCAT, respectively. In addition, the extra 6 Histidine residues are unlikely to be well structured and therefore are unlikely to affect the function of the recombinant proteins. Taken together, our expression and two-steps purification systems of apoCIII described in this chapter provided the useful tools for the structure-functional studies of this important apolipoprotein and will allow other functional studies to be performed.
Chapter 6
Interaction of ApoCIII Variants with Lipids

6.1 Introduction

Most of the biological functions of apolipoprotein: receptor binding, enzyme activation and lipid transport require their association with lipid. Some apolipoproteins, like apoCIII, although they are secreted from cells in the lipid poor form, still remain bound to lipoprotein particles in the circulation. During its residence in plasma, apoCIII exchanges between different lipoproteins undergoing metabolic transformations together with other apolipoproteins, such as other apoCs, apoE and apoAI. In most cases, apolipoproteins only express their biological functions at the lipid-bound state.

None of the lipoproteins can be synthesised and secreted without the corresponding apolipoproteins. The lipid binding properties of apolipoproteins are particularly important for the formation and stabilisation of the lipoprotein structure, as well as the metabolism of the proteins themselves. Apolipoproteins in solution have been shown not to have a stable three-dimensional structure and could be denatured much more easily than most of the water-soluble globular proteins (Tall et al., 1975). In the presence of phospholipid dispersion, however, these apolipoproteins bound to the lipid surfaces, markedly increased in α-helical structure, and became stabilised towards heat or chemical denaturation (Tall et al., 1975; Pownall et al., 1977).

Because of the central role of the apolipoproteins-lipid interaction in defining the structure and function of apolipoproteins and in modulating the metabolism of lipoproteins, much research has been done on this subject. However, the majority of the work has been done on apoAI and apoE. The major breakthrough during these studies came in 1974, when the segments of apolipoprotein sequences that form an amphipathic α-helical structures were identified. It has been shown that the α-helix with hydrophobic residues on one side interact with the fatty acid chain of phospholipids and polar residues on the other side, provide the interface with water
The structural assembly of circulating plasma lipoproteins is mainly due to the phospholipid-apolipoprotein interactions in the outer polar layer of these molecules (Mahley et al., 1984). The role of the amphipathic α-helical structure as the fundamental lipid binding motif of apolipoproteins has been confirmed by the synthesis of potentially amphipathic peptides that rapidly bind lipid (Sparrow, Jr., 1981), and the direct observation of amphipathic α-helical segments in the crystallographic studies of an insect apolipoprotein (Breiter et al., 1991) and the N-terminus of human apoE (Wilson et al., 1991).

As an exchangeable apolipoprotein, apoCIII binds to almost all the lipoprotein particles with the exception of LDL. Several studies aiming to identify the lipid binding properties of apoCIII have already been performed. However, the structure-functional relationship involved in its lipid binding properties is not fully understood. Using the thrombin-cleaved apoCIII fragments, the C-terminus of apoCIII was shown to be important in the lipid binding properties of the protein since a phospholipid complex was formed with the C-terminus of apoCIII (41-79) while the N-terminus of the protein (1-40) failed to do so (Sparrow et al., 1977). Additionally, the C-terminal amphipathic α-helix between residues 50 and 62 was also shown to mediate the binding of apoCIII to phospholipid (Trieu and McConathy, 1995). Similarly the lipid binding domains of apoE and apoAl have been shown to be located at the C-terminus of the protein (De Pauw et al., 1995; Holvoet et al., 1995). Sequence analysis and secondary structure predictions performed by Brasseur and colleagues (Brasseur et al., 1992) applied to apoCIII suggest that there are two amphipathic domains in apoCIII which should be responsible for the protein-lipid interaction, one was defined as peptide 11-28, and the other was peptide 35-52. While the modelling results of the current study illustrate that the two hydrophobic clusters at residues 6-20 and 42-66 localised within the two amphipathic helices domains (See Chapter 3).

In order to map precisely the apoCIII domains that are functionally important for its lipid binding properties, we have expressed recombinant wild type and several site-directed mutants of apoCIII (See Chapter 3) and performed detailed physicochemical studies to clarify further the lipid binding properties of apoCIII. 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) multilamellar vesicles (MLV) were employed for the lipid moiety. DMPC is an artificial phospholipid and has been
commonly used in the structure-functional studies involved in the lipid binding properties of variant apolipoproteins. We chose to use these particles since they are more homogeneous than the lipoproteins isolated from individual donors and apoCIII deficient plasma was not available and endogenous apoCIII would affect the results. The previous studies have shown that the mechanism of the interaction of apoAl with the HDL surface and DMPC MLV is very similar, since the results obtained with the DMPC binding assay using recombinant apoAl are in agreement with the results from the HDL binding assay. Using of DMPC MLV is therefore a good model to perform the lipid binding properties of apoCIII since HDL is the major carrier of apoCIII in the plasma and contains 60% of the apoCIII. The results from this model can at least reflect the interaction of apoCIII between the phospholipid on HDL particles.

The studies described in this and next chapter were performed in Department of Biochemistry, University of Gent (Belgium) in the lab of Professor Maryvonne Rosseneu.

6.2 Lipid Binding Properties of the ApoCIII Variants

Lipid binding properties of the recombinant apoCIII variants were performed to assess the interaction of the apoCIII variants (including GST-WT-apoCIII, GST-apoCIII-T23 and GST, as well as the C-terminal 6xHis tag fused WT-apoCIII, apoCIII-T23, apoCIII-T9L20, apoCIII-A21 and apoCIII-64A65A) with DMPC as described in section 2.2.9.2. Briefly, it was carried out by monitoring the turbidity change of DMPC MLV (obtained as described in section 2.2.9.1) at 325 nm after being mixed with the recombinant apoCIII proteins as a function of the temperature through the transition temperature of DMPC MLV (23.5 °C) from 16 °C to 36 °C. The principle of these experiments is that when the proteins bind to DMPC MLV, the turbidity of DMPC MLV will largely decrease due to the formation of discoidal complexes between the protein and DMPC.

The study was first performed using GST fusion proteins (GST-WT-apoCIII, GST-apoCIII-T23 and GST) and these proteins did not bind to DMPC MLV based on the lack of a large turbidity change as a function of temperature (Figure 6.1) while plasma apoCIII and WT-apoCIII-6xHis fusion protein did. These results indicated
Figure 6.1 Turbidity decrease of DMPC mixed with GST-apoCIII fusion proteins as a function of the temperature. 80 μg of DMPC was mixed with 40 μg of GST-apoCIII fusion proteins in 5 mM NH₄HCO₃ (pH 8.0). The turbidity decrease was expressed as percentage of the initial O.D. value at 325 nm.
that the lipid binding regions of apoCIII in GST-apoCIII proteins were not exposed. According to our previous conclusion made in Chapter 5 (section 5.2.5) that the N-terminal of apoCIII may not be exposed under the native conditions, a further conclusion can therefore be made that most of the apoCIII, including the most potentially lipid binding domain located at C-terminal of apoCIII, were also be masked by the GST protein. GST-apoCIII fusion proteins therefore failed to function in both the LPL assays and lipid binding. All the following lipid binding experiments involved in study of the structure-functional relations of apoCIII were performed using the recombinant apoCIII-6xHis fusion proteins.

When all the recombinant apoCIII-6xHis fusion proteins were used in the study by mixing with DMPC MLV, a similar large decrease in the turbidity of DMPC MLV at 325 nm as a function of temperature was observed that was maximal around the transition temperature of the DMPC (23.5 °C) for all wild type and mutant apoCIII-6xHis proteins (Figure 6.2), indicating the recombinant apoCIII-6xHis fusion proteins bound to DMPC successfully. As these recombinant proteins were expressed using E. coli cells, and the fact that proteins expressed in E. coli are not glycosylated suggests that the glycosylation of apoCIII is not necessary for its lipid binding properties. Additionally, with all apoCIII proteins a similar decrease in the turbidity of the DMPC solution was observed that was maximal around the transition temperature of the DMPC (normally observed at 23.5 °C). The resulting apoCIII:DMPC discoidal complexes were fractionated on a Superose 6 PG gel filtration column in the FPLC system as described in section 2.2.9.4, the apoCIII content of these fractions was monitored by measuring the Trp emission at 330 nm. These results were reproducible, and results from a representative experiment are presented in Figure 6.3. In all the gel filtration runs no free lipid could be detected which would typically elute within the void volume of the column (below the elution volume of 20 ml). All the apoCIII:DMPC complexes eluted as homogeneous peaks, although the amount of unbound apoCIII and the size of the discoidal complexes formed was quite different for each protein. The elution volume of the complexes varied from 26 ml (calculated stokes radius 80 Å or 733 kDa) for the largest complex formed with the apoCIII-A64A65 variant, to 29.5 ml (calculated stokes radius 54 Å or 185 kDa) for the smallest complex formed with the apoCIII-T9L20 variant. The elution volume of the DMPC complex of wild type apoCIII and mutant apoCIII-A21
Figure 6.2 Turbidity decrease of DMPC mixed with recombinant apoCIII-6xHis fusion proteins as a function of the temperature. 80 μg of DMPC was mixed with 40 μg of apoCIII proteins in 5 mM NH₄HCO₃ (pH 8.0). The turbidity decrease was expressed as percentage of the initial O.D. value at 325 nm.
Figure 6.3 Gel filtration of apoCIII:DMPC complexes in FPLC system. 40 µg of each apoCIII protein was mixed with 80 µg of DMPC to form the apoCIII:DMPC complexes. The complexes were analysed by gel filtration on a Superose 6 HR column followed by the Trp fluorescence emission measured at 330 nm as a function of the elution volume.
Table 6.1 Ratio of apoIII bound to DMPC/free protein upon the complex forms

<table>
<thead>
<tr>
<th></th>
<th>WT- apoCIII</th>
<th>apoCIII- T9L20</th>
<th>apoCIII- A21</th>
<th>apoCIII- T23</th>
<th>apoCIII- A64A65</th>
</tr>
</thead>
<tbody>
<tr>
<td>bound/free</td>
<td>3.6</td>
<td>7.8</td>
<td>1.9</td>
<td>2.1</td>
<td>0.51</td>
</tr>
<tr>
<td>size of the complex</td>
<td>61 Å</td>
<td>54 Å</td>
<td>72 Å</td>
<td>72 Å</td>
<td>80 Å</td>
</tr>
</tbody>
</table>

Figure 6.4 Regression line between size of the complexes and ratio of apoCIII bound to DMPC/Free protein The size of the apoCIII:DMPC complexes was presented as elution volume (ml). The amount of the DMPC bound protein and the free protein were calculated by the area under the curve as determined from the gel filtration elution profiles. The ratio of the DMPC bound apoCIII and the free protein during the complexes forming were also listed in Table 6.1
was 29 ml and 27.3 ml, respectively, corresponding to stokes radii of 61 and 72 Å. The naturally occurring mutation apoCIII-T23 formed a complex with the same size as apoCIII-A21:DMPC complex. The fraction eluting at 38 ml corresponds to unbound apoCIII, which due to the small MW of the protein, is well separated from the protein-phospholipid complexes. Although the differences of the sizes are small, in terms of molecular weight these represent a large difference. Additionally, apoCIII-A64A65 was associated with the highest amount of unbound/free apoCIII protein while apoCIII-T9L20 resulted in the lowest amount of the unbound /free protein among all the apoCIII variants, despite the fact that identical amounts of protein, lipid and protein:lipid incubation ratios were used for the preparation of the complexes. A clear correlation \( r=0.9 \) between the elution volume (i.e. stokes radius) and the ratio of bound /free apoCIII, as presented in Table 6.1, could be observed and this is also represented in the Figure 6.4.

6.3 Physicochemical Characteristics of the Lipid-Free and Lipid-Bound ApoCIII Variants

It is generally accepted that the CD spectrum of a protein is a direct reflection of its secondary structure (Johnson, Jr., 1988). ApoCIII proteins and apoCIII protein with 50% 2,2,2-Trifluoro-Ethanol (TFE) mixtures were determined by CD measurements between 184 and 260 nm on a Jasco 600 spectropolarimeter at room temperature. All the samples are placed in a 0.1 cm pathlength quartz cell and their secondary structures were obtained by curve-fitting on the entire ellipticity curve as described in section 2.2.10.1. (Figure 6.5 and Table 6.2). All the apoCIII variants showed fairly random structure in buffer solution (5 mM NH₄HCO₃ (pH 8.0)) with typical minimal around 200 nm (Figure 6.5 (a)). The propensity to form a α-helical structure was demonstrated by adding 50% TFE. The large increases in secondary structure are illustrated in Figure 6.5 (b). In all spectra the two typical minima were approximately 208 and 222 nm, and the single maximum at 190 nm was observed. These wavelengths are typical features of a α-helical structure. The spectra were analysed according to the method described in section 2.2.10.1, and the results are summarised in the Table 6.2. For all apoCIII wild type and mutant proteins the α-
Figure 6.5 CD spectra of free apoCIII proteins (a) and apoCIII/TFE mixtures (b). All the spectra were recorded at room temperature with a 0.1 cm cell path length between 184 and 260 nm at 0.2 mg/ml of protein, nine spectra averaged.
α-helical content of apoCIII after binding to lipid increased by about 25-54%, illustrating that as a result of the binding to the lipid, the secondary structure of the proteins was stabilised. The increase in the α-helical content was most pronounced for the apoCIII-A64A65 variant (54%), followed by apoCIII-A21, apoCIII-T9L20 (31%) and apoCIII-T23 (30%) and less pronounced for wild type apoCIII (25%) demonstrating the different abilities of each apoCIII variants to fold as an α-helix.

**Table 6.2 Amphipathicity structure of apoCIII proteins determined by CD in the presence or absence of 50% TFE**

<table>
<thead>
<tr>
<th></th>
<th>WT-apoCIII</th>
<th>apoCIII-T9L20</th>
<th>apoCIII-A21</th>
<th>apoCIII-T23</th>
<th>apoCIII-A64A65</th>
</tr>
</thead>
<tbody>
<tr>
<td>% α in free protein</td>
<td>20</td>
<td>20</td>
<td>23</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>% α helix 50% TFE</td>
<td>45</td>
<td>51</td>
<td>54</td>
<td>50</td>
<td>74</td>
</tr>
<tr>
<td>increased % α helix</td>
<td>25</td>
<td>31</td>
<td>31</td>
<td>30</td>
<td>54</td>
</tr>
</tbody>
</table>

The stability of the lipid free apoCIII proteins and the apoCIII:DMPC complexes (made as described in section 2.2.9.3 and isolated as described in section 2.2.9.4) was estimated by following the decrease in their α-helical content measured by CD (molar ellipticity at 222 nm) after addition of increasing amounts of GdnHCl as described in section 2.2.10.2. The concentrations of GdnHCl at which 50% of the signal had decreased (the midpoints) are summarised in Table 6.3. The midpoints of all the lipid free apoCIII proteins were below 2 M; 1 M for apoCIII-T9L20, 1.75 M for apoCIII-A64A65, 1.25 M for wild type apoCIII, 1.5 M for apoCIII-A21 and apoCIII-T23. Once associated with DMPC, the apoCIII proteins were stabilised and protected from denaturation by the phospholipid, resulting in a shift of each apoCIII:DMPC complex midpoint towards higher GdnHCl concentrations, as shown in the Table 6.3. The midpoint of the wild type apoCIII:DMPC complex was 3 M and
2.4 M for both apoCIII-A21:DMPC and apoCIII-T23:DMPC complex. ApoCIII-A64A65:DMPC complex denatured more readily with the lowest midpoint of 2 M, suggesting a less stable lipid-associated complex, while the apoCIII-T9L20:DMPC complex showed the highest midpoint of 3.4 M after DMPC binding, indicating the formation of the most stable apoCIII:DMPC complex. The association of apolipoproteins with phospholipid interfaces was studied and described by Pownall and colleagues (Pownall et al., 1987). In this model, the denaturation of apolipoprotein-phospholipid complexes by GdnHCl was shown to involve two steps. Firstly, the protein unfolds but remains bound to the lipid surface by hydrophobic interactions. Secondly, higher GdnHCl concentrations and longer times eventually lead to desorption of the apolipoprotein. When an apolipoprotein binds to lipid efficiently, the above two-step denaturation will be reflected as a two-phase denaturation as a function of increasing GdnHCl concentration. In the case of an apolipoprotein binding to lipid less well, the first step denaturation will finish rather rapidly and the intact denaturation will be reflected as one-phase denaturation. In our study, the fact that two-phase denaturation occurred for wild type apoCIII:DMPC and apoCIII-T9L20:DMPC complexes while one-phase denaturation occurred on all the other apoCIII:DMPC complexes, indicates that WT-apoCIII and apoCIII-T9L20 show the strongest lipid binding properties compared to the other apoCIII variants, and is consistent with the results obtained from other experiments performed in this study.

Table 6.3 Stability of free recombinant apoCIII proteins and the apoCIII:DMPC complexes. The stability of each sample was presented as M½, the concentration of GdnHCl (M) required to obtain 50% reduction of the CD signal measured at 222 nm as a measurement of protein denaturation.

<table>
<thead>
<tr>
<th></th>
<th>WT-apoCIII</th>
<th>apoCIII-T9L20</th>
<th>apoCIII-A21</th>
<th>apoCIII-T23</th>
<th>apoCIII-A64A65</th>
</tr>
</thead>
<tbody>
<tr>
<td>free protein</td>
<td>1.25</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
<td>1.75</td>
</tr>
<tr>
<td>apoCIII:DMPC</td>
<td>3.0</td>
<td>3.4</td>
<td>2.4</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Δ M½</td>
<td>1.75</td>
<td>2.4</td>
<td>0.9</td>
<td>0.9</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Figure 6.6 Denaturation of free apoCIII (a) and the lipid bound apoCIII (b) proteins by GdnHCl. The denaturation was performed by incubating the apoCIII samples with the increased concentration of GdnHCl (0-6 M) in 5 mM of NH₄HCO₃ (pH 8.0) at 4 °C for 12 hours. The samples were monitored by the change in molecular ellipticity (deg. cm⁻¹.mol⁻¹) that measured at protein concentration of 0.2 mg/ml by CD at 222 nm as a function of the GdnHCl concentration.
When apoCIII bound to lipid, the maximal Trp emission wavelengths of the protein could be decreased, as shown in Figure 6.7. This decrease is called blue shift and is due to the reduced exposure of tryptophan residues in apoCIII protein to the solvent after the formation of protein:DMPC complex and indicates a transfer of these residues to a more hydrophobic environment. The maximal Trp emission wavelengths of the free apoCIII proteins and the apoCIII:DMPC complexes were measured as described in section 2.2.10.3 and summarised in Table 6.4. The maximal wavelength of the Trp emission is 343 nm for all the lipid free proteins and shifted to 333 nm for the lipid-bound apoCIII proteins in the apoCIII:DMPC complexes. In the case of the apoCIII-T9L20 an even larger blue shift of 12 nm was observed. This result illustrated that tryptophans of apoCIII-T9L20 are buried in a more hydrophobic environment due to the stronger lipid binding.

![Fluorescence spectra of free WT-apoCIII protein and lipid bound WT-apoCIII protein.](image)

**Figure 6.7 Fluorescence spectra of free WT-apoCIII protein and lipid bound WT-apoCIII protein.** Fluorescence emission spectra of free WT-apoCIII (25 μg/ml) and lipid bound WT-apoCIII (WT-apoCIII:DMPC complex) (60 μg/ml) in 5 mM NH₄HCO₃ (pH 8.0) were recorded at wavelengths between 300 and 450 nm with an excitation wavelength at 290 nm. The maximal fluorescence emission wavelengths of the Trp residues for the two samples are labelled as indicated.
Table 6.4 Blue shift of the apoCIII variants after bound with DMPC

<table>
<thead>
<tr>
<th></th>
<th>WT-apoCIII</th>
<th>apoCIII-T9L20</th>
<th>apoCIII-A21</th>
<th>apoCIII-T23</th>
<th>apoCIII-A64A65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δλ (nm)</td>
<td>10</td>
<td>12</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

From these observations we can conclude that the substitution of F64A/W65A largely diminished the ability of apoCIII to bind to DMPC, however, it contained the highest portion of amphipathicity structure of the protein, determined by CD in the presence of lipid. We can conclude that although the amphipathic structure can largely determine the lipid binding properties of the protein, its intact lipid binding affinity is also contributed by other factors, such as the hydrophobic interaction of the non-polar helix face of the protein and the phospholipid acyl chains. Compared to the other apoCIII variants, apoCIII-F64A/W65A forms a complex with the least stability, indicating that these two aromatic residues are very important for the lipid binding properties of apoCIII. Although located in the N-terminus of the protein, the redistribution of the N-terminal helix of apoCIII (L9T/T20L) increased the ability of apoCIII to bind to DMPC significantly and resulted in the formation of the more stable apoCIII-DMPC complex. In contrast, neither apoCIII-K21A nor apoCIII-A23T showed severely altered lipid-binding abilities of apoCIII.

6.4 Discussion and Conclusion

All the soluble apolipoproteins, including apoCIII, have a similar gene arrangement resulting in repeated blocks of functionally identical amino acids, which have the characteristics of amphipathic α-helices (Li et al., 1988; Luo et al., 1986) which are involved in the lipid-binding properties of the apolipoproteins. The amphipathicity of apolipoprotein is correlated with the ability of the protein to interact with phospholipid. All the apoCIII variants involved in our lipid binding studies showed efficient lipid binding abilities, suggesting that their amphipathic α-helical structures were maintained upon introducing the mutations. Furthermore, the
fusion with the 6xHis tag is unlikely to effect the lipid binding functions since these tags have little secondary structure. However, the differences in their physicochemical properties that further corresponded to their lipid binding properties, indicated that although several structural domains (where the apoCIII mutation was located) correlated with the lipid binding properties of the apoCIII, some domains were more important for the lipid binding properties of protein than the others. The new information of the structure-functional relations of apoCIII obtained from our studies will be described below.

Several studies have concluded that the C-terminus of apoCIII was involved in the lipid binding properties (Sparrow et al., 1977; Trieu and McConathy, 1995). These results were confirmed by our data since the association with the DMPC MLV indicated that the apoCIII-A64A65 showed severely diminished lipid binding properties. Sparrow and co-workers also concluded that the N-terminus of apoCIII (1-40) was not involved in the lipid binding (Sparrow et al., 1977). Our results suggested otherwise, since the mutant apoCIII-T9L20 showed significantly increased lipid-binding properties compared to wild type apoCIII, the contribution at the N-terminus to overall lipid binding is therefore important. Based on the protein modelling results, peptide 6-20 of apoCIII is a tilted peptide, which can associate with phospholipid at an angle of 45°; the substitution by L9T/T20L alters this angle to 0°. Our results indicate that this change located at the N-terminus of apoCIII influences the lipid binding properties of the whole protein and the overall stability of the protein-phospholipid complexes. The rearrangement of leucine residues appears to be a key factor for lipid binding of apoCIII. This observation is similar to the results obtained from the X-ray crystallographic studies of apoE (Wilson et al., 1991).

The size of the discoidal apoCIII:DMPC particles dependent on the lipid binding properties of the protein variants. The stronger the lipid binding properties of the apolipoprotein the more protein bound to lipid and resulted in a smaller size lipid particle with tighter interactions between the protein and lipid. Our results obtained from gel filtration column were in agreement with this fact. Whereas the wild type apoCIII formed complexes with Stokes radii comparable to those previous reported by others (Jonas, 1992), the size of the complexes formed with the poor lipid binder (apoCIII-A64A65) was larger than that of wild type apoCIII particles, while the
apoCIII mutant with enhanced lipid binding properties (apoCIII-T9L20) formed discs with smaller radii as compared to wild type apoCIII. ApoCIII-A21 and apoCIII-T23, the two variants with the similarly moderateness of diminished lipid binding properties, formed discs with slightly bigger radii compared to wild type apoCIII. The relationship between size of discoidal complexes formed and lipid-binding properties of apolipoprotein mutants was also observed in studies on apoAI mutants (Holvoet et al., 1995).

Pownall and colleagues reported an increase in α-helical content of the apolipoprotein going from an aqueous solution to a lipid bound state, and is well recognised as a common feature of apolipoproteins (Pownall and Massey, 1986). The interaction of apolipoproteins with fluid lipid interfaces is driven by α-helix formation between the hydrophobic interaction of the non-polar helix face of the protein and the fatty acid chain of phospholipids. Previous reports (Jonas, 1992) showed on average a 26% α-helix in solution and 65-70% α-helix in lipid bound apoCIII. Careful analysis and deconvolution of the entire CD spectra of the apoCIII proteins (with and without 50% TFE) showed a comparable increase in α-helix in the presence of the helix promoting solvent TFE for the wild type apoCIII, apoCIII-T9L20, apoCIII-A21 and apoCIII-T23. By contrast, apoCIII-A64A65 showed a very large increase in α-helical structure (54%) in TFE. This is due to the fact that the mutation of those two bulky hydrophobic residues located at the C-terminal extremity of the predicted second amphipathic helix (as mentioned in Chapter 3), could drastically improve the propensity of this segment to fold as a α-helix, and therefore favour α-helix formation. However as a consequence of these mutations, the lipid binding affinity of this mutation was severely decreased, suggesting that these C-terminal hydrophobic residues in apoCIII have important functions in the initial interfacial recognition between the polypeptide and the fatty acid chain of phospholipid during the initial phases of the lipid binding.

The binding of apolipoproteins to the phospholipid interface is also accompanied by changes in their spectral properties (CD and Trp fluorescence emission). The intrinsic fluorescence spectra of the apolipoproteins are dominated by the contribution of Trp fluorescence, which is highly sensitive to the polarity of the environment. In aqueous buffer the maximum of Trp fluorescence emission for all
apoCIII proteins tested was 343 nm, which is consistent with previous (Jonas, 1992). These data also suggest that the Trp residues in the recombinant apoCIII are fully exposed to the aqueous buffer and the recombinant apoCIII proteins are not in a highly aggregated state. The shift of fluorescence maximum emission to lower wavelengths (blue-shift) in the presence of lipids therefore indicates shielding of the Trp from the water and the replacement of the local Trp environment by lipid molecules. In the case of the apoCIII proteins this shift was quite large (10 nm) and even larger (12 nm) for the variant with the enhanced lipid binding property, apoCIII-T9L20, suggesting an even deeper insertion of this mutant in the lipid phase. These observations are consistent with the higher lipid binding affinity of this mutant and suggest a tight association with the phospholipids for this particular mutant.

In our studies, the stability of the free and lipid bound apoCIII was detected by GdnHCl induced denaturation of lipid bound and lipid free apoCIII which was followed by measuring the disappearance of the α helical structure in the polypeptide. The GdnHCl concentration at which both free and lipid bound proteins denatured is comparable to the values reported for other apolipoproteins such as apoE and apoAI (De Pauw et al., 1995). The difference in denaturation behaviour between the wild type apoCIII and some of the mutants suggested the differences in the overall stability of the protein folds. Lipid bound wild type apoCIII and apoCIII-T9L20 mutants denatured in a two-phase manner as reported for apoE (De Pauw et al., 1995), suggesting that the unfolding of the lipid bound apoCIII occurred through stable intermediates. Moreover, apoCIII-T9L20:DMPC complex required a higher concentration of GdnHCl in order to obtain a 50% reduction in the α-helix signal suggesting that this mutant has a higher overall stability when bound to lipid which is probably due to its higher affinity for lipids, as evidenced by the smaller size of the complex it formed with DMPC and the reduced bound/free apoCIII ratio; as well as its deeper insertion in to the lipid phase as evidenced by the Trp emission behaviour of this mutant. In contrast the apoCIII-A64A65 mutant contains a high-percentage of α helix, however the mono-phase denaturation pattern of the lipid bound complex of this mutant suggests that the protein-lipid association is less tight and more prone to GdnHCl induced denaturation. Not surprisingly, since apoCIII-A21 and apoCIII-T23 showed the diminished lipid binding properties, their lipid bound complexes showed the mono-phase denaturation pattern as the function of GdnHCl concentrations and
required a lower concentration of GdnHCl in order to obtain a 50% reduction in the α-helix signal compared to wild type apoCIII.

Taken together our data from the structure-functional studies of apoCIII, show for the first time that the hydrophobic residues F64 and W65 are crucial for the lipid binding properties of apoCIII and they are probably involved in the C-terminus amphipathic helices of apoCIII. Apart from the amphipathicity of the protein, the lipid binding property of apoCIII is largely contributed by the initiation of lipid binding between the apoCIII protein and the fatty acid chain of phospholipid during the initial phases of the lipid binding. The C-terminal hydrophobic residues in apoCIII therefore have important functions in the lipid binding properties of the protein due to both the involvement of the amphipathic helical structures and their initiation of lipid binding. Additionally, the fact that the redistribution of the N-terminal helix of apoCIII (L9T/T20L) enhances the stability of the lipid-bound protein, suggests that the N-terminal domain of apoCIII is also necessary for the efficient function of lipid binding of the protein and/or normal lipoprotein metabolism.
Chapter 7
Interaction of ApoE and ApoCIII Variants

7.1 Introduction

ApoCIII is the most abundant of the three C apolipoproteins. Together with apoE, apoCIII plays a key role in TG metabolism and the apoCIII and apoE contents of TRL determine their in vivo clearance rate. The apolipoproteins can exchange between chylomicrons, chylomicron remnants, VLDL and can rapidly transfer to HDL (Havel et al., 1973). ApoCIII and apoE are normally associated with the same lipoprotein particles in vivo and play the opposing roles in the processes involved in the TRL clearance. ApoE in the TRLs can favour their binding to HSPG and therefore increase their LPL-mediated lipolysis and remodelling at the endothelial cell surface, while apoCIII prevents this process (See section 1.4.1.1 and 1.4.1.3). Additionally, apoE was shown to mediate the receptor uptake of TRL by receptors on hepatocytes while apoCIII was shown to inhibit this process possibly by impairing the interaction of apoE on the lipoproteins with the cell receptors, mainly LRP (Windler and Havel, 1985; Kowal et al., 1990). Confirmation of this hypothesis comes from transgenic mice studies. The overexpression of human apoCIII results in hypertriglyceridemia, with a positive linear relation between apoCIII levels and TG concentration (Ito et al., 1990; Aalto Setala et al., 1996; de Silva et al., 1994). Crossbreeding of apoCIII transgenic mice with apoE transgenic mice normalises the plasma TG levels (de Silva et al., 1994), concludes that hypertriglyceridemia in the apoCIII overexpressor was due to the low amount of apoE relative to apoCIII on the TRL. However, in the studies reported by Ebara, apoCIII transgenic mice were crossbred with apoE knock out mice, and showed largely increased plasma TG levels compared to apoE knock-out mice, mainly due to the marked increase in TG-rich lipoproteins, chylomicrons and VLDL. As there was no significant increase in TG production in apoCIII transgenic/apoE knock out mice, the predominant mechanism of apoCIII induced hypertriglyceridemia appear to be due to the decreased association of TRL with cell-surface glycosaminoglycans, and consequent decreased lipolysis in vivo, independent of apoE (Ebara et al., 1997). Therefore, the interaction between the
two apolipoproteins at the lipoprotein surface, particularly the apoE/apoCIII ratio on the lipid particle is a key determinant for the regulation of plasma TG level. In agreement with this, the studies reported by Haubenwallner showed that administration of various fibrates, the TG lowering drugs, lead not only to the decrease in plasma apoCIII levels in rats, but also increased the plasma apoE/apoCIII ratio (Haubenwallner et al., 1995).

Due to the similar gene structure in most of the soluble apolipoproteins, apoCIII and apoE have the characteristic amphipathic α-helices (See section 1.1.2.2). Common lipid binding properties could be shared between the two apolipoproteins, and thus they might compete for the lipid binding and therefore displace each other from the lipid particles. The \textit{in vitro} displacement of apoE by apoCIII has recently been confirmed by adding purified human apoCIII to human plasma at levels observed in hypertriglyceridemic subjects (at concentration up to four times the intrinsic apoCIII concentration) and incubating under specific conditions (2 hours, 37 °C). Addition of apoCIII to plasma almost completely displaced apoE from small VLDL particles to HDL, while this displacement was not complete with larger VLDL particles, since VLDL contain tightly bound apoE which are not displaceable (Breyer et al., 1999). In order to have a better understanding of the structure-functional relationship of apoCIII and its involvement in the interactions between apoCIII and apoE, we followed the \textit{in vitro} displacement of apoE from the apoE enriched lipid particles by our recombinant apoCIII variants. The recombinant apoCIII proteins involved in this study included wild type apoCIII, the naturally occurring mutation apoCIII-T23, and the variants generated according to the protein modelling prediction apoCIII-A21, apoCIII-T9L20 and apoCIII-A64A65. We chose the discoidal apoE complexes, apoE:DPPC to perform these experiments because these particles are more homogeneous than the lipoproteins isolated from individual donors and apoCIII deficient plasma was not available. Furthermore, endogenous apoCIII would affect the results.
7.2 Displacement of ApoE by ApoCIII Variants from Discoidal ApoE:DPPC Complexes

The displacement of apoE by apoCIII variants from apoE:DPPC complex was performed by incubating 250 µg of the apoE:DPPC complex with the equal amount of apoCIII variant proteins at room temperature for 2 hours as described in section 2.2.11. The capacity of apoCIII variants to displace apoE from the reconstituted apoE:DPPC complexes was followed by analysing gel filtration profiles of the resulting apoE:apoCIII:DPPC mixtures from a Superose 6PG column in FPLC system (See section 2.2.9.4) to check the integrity of the original apoE:DPPC complexes and to identify the distribution of apoE and apoCIII on the particles. The resulting complexes were still eluted at their original elution volume of 29 ml. Free proteins in the mixture, including the displaced apoE and unbound apoCIII, that were not associated with the complex, were eluted at higher elution volumes of 37 ml (Figure 7.1). Western blotting (See section 2.2.6.4, protocol 2) was used to determine...
the distribution of both apoE and apoCIII in the samples from the gel filtration fractions (data not shown). The apoCIII variants were found associated with apoE:DPPC complex. Free apoE displaced from its original complex was detected at higher elution volumes, in the free protein fractions. These results illustrated that the fraction of the apoE from the complex had been displaced by apoCIII, and apoCIII had been incorporated into the apo E:DPPC complex. All the apoCIII variants showed the capacity to displace apoE from the apoE:DPPC complex.

7.3 Quantification of ApoCIII and ApoE Proteins in the ApoCIII Displaced Lipid Particles

In order to compare the ability of each apoCIII variants to displace apoE from the apoE:DPPC complexes, it was necessary to accurately detect the amount of apoCIII and apoE protein in the apoE:DPPC complexes upon the displacement by identical amount of apoCIII variants, and this allowed us to find out if there were any

![Graph]

**Figure 7.2 Protein component in the apoE:DPPC complex after displaced by WT-apoCIII.** The apoE:WT-apoCIII:DPPC complex was analysed by gel filtration on a Superose 6PG column in FPLC system. The concentrations of both apoE and apoCIII from the gel filtration profiles were detected by ELISA.
difference between the amount of two proteins in the resulting particles. The quantification was performed by analysis of both proteins in the individual fractions from the gel filtration runs (as described in section 2.2.9.4) and determined by ELISA using polyclonal antibodies of apoCIII and apoE as described in section 2.2.6.6. Consistent with the Western-blotting results, in all the displacement experiment, apoE protein displaced from the complex was detected in the fractions of free protein while apoCIII protein was found in the fractions of apoE:DPPC complex, as shown in Figure 7.2.

The concentration of apoCIII variants bound to the apoE:DPPC complex is presented in Figure 7.3 (a). The concentration of apoCIII-A21 was 65 μg/ml, similar to WT-apoCIII of 62 μg/ml. ApoCIII-T23 and apoCIII-T9L20 were present at the similar concentrations in the complexes, 43 μg/ml and 46 μg/ml, respectively. For apoCIII-A64A65, the variant with diminished lipid binding properties, the concentration was 35 μg/ml, which was lower when compared with all the other apoCIII variants. The amount of apoE displaced from the complex (shown in Figure 7.3 (b)) was also quite different among the variants. The concentration of apoE displaced by WT-apoCIII and apoCIII-A21 was 16 μg/ml and 20 μg/ml, respectively in the free protein fractions, corresponding to 13.2% and 16.7% of the total apoE displaced. While the concentration of apoE displaced by the naturally occurring mutation apoCIII-T23 was 25.6 μg/ml correspond to 19.3% of the original apoE. The concentration was much more pronounced for the apoCIII-T9L20 (31.9 μg/ml, correspond to 31.2% of the original apoE) and very reduced for the apoCIII-A64A65 (10.5 μg/ml, correspond to 8.4% of the original apoE) variants. These results suggest that lipid-binding affinity of the apoCIII variants determined the ability to displace apoE from its complex.

The ratios of apoE/apoCIII in the apoCIII:apoE:DPPC lipid particle complexes have been compared and shown in Table 7.1. This ratio is an important factor for the hepatic uptake of the lipid particle by the cellular receptors in vivo.
Table 7.1 ApoE/apoCIII ratio (mol/mol) (%) in the apoE:DPPC complex after displaced by apoCIII variants.

<table>
<thead>
<tr>
<th>apoE/apoCIII in complexes (mol/mol) (%)</th>
<th>WT- apoCIII</th>
<th>apoCIII-T9L20</th>
<th>apoCIII-A21</th>
<th>apoCIII-T23</th>
<th>apoCIII-A64A65</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>107</td>
<td>92</td>
<td>134</td>
<td>187</td>
<td></td>
</tr>
</tbody>
</table>

7.4 Discussion and Conclusion

Both apoCIII and apoE can interact with phospholipid (See Chapter 6), and thus allow the possible competition occurred on the lipid surface between two proteins. This hypothesis has been confirmed by the indication of the common binding sites for various apolipoproteins on DMPC vesicles (Cardin et al., 1982). Their binding to lipid particles is competitive, reversible and in equilibrium. This is likely to be the same for DPPC due to its structure is similar to that of DMPC. Consistent with the previous results obtained by Breyer et al (Breyer et al., 1999) which showed the displacement of apoE by apoCIII from VLDL to HDL in plasma, our data also demonstrate that in our in vitro model apoCIII is capable of displacing apoE from apoE enriched particles, apoE:DPPC. The displacement between the two apolipoproteins occurred without the concomitant dissociation of lipid from the lipid particles, since no free lipid was seen when apoCIII displacement of apoE from the complex was analysed by gel filtration. The displacement of apoE by apoCIII largely depends on the lipid binding properties of apoCIII variants.

As observed from our data, apoCIII-K21A and apoCIII-T23, the apoCIII variants with similar lipid binding properties as wild type (see Chapter 6), replaced similar amounts of apoE (correspond to the 16.7% and 19.3% of the total apoE) from the apoE:DPPC complex compared to wild type (13.2%). ApoCIII-T9L20, the variant with strongest lipid binding properties, replaced 31.2% of apoE from the apoE:DPPC complex although the comparable amount of apoE:DPPC complex and apoCIII variants were used to perform all the displacement. It is noticeable that only
8.4% of apoE were displaced by apoCIII-A64A65, the apoCIII variant with the highest percentage of α-helix structure upon binding to phospholipid and with diminished lipid binding properties, due to the elimination of the two consecutive C-terminal aromatic residues. This result adds to our knowledge of the displacement of apoE by apoCIII from the lipid particle surface by suggesting that it is mainly dependent on the lipid binding properties of the apoCIII, rather than the content of amphipathic helix, the common structures between the two proteins, in apoCIII, although the helical structures are largely corresponded to the lipid binding properties of the proteins. The initial interaction between lipid and protein is therefore shown to be rather important for the interaction between the two apolipoproteins on the lipid particles.

It is interesting that the amount of apoCIII protein on the lipid particles of each apoCIII variants are not always inversely correlated with the amount of apoE protein displaced from the lipid particles. WT-apoCIII and apoCIII-A21 corresponded to the highest apoCIII concentration in the complex (Figure 7.3, a), however, apoCIII-T9L20 displaced most apoE from the apoE:DPPC complex (Figure 7.3, b). Consequently, although the displacement of apoE by apoCIII has been shown mainly to be dependent on the lipid binding properties of apoCIII (Chapter 6), the apoE/apoCIII ratio in the apoE:DPPC lipid particles after the displacement of apoE is not consistent with their lipid binding properties either (apoCIII-T9L20 showed the highest lipid binding ability, therefore likely resulted in the lowest apoE/apoCIII ratio of the lipid particles upon the displacement of apoCIII). However, the relevant apoE/apoCIII ratio (%) of apoCIII-T9L20 is 107, which is higher than that of WT-apoCIII (100) and apoCIII-A21 (92) and lower than that of apoCIII-T23 (134) and apoCIII-A64A65 (187). This could be because the apoE displacement by apoCIII from the lipid particle is also due to the interaction between these two apolipoproteins, namely the interaction of apoE with apoCIII and apoCIII with apoCIII on the surface of the lipid particles. In other words, the binding ability of an apolipoprotein to the phospholipid could also be dependent on their steric structure and their compatibility with other apolipoprotein on the lipid particles. Some apoCIII variants have the conformations which may favor this sort of compatibility while some may not. Since the mechanism of the protein-protein interaction may not be the same as the lipid-protein interaction, the apoE/apoCIII ratio is therefore not consistent
with their lipid binding properties. Further support for this hypothesis comes from the recent studies performed by MacPhee et al (MacPhee et al., 1999). In this study, the ability of apoCII to form self-association complexes on the lipid surface has been shown and suggests as a common property of amphipathic α-helices in apolipoproteins. Apart from the forming of the self-association complexes of apolipoproteins, this association may therefore also occur between different apolipoproteins (MacPhee et al., 1999). Similarly, the association of apoCIII and apoE may also be present on the apoE:DPPC particles, and apoCIII-A64A65 is likely to associate with apoE more tightly, due to its high percentage of α-helix structure upon binding to phospholipid. The apoCIII-apoE association favors the ability of more protein to be present on the lipid particles and affects the distribution of the two apolipoproteins on the apoE:DPPC complex. Conversely, apoCIII-T9L20, the best variant at displacing apoE from the lipid particles, did not lead to the highest amount of apoCIII on the lipid particles, which could be because its conformation may not favor the compatibility of both proteins on the lipid particles. Previously, most attention has been paid to the lipid binding properties of the apolipoproteins, to explore their interaction on the lipid particles, our data now suggests a possible alternative mechanism, namely the interaction between homologous or heterologous apolipoproteins that might influence the protein content on the lipid particle. However, the full investigation of protein-protein interactions between the apolipoproteins has not been carried out in our studies.
Figure 7.3 Distribution of apoCIII and apoE proteins after the displacement of apoE from the apoE:DPPC complex by apoCIII variants. ApoE:DPPC complexes that displaced apoCIII variants were analysed on a Superose 6PG column. The concentrations of both apoE and apoCIII from the gel filtration profiles were detected by ELISA.
Chapter 8
LPL Inhibition by ApoCIII Variants

8.1 Introduction

LPL and its lipolysis of TGs play a central role in lipoprotein metabolism and thereby is involved in the presence, severity and progression of atherosclerosis. Accumulating evidence indicates that many TRL remnants are associated with the progression of atherosclerosis (Hodis, 1999). Thus LPL, central to the conversion of TRLs to remnants plays a crucial role in this progress. Additionally, since HDL particles are formed in the circulation as a by-product of lipolysis of chylomicron and VLDL, the surface lipids and apolipoproteins transferred to HDL are obtained from the LPL-mediated hydrolysis of TRL, and the amount of HDL cholesterol is modulated by the concentration of TG mainly contained in VLDL (see section 1.1.3). Therefore the fate of plasma TG and cholesterol largely depends on the proper function of LPL mediated lipolysis.

ApoCIII has been well recognised as the most specific and thus the most efficient inhibitor of LPL according to both the in vivo (Ordovas et al., 1989; Maeda et al., 1994) and the in vitro (Wang et al., 1985; Lambert et al., 1996) studies. In the patients with a genetic deficiency of apoAI and apoCIII, the VLDL turnover studies showed that their fractional catabolic rate (FCR) was six to seven folds increased compared to normal. Addition of purified apoCIII to the serum from these patients resulted in a 20-50% reduction in the maximal LPL activity compared to the addition of the serum alone (Ginsberg et al., 1986). In agreement with this, the lower fasting plasma TG levels and the protection from the postprandial hypertriglyceridemia as well as an increased rate of chylomicron clearance were observed in the apoCIII knockout mice (Maeda et al., 1994). However, the functional domain of apoCIII and the mechanism involved in its LPL inhibitory effect are still controversy. Catapano (Catapano, 1987) examined the inhibitory potential of apoCIII using bovine milk LPL and egg phosphatidylcholine-trioleoylglycerol as substrate. This study showed that a threefold molar excess of apoCIII decreased LPL activity by 25% and that LPL
inhibitory effect could be localised in the C-terminal domain of apoCIII between residues 41 and 79, which also binds phospholipids (Sparrow et al., 1977). However, in vitro study performed by McConathy using bovine milk LPL and dioleoyl phosphatidylcholine-emulsified glycerol tri [9,10-\textsuperscript{3}H] olate as substrate showed the loss of 79% of LPL-inhibition after CNBr cleavage of the N-terminal portion of apoCIII (amino acids 1-40), suggesting that the N-terminus of the protein were the primary domain modulating LPL activity. Using the synthetic apoCIII peptides, this study also demonstrated that the segment containing amino acids 1-7 and amino acids 17-25 of apoCIII are particularly important for the LPL inhibitory effect (McConathy et al., 1992).

ApoCIII has been suggested to inhibit LPL by displacing the natural activator of LPL, apoCII from the lipoprotein particles (as mentioned in section 1.4.1.3). In vitro studies indicate that apoCIII/apoCII ratio of 20 is essential for LPL inhibition. Additionally, as mentioned in section 1.4.1.3, the apoCIII/apoCII ratio on the TRL is an important factor for their clearance from plasma, and this ratio is higher in hypertriglyceridemia than normal. However, more recently studies have suggested that apoCIII acts as a direct non-competitive inhibitor of LPL and can therefore directly inhibit LPL rather than through its interaction with apoCII (Wang et al., 1985; Lambert et al., 1996). Several possible interactions have therefore to be considered to understand the mechanism of LPL inhibition by apoCIII, including the interactions of protein-lipid (apoCIII + lipid particles), protein-protein (apoCIII + apoCII or LPL) and protein-protein-lipid (apoCIII + apoCII/LPL + lipid particles). Several questions also arise whether for example does apoCIII affect the lipid structure and turn it into a worse substrate for LPL? Does apoCIII displace apoCII? Does apoCIII change the conformation of apoCII and LPL? Does apoCIII decrease the affinity of LPL for lipid substrate? In order to have a better understand of these questions we have undertaken the detail studies to clarify the LPL inhibition of apoCIII using the recombinant apoCIII proteins of wild type and the naturally occurring A23T variant as well as the apoCIII variants generated according to the protein modelling results.
8.2 LPL Inhibition by GST-apoCIII Fusion Proteins

LPL inhibition by apoCIII has been first tested on bovine LPL with the recombinant fusion proteins of GST-WT-apoCIII and GST-apoCIII-Thr23 as well as the recombinant GST protein, LPL activity was measured using KABI-intralipid as substrate. These experiments were performed by our collaborators in the lab of Prof. Gunilla Olivecrona, at Umeå in Sweden. None of these proteins showed any effect on LPL activity (data not shown) with the concentration from 0 to 40 μM of apoCIII, while human plasma apoCIII showed very efficient LPL inhibition in the same assay. These results strongly suggested that the LPL inhibitory domains of apoCIII in the GST-apoCIII fusion proteins were not functional since they were not exposed under native conditions. This is probably because that the N-terminal domain of apoCIII was actually covered by GST protein, which is in accordance with our previous hypothesis mentioned in section 5.2.5. The LPL inhibition studies were thus carried out using recombinant apoCIII-6xHis fusion proteins.

8.3 LPL Inhibition by ApoCIII-6xHis Variants

The inhibitory effect of all the recombinant apoCIII variants was tested in the bovine LPL assay using [3H] labelled Intralipid as substrate as described in section 2.2.12. Comparing the Io% of every sample, inhibition of the recombinant wild type apoCIII was two folds lower than the plasma human apoCIII in all cases (data not shown). All the recombinant apoCIII variants were able to inhibit LPL activity efficiently, both in the absence and in the presence of human apoCII (Figure 8.1). However in the presence of apoCII, the LPL inhibition was less pronounced at lower apoCIII concentrations (0-1.5 μM), with the recombinant wild type apoCIII showed an increase in LPL activity at these concentrations. From apoCIII concentrations of 3 μM, the LPL inhibition by all the apoCIII variants was well pronounced. LPL activity was fully inhibited at apoCIII concentration of 10 μM. In the absence of apoCII, all the apoCIII variants showed effective inhibition even at low apoCIII concentrations. In the absence of apoCII Io% of apoCIII-A64A65 was 1.3 μM and all the other apoCIII variants had the similar Io% around 2 μM. In the presence of apoCII, Io% for apoCIII-A64A65 was 2 μM and for the other apoCIII variants it was 4.4 μM. ApoCIII-
Figure 8.1 Inhibition of LPL by apoCIII variants. Bovine LPL (15 ng) was incubated for 30 minutes at 25 °C in a total volume of 200 μl with 3H-labelled Intralipid™ in the presence (a) and in the absence (b) of 40 ng human apoCII protein. Each data point is the mean of triplicate samples. LPL activity is expressed as nmol fatty acids released per minute and ml of incubation.
64A65A was therefore shown to be the most efficient inhibitor of LPL. These results represent the mean from three experiments.

**Table 8.1** $I_{50}$ of each apoCIII variants in the presence and absence of apoCII

<table>
<thead>
<tr>
<th>apoCIII variants</th>
<th>$I_{50}$ (µM) (n=3)</th>
<th>with apoCII</th>
<th>without apoCII</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-apoCIII</td>
<td>4.4±0.10</td>
<td>2.0±0.21</td>
<td></td>
</tr>
<tr>
<td>apoCIII-T9L20</td>
<td>4.4±0.05</td>
<td>2.0±0.23</td>
<td></td>
</tr>
<tr>
<td>apoCIII-A21</td>
<td>4.4±0.05</td>
<td>2.0±0.23</td>
<td></td>
</tr>
<tr>
<td>apoCIII-T23</td>
<td>4.4±0.12</td>
<td>2.0±0.12</td>
<td></td>
</tr>
<tr>
<td>apoCIII-A64A65</td>
<td>2.0±0.14</td>
<td>1.3±0.08</td>
<td></td>
</tr>
<tr>
<td>plasma human apoCIII</td>
<td>7.5±0.13</td>
<td>7.9±0.14</td>
<td></td>
</tr>
</tbody>
</table>

**8.4 Discussion and Conclusion**

The previous results obtained from the study using synthetic apoCIII peptides suggested that the N-terminal domains of apoCIII, in particular the segments containing amino acids 1-7 and amino acids 17-25, are important in the modulation of LPL activity (McConathy et al., 1992). In the current study, the N-terminal substitutions of apoCIII, apoCIII-A23T, apoCIII-K21A and apoCIII-L9T/T20L, showed similar LPL inhibition compared to recombinant wild type apoCIII, suggesting that these amino acid changes, although located in the N-terminus of apoCIII, did not affect LPL inhibition. These data therefore do not provide any further insight as to whether the N-terminus of apoCIII is involved in LPL inhibition. Since the recombinant apoCIII protein lack the carbohydrate moiety and these proteins inhibited LPL more efficiently than plasma apoCIII, the $O$-glycosylation of plasma apoCIII is thus suggested to modulate LPL inhibition.
ApoCIII-A64A65, the variant that bound least well to DMPC particles (see Chapter 6) displayed the most efficient LPL inhibition, strongly suggest that LPL inhibition by apoCIII is unlikely to depend entirely on lipid binding, leading to the displacement of apoCII or LPL from the lipid substrate. Furthermore, the enhanced lipid binding of apoCIII-T9L20 did not appear to alter the LPL inhibition, suggesting that these two functions are independent. This is consistent with the notion that apoCIII inhibition of LPL lipolysis is due to the protein-protein interactions rather than the protein-lipid interactions (Wang et al., 1985). The involvement of the C-terminus of apoCIII in LPL inhibition supports the study reported by Catapano (Catapano, 1987) using bovine LPL and an egg phosphatidylcholine-trioleoylglycerol substrate which concluded that the inhibitory effect of apoCIII could be localised to the C-terminal domain of apoCIII.

Our studies allow us to speculate on the mechanism of LPL inhibition by apoCIII. All the apoCIII variants were able to inhibit LPL activity either induced or not induced by apoCII, although with different \( I_{50} \). In the presence of apoCII, low concentrations of apoCIII inhibits LPL less efficiently and in fact at low concentration of wild type apoCIII, LPL activity even increased. While in the absence of apoCII, all the apoCIII variants can inhibit LPL efficiently even at low concentration. The different pattern of the LPL inhibition by apoCIII and the different \( I_{50} \) of each apoCIII variant in the two cases, suggest that the two mechanisms of the LPL inhibition by apoCIII are different. In the absence of apoCII, apoCIII may inhibit LPL through the direct apoCIII-LPL interaction, in the presence of apoCII this inhibition could be contributed by a combination of the interaction of apoCIII-LPL, apoCIII-apoCII or apoCIII-LPL-apoCII. Alternatively, the most efficient LPL inhibition of apoCIII-A64A65 could be explained by its lowest lipid biding affinity: since apoCIII-A64A65 bound to the lipid substrate poorly, increased amounts of free apoCIII-A64A65 would be available for the direct protein-protein interaction with LPL (or apoCII) and thus inhibit LPL activity more efficiently. However the binding site of apoCIII on LPL and apoCII was not reported yet.

Taken together, the LPL assays using the site-directed mutations of apoCIII described in this chapter do not provide any further insight as to whether the N-terminus of apoCIII is involved in LPL inhibition, however, our results suggest the
involvement of the C-terminus of apoCIII in LPL inhibition. Although this portion of protein can bind to phospholipid very well, the LPL inhibition of apoCIII is unlikely to depend entirely on its lipid binding properties. In other words, it is not due to the displacement of apoCII or LPL from the lipid substrate, rather than the protein-protein interactions between apoCIII-apoCII, apoCIII-LPL or apoCIII-apoCII-LPL. However, the entire mechanism is not fully understood.
Chapter 9
Discussion and Conclusion

The studies reported by Austin and the study reported by Davignon and Cohn strongly support the hypothesis that high plasma TG level is an independent risk factor for CHD (Austin et al., 1998; Davignon and Cohn, 1996). However, accumulating evidence suggests that TGs themselves may not be directly atherogenic but some of the lipoprotein classes transporting TGs or reflecting abnormalities in TG metabolism, such as the TRL remnants of different size and apolipoproteins composition, are more likely to be related to progression of atherosclerosis (Hodis, 1999). For example, smaller VLDL and IDL are shown to be atherogenic while larger VLDL is not.

ApoCIII plays a key role in TG metabolism, both its concentration in the plasma and distribution to the different lipoprotein particles are believed to be a marker of TRL metabolism. It can therefore largely affect the properties of the TRL remnants and thus can be strongly related to the progression of atherosclerosis. However, the studies of the structure-functional relationships of apoCIII have not been performed systematically before. The studies involved in this thesis described the first \textit{in vitro} expression-purification system of recombinant apoCIII protein and the first structure-functional studies using recombinant WT-apoCIII and apoCIII mutations.

Naturally occurring mutations in apolipoprotein genes, particularly apoE, apoB and apoCII have provided insight into the structure-functional relationship of the protein (Talmud, 1992). However, such mutations are rare in apoCIII, including apoCIII-A23T, the apoCIII variant identified in our lab, there are five apoCIII variants found so far (Jong et al., 1999). The current studies on these variants have not shown the clear associations between these mutations and the altered lipoprotein metabolism mainly due to the small number of the carriers and they do not provide sufficient insight into the structure-functional relationship of the protein. The identification of the novel apoCIII-A23T variant in Mayan Indians prompted this thesis and the structure-functional studies of apoCIII described here. The recombinant wild type apoCIII and the apoCIII-A23T were primarily tested. In addition, several recombinant apoCIII
variants were generated according to the protein modelling results, designed to alter specific amino acid residues that implicated in both LPL inhibition and the lipid binding properties of the protein by site-directed mutagenesis reactions. Their lipid binding properties and LPL inhibition have been investigated, since we believe they are the two key factors related to the apoCIII function. Our findings have shed light on the functional domains of apoCIII and may reflect on the important properties of apoCIII in lipid metabolism in vivo and can help to explain the apoCIII deficiency and the low TG levels in the carriers of apoCIII-A23T mutation. Additionally, the successful expression and purification of the apoCIII variants, and the establishment of the in vitro system for the functional studies of apoCIII involved in this thesis, will also allow the other functional domains of this important apolipoprotein to be examined.

The mechanisms by which apoCIII regulates the TRL metabolism is complex. Both in vitro and in vivo results have shown that apoCIII is involved in the LPL inhibition, the central role in the lipoprotein metabolism (Ginsberg et al., 1986; Wang et al., 1985; Brown and Baginsky, 1972), and reduces the TG hydrolysis as well as the remodelling of the TRL. The latter has been shown to largely affect the receptor uptake of these lipid particles. Furthermore, the interaction between apoCIII and other apolipoproteins on the lipid particles has been shown to retard the receptor-mediated uptake of TRL by the liver. These interactions can result in the displacement of some apolipoproteins (such as apoE or maybe apoCII) on the lipid particles, or only result in the conformation change of the apolipoproteins (such as apoB). The displacement of the apolipoproteins by apoCIII can only occur in the soluble apolipoproteins, due to their similarity of the structures. All the soluble apolipoproteins, including apoCIII, have a similar gene arrangement resulting in the amphiphatic α-helices (Li et al., 1988; Luo et al., 1986). The amphiphathic helices in all apolipoproteins promote their binding to a lipid interface with varying affinities (Dolphin, 1992) and this might be the basis for the apoCIII function, such as the displacement of other apolipoproteins from the lipoprotein particles by excess apoCIII. The lipid binding properties of apoCIII can therefore directly affect both the distribution of apoCIII on the surface of different lipoproteins which reflect its stability in the plasma due to the protection this protein obtains from the lipid, and the apolipoprotein composition of each of the lipoprotein thus affecting their metabolism. In order to work out the key structural
elements of apoCIII involved in the displacement of apolipoproteins, we have performed the displacement of apoE from the apoE enriched lipid particles by the apoCIII variants. From our results, the ability of apoCIII to displace apoE from the lipid particles depends on its lipid binding properties that largely depend on its amphipathicity. Apart from this, the protein composition of the lipid particles (can be reflected as apoE/apoCm ratio) after the displacement could also be affected by the protein-protein interaction, namely the interaction between homologous or heterologous apolipoproteins on the surface of the lipid particles. Additionally, this interaction probably relates to the amphipathic α-helical structures of each protein, since these structural motifs can favour the association between the apolipoproteins and therefore favour the compatibility of these proteins. The amphipathic α-helices of the apolipoproteins are therefore revealed as the key structural elements involved in the interaction between these proteins on the surface of the lipid particles, although the detailed mechanism could be rather complicated.

*ApoCIII-Thr23: Explanation for ApoCIII Deficiency and Lower Plasma TG Levels in Carriers*

Extrapolation of *in vitro* data to the *in vivo* situation must be carried out with caution. However, the results from the structure-functional studies have provided an explanation for the apoCIII deficiency and the low TG levels in the Mayan Indian carriers of apoCIII-A23T mutation. The molecular modelling results demonstrated that residue 23 lies in an amphipathic helix that is important in lipid binding but not in direct inhibition of LPL. Our results are consistent with the molecular modelling prediction. Comparing to wild type apoCIII, the results obtained from our studies suggest that apoCIII-A23T shows no effect on LPL inhibition, it binds phospholipid (DMPC) less well and forms less stable complex. We can therefore speculate that *in vivo*, in the A23T carriers, apoCIII-A23T might be catabolised more rapidly in plasma due to its lower lipid binding affinity, suggesting that free apoCIII T23 would be rapidly degraded and resulting in low plasma apoCIII levels. This could result in TRL particles with a higher apoE/apoCIII ratio (34% higher compared with wild type apoCIII) that might be cleared faster by cellular receptors and thus result in low TG levels. The lower plasma TG levels seen in two of the three carriers of the variant
might reflect the increased cellular uptake of the TRL particles mediated by apoE, rather than the reduced ability of apoCIII-A23T to inhibit LPL activity, which would result in an increased catabolic rate of TRL. Additionally, the higher apoE/apoCIII ratio of the lipid particles could increase their binding to HSPG and therefore increase their lipolysis at the cell surface therefore result in the lower plasma TG levels in the carriers. The difference of the binding capacity between the apoCIII-T23 and wild type apoCIII containing lipid particles with HSPG should be compared in future experiments (See future work). The third A23T carrier had raised plasma TG and cholesterol levels when compared to the mean values for the Mayan control samples and a very low BMI. Difference in the lipid levels could not be explained by apoE genotype since that all three carriers had the same apoE3 genotype. This suggests that other unidentified genetic or environmental factors (for example diet or smoking) may be modulating the effect of the A23T variant.

It is surprising that in the heterozygous state this apoCIII-T23 variant appears to act in a dominant manner. This might reflect the co-inheritance of other unidentified mutations affecting the clearance of TRL. In vitro studies using short peptidase of apoE suggested that apoE may function as a dimer when acting as a ligand for lipoprotein binding to the LDLR (Dyer et al., 1991). Similarly, an alternative hypothesis is that apoCIII normally also act as a multimer and hetero-multimers containing both apoCIII-A23 (wild type apoCIII) and apoCIII-T23, that may be unstable or their associations with lipids may be weak, resulting in a dramatic increase in apoCIII catabolism and cause apoCIII deficiency in heterozygous individuals. Unfortunately, because of the geographic location of the only identified carriers of this variant, it is not possible to confirm these predictions in vivo.

Site-directed Mutations of ApoCIII

The main insight from this study into the structure-functional relationship of apoCIII has been obtained from the results of the functional studies of the apoCIII variants generated according to the molecular modelling predictions.

The variant apoCIII-L9T/T20L showed the greatest increase in lipid binding properties of all the variants and the greatest stability of the lipid-bound apoCIII
variant compared to wild type apoCIII. However, the double amino acids changes did not effect LPL inhibition despite their location at the N-terminus of the protein. The other N-terminal substitution, apoCIII-K21A, that was designed to alter the LPL inhibition properties of apoCIII, showed no effect on LPL inhibition either and its lipid binding properties were very similar to that of the naturally occurring variants apoCIII-T23. The displacement of apoE from the apoE:DPPC complex by these apoCIII variants was shown to be mainly dependent on their lipid binding affinities, since more apoE was displaced by apoCIII-T9L20 compared to apoCIII-K21A and wild type apoCIII. The latter two variants displaced similar amounts of apoE from the apoE:DPPC particles. However, the apoE/apoCIII ratio in the resulting apoE:DPPC complexes after the displacement by both apoCIII-K21A and apoCIII-T9L20 was comparable to that of wild type apoCIII.

The C-terminal substitution, apoCIII-F64A/W65A, largely diminished the ability of apoCIII to bind to DMPC. The residues F64 and W65 are possibly involved in the C-terminus amphipathic helices of apoCIII, suggesting the more extended amphipathic domain of the protein than the previous results from the sequence predictions. The fact that this poor lipid binder is the most efficient LPL inhibitor, suggests that the LPL inhibition by apoCIII is independent of its lipid binding affinities, but rather depends on the protein-protein interaction between apoCIII, apoCII and LPL. Compared to all the other apoCIII variants, the apoE displacement from the apoE:DPPC complex by apoCIII-A64A65 was the least efficient, probably due to its lowest lipid-binding ability, which result in a high apoE/apoCIII ratio (87%) in the resulting complex.

Apart from apoCIII-A64A65, both the amount of apoCIII and the apoE/apoCIII ratio in the apoE enriched lipid particles was not always consistent with their lipid binding properties after the displacement of apoE by the apoCIII variants. Our results suggest the possible interactions of the two apolipoproteins on the surface of the lipid particles were also shown to contribute to the displacement of apoE by apoCIII and to be dependent on the \( \alpha \)-helical structures of both of the apolipoproteins. Once again, the amphipathic structure of the apolipoproteins has been shown to be the key structure that determines function.
Chapter 10

Future Work

Although the results from the current studies have provided additional understanding of apoCIII structure-function relations and some insight into the important in vivo properties of apoCIII, the structure-functional relations of apoCIII have still not been fully elucidated. Further studies are required to provide a better understanding of this protein.

The involvement of the N-terminus of apoCIII in LPL inhibition still remains unclear, and it is unknown if there are such domains present or not. Additionally, the functional domains located at the C-terminus of apoCIII was not fully studied either. In order to clarify these points and have a better understanding on the structure-functional relationships of apoCIII, more experiment have to be performed by generation more apoCIII variants in both N-terminus and C-terminus of the protein according to the protein modelling results, and comparing the differences of their functional studies.

Since the apoE/apoCIII ratio of the TRL is a key factor of their metabolism, further investigation of the interaction between apoE and apoCIII will be performed. The future studies should include the interactions of apoCIII with phospholipid, apoCIII with apoE and apoCIII with apoCIII, the results will be helpful in understanding the metabolism of TRL, and could also provide a guide to drug design. Additionally, the effect of apoCIII on the binding of lipoprotein particles to HSPG on the endothelial cells should also be studied, the heparin-conjugated agarose column has been described as an ideal model to investigate this (Shachter et al., 1996). The binding experiments should be performed using both the apoCIII:DMPC particles and the apoE enriched lipid particles after displacement by the apoCIII variants, since apoE favour the interactions while excess apoCIII prevent them. The above lipid particles can be further used to detect their receptor mediated uptaken in the in vitro system. The results from these studies will help us to understand the initial event in the lipoprotein TG lipolysis mediated by LPL at the cell surface.
In collaboration with Dr. Miek Jong working in Leiden, Holland, we will use adenovirus mediated transfer of WT-apoCIII and site-directed apoCIII mutants to test their in vivo effect in different knockout mouse models eg apoC3 and apoE knock out mice. In this way we will be able to complement our in vitro studies with in vivo studies.

Finally, the crystallographic studies using wild type apoCIII and apoCIII-T9L20 are in progress. Since we have been able to express large amounts of apoCIII and purified to a high degree, we are collaborating with Dr. Renos Sava working in Birkbeck College, London to co-crystallise the apoCIII-DMPC particles. Currently, we have obtained some small crystals from both proteins, however, these crystals are not suitable for the further analysis yet. This study will be continued and the final results from this study should provide a clear view on the three-dimensional structures of apoCIII and will give us a much better understanding of the structure-functional relations of this protein.
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Characterization of Recombinant Wild Type and Site-Directed Mutations of Apolipoprotein C-III: Lipid Binding, Displacement of ApoE, and Inhibition of Lipoprotein Lipase

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ABSTRACT: The physicochemical properties of recombinant wild type and three site-directed mutants of apolipoprotein C-III (apoC-III), designed by molecular modeling to alter specific amino acid residues implicated in lipid binding (L9T/T20L, F64A/W65A) or LPL inhibition (K21A), were compared. Relative lipid binding efficiencies to dimyristoylphosphatidylcholine (DMPC) were L9T/T20L > WT > K21A > F64A/W65A with an inverse correlation with size of the discoidal complexes formed. Physicochemical analysis (Tryp fluorescence, circular dichroism, and GuHCl denaturation) suggests that L9T/T20L forms tighter and more stable lipid complexes with phospholipids, while F64A/W65A associates less tightly. Lipid displacement properties were tested by gel-filtrating apoC-III lipid complexes with the various apoC-III variants. All apoC-III proteins bound to the apoE: DPPC complexes; the amount of apoE displaced from the complex was dependent on the apoC-III lipid binding affinity. All apoC-III proteins inhibited LPL in the presence or absence of apoC-II, with F64A/ W65A displaying the most inhibition, suggesting that apoC-III inhibition of LPL is independent of lipid binding and therefore of apoC-II displacement. Taken together, these data suggest that the hydrophobic residues F64 and W65 are crucial for the lipid binding properties of apoC-III and that redistribution of the N-terminal helix of apoC-III (L9T/T20L) enhances the stability of the lipid-bound protein, while LPL inhibition by apoC-III is likely to be due to protein:protein interactions.

Apolipoprotein C-III is a 79 amino acid glycoprotein synthesized in the intestine and liver as components of both very low-density lipoproteins (VLDL) and high-density lipoproteins (HDL). The strong positive correlation between plasma apoC-III levels and plasma triglyceride levels (Tg) and postprandial clearance (1-4) strongly suggests that apoC-III plays a major role in Tg metabolism. The distribution of apoC-III between HDL particles and non-HDL particles is a strong predictor of coronary atherosclerosis (5), and it has been suggested that increased apoC-III levels lead to an accumulation of apoB-rich particles (LpB:C-III) and result in a shift in the apoC-III distribution between VLDL and HDL, which leads to the decreased clearance and thus higher levels of circulating Tg-rich particles (6).

The amphipathic helices in all apolipoproteins promote binding to the lipid interface but with varying affinity (7), and this might form the basis for the action of apoC-III, such that excess apoC-III might lead to the displacement of other apoproteins from the lipoproteins. It has been suggested that the ability of apoC-III to displace apoC-II from the lipoprotein particles would reduce apoC-II activation of LPL (8) and increased plasma levels of apoC-III present in hypertriglyceridemic individuals would alter the apoC-III:C-II ratio, reducing LPL activation. In vitro studies show that a 3-fold molar excess of apoC-III reduced LPL activity by 25% (9). It has also been proposed that apoC-III acts as a direct noncompetitive inhibitor of LPL and this would require the presence of an apoC-III binding site on LPL (10). Sparrow et al. showed that the inhibitory effect of apoC-III could be localized to the C-terminal domain between residues 41 and 79 (11). However, McConathy et al., using synthetic peptides of apoC-III, concluded that the N-terminus was the primary domain modulating LPL activity (12). Thus, there is some discrepancy as to the mechanism of apoC-III inhibition of LPL and the domains responsible for this inhibition.

A second outcome of raised plasma apoC-III levels is the displacement of apoE from lipoprotein particles, thus reducing the apoE-mediated clearance of Tg-rich remnant lipoproteins. Elevated Tg in human APOC3 transgenic mice reflects the increased number of VLDL particles in the...
circulation which contain more Tg and apoC-III and less apoE, thereby diminishing apoE-mediated lipoprotein uptake (13). ApoC-III overexpression also reduces VLDL glycosaminoglycan binding, decreasing lipolysis at the cell surface (14). The displacement of apoE by apoC-III has recently been confirmed in vitro, but this is modulated by the size of the lipoprotein particles, with displacement of apoE by apoC-III from small LDL particles being more efficient than from large VLDL particles (15).

Naturally occurring mutations in apolipoprotein genes, particularly in apoE, apoB, apoC-II, and apoA-I (16, 17), have provided insight into the structure/function relationship of these proteins; however, such mutations are rare in apoC-III. A deletion of the APOAI-C3-A4 gene locus identified in one family was associated with increased conversion of VLDL to LDL and HDL (18). Only four rare structural variants of apoC-III have been identified, but not all of these show clear association between the mutation and altered lipoprotein metabolism. Carriers of the Gly34Lys mutation have mildly elevated plasma apoC-III and Tg levels, and this could reflect the additional charge that might enhance lipid binding and/or alter the effect on LPL (19). Asp34Asn (20) and Lys34Glu (21) mutations result in the change or loss of a charge, but Asp34Asn carriers have normal lipid levels with increased VLDL-apoC-III content while Lys34Glu is associated with 30–40% lower apoC-III levels and with reduced Gly34-apoC-III on VLDL and HDL, resulting in enrichment of HDL with apoE creating atypically large HDL particles. The Thr34Ala variant, which disrupts glycosylation, is associated with a normal lipid profile, suggesting that glycosylation does not have a profound impact on apoC-III function (22, 23).

Despite the large body of literature characterizing the function of apoC-III, this topic still remains a subject of debate. To study this, we have expressed recombinant wild type and site-directed mutations of apoC-III and undertaken detailed physicochemical studies to clarify further the lipid binding properties, apoE displacement, and inhibition of LPL by apoC-III.

EXPERIMENTAL PROCEDURES

Materials. Dimyristoylphosphatidylcholine (DPMC), dipalmitoylphosphatidylcholine (DPPC), bovine serum albumin (BSA), bovine lipoprotein lipase (LPL), and ampicillin were obtained from Sigma (Irving, U.K.). GdnHCl was the highest purity and obtained from C. Roth, GmbH, Karlsruhe, Germany. Unless specified, all other reagents are from Sigma (p.a. grade). Glycerol, triphosphate, dNTP, and low molecular weight markers were purchased from Amersham-Pharmacia, U.K. All the primers, W-1 detergent, and DNA Taq polymerase were obtained from Gibco BRL, Paisley, U.K. The E. coli strains B834 (DE3) and BL21 (DE3) and pET23b vector were provided by Novagen, Biomega, Cambridge, U.K. QuickChange Site-Directed Mutagenesis Kit was made by Stratagene, La Jolla, CA. All the related products, competent cells, and enzymes involved in the mutagenesis reaction were also provided in the kit. ApoE used for the preparation of the DPPC complexes is recombinant apoE3 prepared as previously described (24).

Molecular Modeling of Wild-Type ApoC-III and Mutants. (A) Hydrophobic Cluster Analysis (HCA). This method is based on a 2D helical plot of a given sequence (25). The sequence is written on an α-helix-like cylinder that is cut parallel to the main axis and unrolled. The unfolding of this cylinder thus separates adjacent residues, and the plot is duplicated to restore all amino acids in the vicinity. The hydrophobic residues are circled and hatched, allowing the detection of hydrophobic clusters whose size, shape, and composition can be analyzed (25). Long horizontal clusters are indicative of helices, while short vertical or mosaic clusters indicate the presence of β strands or turns.

(B) 3D Construction of Peptides. 3D construction of the peptides was carried out as previously described (26, 27). The method accounts for the contribution of the lipid–water interface, the concomitant variation of the dielectric constant, and the transfer energy of atoms from a hydrophobic to a hydrophilic environment (28).

(C) Secondary Structure Prediction. The secondary structure prediction is carried out at the NPS@ (Network Protein Sequence Analysis) web site (http://phil.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_secons.html). Different predictive methods are used to obtain the consensus prediction: SOPMA (29), PHD (30, 31), Predator (32), GORIV (33), DPM (34), DSC (35), and SIMPA96 (36, 37).

All calculations were performed on Pentium-Pro processors, using PC-TAMMCH+ (Theoretical Analysis of Membrane Membrane Organization) and PC-NPRO+ (Protein Plus analysis) softwares. Graphs were drawn with the WinMGM program (Ab Initio Technology, France).

Generation of pET23b/APOC3 Construct. The human APOC3 cDNA (a kind gift from Dr. John Taylor, San Francisco, CA) in the pBSSK vector (Stratagene) was used as a template for the amplification of the APOC3 gene by PCR. This was performed in a 50 μL reaction containing 50 ng of plasmid DNA pBSSK/APOC3 as template, 250 ng of each primer, and 1 unit of DNA polymerase in a buffer recommended by the manufacturers including 0.2 mM each dNTP and 1.5 mM MgCl2. Two primers, the 5′ forward primer with an internal NdeI (underlined) site (5′-GGGAATTCCATATGTCAGAGGCGAGGCTATGCA-3′) and a 3′ reverse primer designed to append the sequence for a S' extension site (underlined) (5′-GTTGGGCTTGGCGACCTCGGGCTGAAATG-3′), were used for PCR, using the following conditions: the initial cycle of 94 °C, 5 min; 66 °C, 1 min; and 72 °C, 2 min was followed by 35 cycles of 94 °C, 30 s; 66 °C, 1 min; and 72 °C, 1 min. Reactions were performed in an automated Omigen PCR machine (Hybaid Ltd., Middlesex, U.K.). The resulting 264 bp human APOC3 fragment was digested with NdeI and XhoI, and subcloned into a similarly digested pET23b vector following standard methodology. The ligation mixtures were transformed into competent cells of the XL1Blue strain of E. coli (Promega, Southampton, U.K.), and the transformed cells were selected with ampicillin (75 μg/mL) on LB-agar plates. The entire APOC3 cDNA sequence was sequenced in both directions, using an ABI 377 prism DNA sequencer (Perkin-Elmer).

Generation of ApoC-III Variants. All the APOC3 variants were generated by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). In a 50 μL reaction, 50 ng of plasmid DNA pET23b/APOC3 was used as a template, and two synthetic oligonucleotide primers that contained the
obtained as previously described (38), and 40 µg of apoC-III protein was added to 80 µg of DMPC vesicles in a 10 mM Tris-HCl, 150 mM NaCl buffer, pH 8.0, containing 8.5% KBr, 0.01% NaN3, and 0.01% EDTA, in an Uvikon 931 spectrophotometer.

Preparation and Isolation of Phospholipid:ApoC-III Complexes. ApoC-III:DMPC complexes were prepared by incubation of the recombinant apoC-III proteins with DMPC multimellar vesicles, at DMPC:protein (w/w) ratios of 2:1 at 25 °C for 16 h. The complexes were isolated by gel filtration on a Superose 6 HR column (Pharmacia) in 10 mM Tris-HCl, 150 mM NaCl buffer, pH 7.6, and 0.2 g/L NaN3 in an FPLC system (Waters). Complexes were detected by measuring the absorbance at 280 nm and the tryptophan (Trp) fluorescence emission at 330 nm (excitation at 295 nm). The Superose 6 HR column was calibrated with a set of protein standards ranging from thyroglobulin (molecular mass 668 kDa) to cytochrome c (molecular mass 65 kDa) (Pharmacia). The resulting calibration curve was used for the determination of the molecular mass or Stokes radii of the particles eluting from the column.

Fluorescence Measurements. The maximum emission wavelength of the Trp fluorescence measurement was performed on an Aminco Bowman Series 2 spectrophotometer (39). The lipid-free apoC-III protein at a protein concentration of 25 µg/mL and apoC-III:DMPC complexes at a protein concentration of 60 µg/mL were measured at room temperature. Emission spectra were recorded between 300 and 450 nm, with the excitation wavelength set at 295 nm.

Circular Dichroism Measurements. Circular dichroism (CD) spectra of each recombinant apoC-III protein in the presence or in the absence of 2,2,2-trifluoroethanol (TFE) were measured on a Jasco 600 spectropolarimeter at room temperature. Measurements were carried out at a protein concentration of 0.2 mg/mL in 10 mM Na2HPO4/NaH2PO4 buffer, pH 7.5. Nine spectra were collected and averaged for each sample. For the protein and protein/TFE mixtures, the secondary structures were obtained by curve-fitting on the entire ellipticity curve between 184 and 260 nm, using the software available on the Internet (http://bioinformatik. biochemtech.ini.halle.de/cdnn/).

For the denaturation experiments, the lipid-free apoC-III proteins and apoC-III:DMPC complexes, at a protein concentration of 100 µg/mL, were incubated for 12 h at 4 °C in the presence of increasing GdnHCl concentrations between 0 and 6 M prior to the CD measurements. The signal measured at 222 nm and expressed as molar ellipticity is plotted as a function of the GdnHCl concentration (M) in the sample.

Displacement of ApoE from ApoE: DPPC Complexes by ApoC-III Variants. The apoE:DPPC complexes were prepared by the sodium cholate dialysis method as described.

Table 1: Primers Used To Generate the ApoC-III Variants by the in Vitro Mutagenesis Reactions

<table>
<thead>
<tr>
<th>Primers Used</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>3'-F64AW65A</td>
<td>5'-CATGAAGCACCCACCCGAGGCAGAAGTCAC-3'</td>
</tr>
<tr>
<td>3'-F64AW65A</td>
<td>5'-GGGGCCCAAATCCCACCCGCACCAGAACTC-3'</td>
</tr>
<tr>
<td>5'-F64AW65A</td>
<td>5'-AGGAATGCCTTCCTGACTCCACGGATGGAGCC-3'</td>
</tr>
<tr>
<td>3'-F64AW65A</td>
<td>5'-CTGCATAGACGACGCCGTCAGAGGCAC-3'</td>
</tr>
<tr>
<td>5'-T20L</td>
<td>5'-CATCCCTGGGGTCGCCGATGGTCCTTACATGC-3'</td>
</tr>
<tr>
<td>3'-T20L</td>
<td>5'-CTGGTCAGAGAAGACGCCCTGAGGGAGGC-3'</td>
</tr>
</tbody>
</table>

Characterization of ApoC-II Wild Type and Mutants

Expression and Purification of the Recombinant ApoC-III Variants. Plasmid DNA of pET23b/wild-type APOC3 was transformed into competent E. coli B834 cells, and all the other APOC3 variants in pET23b were transformed into E. coli BL21 respectively for optimum expression and used to inoculate an overnight culture in SOC medium containing 75 µg/mL ampicillin. Thirty milliliters of the overnight culture was used to inoculate 1 L of SOC medium in an induction medium (37) for 4 h, and batch-washed 4 times. The recombinant human apoC-IIL fusion protein was identified by electrophoresis on a Tricine—SDS—PAGE (17.5% precast gels) (Bio-Rad, Richmond, CA). The APOC3-K21A and apoC-II DMPC complexes were detected by measuring the absorbance at 280 nm and the tryptophan (Trp) fluorescence emission at 330 nm (excitation at 295 nm). The Superose 6 HR column was calibrated with a set of protein standards ranging from thyroglobulin (molecular mass 668 kDa) to cytochrome c (molecular mass 65 kDa) (Pharmacia). The resulting calibration curve was used for the determination of the molecular mass or Stokes radii of the particles eluting from the column.

Lipid Binding Properties of ApoC-III Variants. Association of recombinant apoC-III variants with lipid was followed by monitoring the turbidity decrease of dimyristoylphosphatidylcholine (DMPC) multimellar vesicles at 325 nm as a function of the temperature. The DMPC vesicles were

desired mutation (underlined) and were complemented to the opposite strands of the vector were extended using Pfu Turbo DNA polymerase. APOC3-K21A was generated using primers 5'-F64A/3' and 3'-K21A while APOC3-5'F64A/3' was generated using primers 5'-F64A/3' and 3'-F64A/3'. APOC3-5'F64A/3' was generated by a two-step mutagenesis reaction, first by introducing the L9 to T9 substitution using primers 5'-L9T and 3'-L9T, and the resulting construct was used as a template to perform the second mutagenesis reaction using 5'-T20L and 3'-T20L, resulting in variant APOC3-5'F64A/3'. All the primers used are listed in Table 1.
Using DPPC at a ratio (w:w) of 2:1 and using recombinant apoE3 purified from E. coli as previously described (24), the protein—lipid mixtures were incubated overnight at 43 °C and then extensively dialyzed against 10 mM Tris-HCl buffer, pH 8, containing 150 mM NaCl and 0.1 g/L Na2-EDTA and 1 mM azide, each time during 24 h at 43 °C, at room temperature, and at 4 °C. The homogeneity of the apoE:DPPC complexes was verified by gel filtration on a Superose 6 PG column where the complexes eluted in one homogeneous peak. The protein concentration in the complexes was determined by optical density measurement at 280 nm, and the phospholipid concentration was determined enzymatically (Biomerieux, Marcy l’Etoile, France). The concentrations of the complexes are expressed by their protein mass in all the following experiments. apoE:DPPC complexes were mixed with the apoC-III proteins at a ratio of 1:1 (w:w) and incubated at room temperature for 3 h. The apoE:DPPC—apoC-III mixture was then separated by gel filtration on a Superose 6 PG column. The protein elution profile was recorded by measuring the Trp emission at 330 nm in each fraction. The content of apoE and apoC-III was measured in each fraction by sandwich ELISA as described (41). Briefly, antibodies were coated onto 96-well ELISA plates; residual binding sites were blocked, and samples or standards were incubated on the plates for 24 h, 37 °C. After excess antigen was washed away, the peroxidase-labeled antibody was incubated for 2 h at 37 °C. After washing, the amount of bound peroxidase was revealed with a chromogenic substrate. The plates were read, and the standard curve and samples were calculated with the software provided with the reader (Biotek, KC4 software). The sensitivity of the assays was approximately 2 ng.

**Detection of apoC-II and apoE Proteins by Western Blotting.** Samples were separated on Tricine—SDS—PAGE (17.5%), or on 15% SDS—PAGE. Proteins were transferred using a wet blot system (Bio-Rad) in a Tris (5 mM)—glycinie (192 mM) buffer (pH 8.3) containing 20% methanol. Proteins were visualized with a rabbit anti-apoC-II antibody or a rabbit anti-apoE polyclonal antibody and a secondary peroxidase labeled goat anti-rabbit antibody. The bound peroxidase was visualized using the CN-DAB-kit (Pierce).

**LPL Inhibition by apoC-III Variants.** Bovine LPL (EC 3.1.1.34) (Sigma, Irvine, U.K.) catalytic activity, in the presence of apoC-III variants, was measured using the emulsion Intralipid (Pharmacia Laboratories, Milton Keynes, U.K.) (100 mg/mL) into which “Philurolan” had been incorporated by sonication (42). The incubation media contained 2% (v/v) of the emulsion, 5% (w/v) BSA (Sigma, Irvine, U.K.), 1.2% NaCl (w/v), 6.3% Tris-HCl (w/v), pH 8.5, and 3 IU of heparin (Sigma, Irvine, U.K.). As a source of apoC-II, human plasma apoC-II (43) was dissolved in 5 M urea, 10 mM Tris-HCl, pH 8.5, at 2 mg/mL. The experiments were optimized for both apoC-II and LPL, to give optimum hydrolysis with minimum apoC-II concentration. The emulsion was preincubated with each apoC-III sample with or without 2 µL of the 1:100 dilution of above apoC-II sample for at least 15 min; then the bovine LPL (10 µL of 1.5 µg/mL) was added in a final volume of 200 µL. The mixture was shaken in a water bath at 25 °C for 30 min. The reaction was stopped by extracting the fatty acids, and the radioactivity was counted (42).

**RESULTS**

**Molecular Modeling of apoC-III Variants.** To obtain a better understanding of the structure—function relationship of apoC-III, molecular modeling was carried out. Consensus secondary structure prediction suggests that apoC-III is made of two helical domains: one in the N-terminal part, from approximately residues 5 to 40, and a second C-terminal helical domain localized at residues 45–60 (Figure 1A). Hydrophobic cluster analysis (HCA, explained under Experimental Procedures) of the entire apoC-III sequence identified two hydrophobic clusters at residues 6–20 and 42–66 localized within the two helical domains as detected from the secondary structure predictions analysis (Figure 1B). The shape and hydrophobic repartition in these clusters indicate that they are amphipathic helices, suggesting a potential role in lipid binding.

The 6–20 and 42–66 peptides were constructed in a 3D model, and their favored orientation at the lipid/water interface (Figure 2A,C) was calculated according to the method described (28). While the 42–66 peptide is clearly amphipathic, with its main axis mostly parallel to the interface (Figure 2C), the N-terminal peptide was tilted toward the interface, due to an uneven segregation of the hydrophilic and hydrophobic residues along the long axis of this particular α-helix (Figure 2C). Another interesting feature of the 42–66 helix was the unusually high content of aromatic residues (Phe, Trp, and to a lesser extent Tyr), especially at the C-terminus of the peptide.

To investigate the respective role of the N- and C-terminal domains of apoC-III, we designed mutants by modifying the amphipathicity and the aromatic content. The mutation in the N-terminal segment was obtained by permutation of the Leu9–Thr20 residues. This permutation avoided changes in hydrophobicity (that could have an effect by itself on the lipid binding properties) and restored amphipathicity, since its mean axis was oriented mostly parallel to the interface (Figure 2B). For the mutation in the C-terminus, Phe64 and Trp65 were mutated to Ala. These mutations changed the amphipathicity and the aromatic content (Figure 2D), as compared to the 42–66 wild-type apoC-III fragment (Figure 2C).

**Expression and Purification of Recombinant apoC-III Variants.** To obtain large quantities of apoC-III proteins to investigate the structure—function relationship, an E. coli expression system was developed that yielded milligram quantities of the recombinant C-terminus His-tagged apoC-III proteins. For optimal expression, wild-type apoC-III was expressed in E. coli B834 while all other apoC-III mutants were expressed in E. coli BL21. After induction by IPTG at 37 °C, the fusion proteins reached about 5% of the total cellular protein. Since the fusion proteins were contained in inclusion bodies, they were purified in the presence of urea, using a combination of Talon affinity agarose and anion exchange chromatography. The resulting apoC-III fusion proteins were shown to be more than 95% pure (data not shown), and approximately 2 mg of the pure recombinant fusion protein was obtained from 1 L of culture medium.

**Lipid Binding Properties of the apoC-III Variants.** DMPC binding experiments were performed to assess the interaction of the apoC-III variants with DMPC multilamellar vesicles. A scan through the transition temperature of DMPC (Figure
**Figure 1:** Sequence analysis of the apoC-III sequence. (A) Secondary structure prediction of apoC-III. Different predictive methods were used (see Experimental Procedures), and a consensus was obtained. Abbreviations: h, α-helix; e, β-sheet; c, coil; t, turn. (B) HCA plot of the apoC-III sequence. Hydrophobic residues (F, Y, W, M, L, I, V) are circled and hatched, red stars are Pro, black diamonds are Gly, white boxes are Thr, and pointed boxes are Ser. Negatively charged residues are red, and positively charged ones are blue. The two boxes indicate the main hydrophobic clusters of apoC-III.
Figure 2: Orientation of the different apoC-III peptides (represented in CPK) at the lipid/water interface (orange plane). Lipid phase is above and water is below. (A) 6–20 peptide; (B) apoC-III-L9T/T20L mutant; (C) 42–66 peptide; (D) apoC-III-F64A/W65A mutant.

Figure 3: Turbidity decrease of DMPC multilamellar vesicles mixed with apoC-III at a w:w lipid:protein ratio of 2:1 expressed as a percentage decrease of the initial OD value at 325 nm as a function of temperature. (♦) WT apoC-III; (▲) apoC-III-K21A; (■) apoC-III-F64A/W65A; (●) apoC-III-L9T/T20L.

3) showed a turbidity decrease due to the formation of discoidal complexes. With all apoC-III proteins a similar decrease in the turbidity of the DMPC solution was observed that was maximal around the transition temperature of the DMPC. The resulting apoC-III:DMPC discoidal complexes were fractionated on a Superose 6 PG gel filtration column.
Charaterization of ApoC-III Wild Type and Mutants

**FIGURE 4:** Gel filtration on a Superose 6 HR column of DMPC/apoC-III complexes with (Φ) WT apoC-III, (▲) apoC-III-K21A, (■) apoC-III-F64A/W65A, and (●) apoC-III-L9T/T20L. Tryptophan fluorescence emission was measured at 330 nm as a function of the elution volume in milliliters. Inset: Regression line between size (elution volume in milliliters) and ratio of apoC-III bound to DMPC/amount of free as determined from the gel filtration elution profiles.

The apoC-III protein in these fractions was monitored by measuring the Trp emission at 330 nm. In all the gel filtration runs, no free lipid could be detected which would typically elute within the void volume of the column (below an elution volume of 20 mL). All the apoC-III:DMPC complexes eluted as homogeneous peaks, although the amount of unbound apoC-III and the size of the discoidal complexes formed were quite different for each protein. The elution volume of the complexes varied from 26 mL (calculated Stokes radius 80 Å or 733 kDa) for the largest complex formed with the apoC-III-F64A/W65A variant to 29.5 mL (calculated Stokes radius 54 Å or 185 kDa) for the smallest complex formed with the apoC-III-L9T/T20L variant. The elution volumes of the DMPC complex of wild-type apoC-III and mutant apoC-III-K21A were 29 and 27.3 mL, respectively, corresponding to Stokes radii of 61 and 72 Å. The fraction eluting at 38 mL corresponds to unbound apoC-III, due to the small molecular mass of the protein, and is well separated from the protein:phospholipid complexes. Remarkably, however, for the different apoC-III proteins the shape and the maximum elution volume for the "lipid free" apo-C-III vary slightly. The differences in elution behavior might represent partly lipidated apoC-III intermediates. Note that the heterogeneity in this fraction is highest for the poorest lipid binder, apoC-III-F64A/W65A. This variant was also associated with the highest amount of unbound/free apoC-III protein while apoC-III-L9T/T20L resulted in the lowest amount of the unbound/free protein among all the apoC-III variants, despite the fact that identical amounts of protein, lipid, and protein:lipid incubation ratios were used for the preparation of the complexes. A clear correlation between elution volume (i.e., Stokes radius) and the bound/free apoC-III ratio could be observed and is represented in the inset of Figure 4 (r = 0.9).

**Physicochemical Characteristics of the Lipid-Free and Lipid-Bound ApoC-III Variants.** The secondary structures of the apoC-III proteins and apoC-III/TFE mixtures were determined by CD measurements (Figure 5 and Table 2). All the apoC-III variants showed fairly random structure in buffer solution with typical minima around 200 nm. The propensity to form an α-helical structure was demonstrated by adding 50% TFE. The large increases in secondary structure are illustrated in Figure 5; in all spectra, the two typical minima were approximately 208 and 222 nm, and the single maximum at 190 nm was observed. These wavelengths are typical features of an α-helical structure. The spectra were deconvoluted according to the method described, and the results are summarized in Table 2. The increase in the α-helical content was most pronounced for the apoC-III-F64A/W65A variant (+54%), followed by apoC-III-K21A and apoC-III-L9T/T20L (+31%) and less pronounced for wild-type apoC-III (+25%) (Table 2), demonstrating the different abilities of each apoC-III variant to fold as an α-helix.

The stability of the lipid-free apoC-III proteins and the apoC-III:DMPC complexes was estimated by following the decrease in their α-helical content measured by CD (molar ellipticity at 222 nm) after addition of increasing amounts of GdnHCl (Figure 6). The concentrations of GdnHCl at which 50% of the signal had decreased (the midpoints) are

<table>
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<tr>
<th>protein</th>
<th>α-helix (%)</th>
<th>β-sheet (%)</th>
<th>random coil (%)</th>
<th>Δ increase % α-helix</th>
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<td>apoC-III wild type</td>
<td>20</td>
<td>41</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>apoC-III-K21A</td>
<td>23</td>
<td>35</td>
<td>42</td>
<td></td>
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<tr>
<td>apoC-III-F64A/W65A</td>
<td>20</td>
<td>45</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>apoC-III-L9T/T20L + TFE 50%</td>
<td>20</td>
<td>41</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>apoC-III wild type</td>
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<td>26</td>
<td>25</td>
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<td>apoC-III-K21A</td>
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<td>24</td>
<td>22</td>
<td>31</td>
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<tr>
<td>apoC-III-F64A/W65A</td>
<td>74</td>
<td>16</td>
<td>10</td>
<td>54</td>
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<tr>
<td>apoC-III-L9T/T20L</td>
<td>51</td>
<td>26</td>
<td>23</td>
<td>31</td>
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Table 3: Physicochemical Characteristics of the Recombinant ApoC-III Variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>Trp $\lambda_{\text{max}}$ (nm), lipid-free</th>
<th>Trp $\lambda_{\text{max}}$ (nm), lipid-bound</th>
<th>$\Delta \lambda_{\text{max}}^*$</th>
<th>[GdnHCl] $\text{I}_{222}^*$ (M), lipid-free protein</th>
<th>[GdnHCl] $\text{I}_{222}^*$ (M), apoC-III:DMPC</th>
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</thead>
<tbody>
<tr>
<td>apoC-III wild type</td>
<td>343</td>
<td>333</td>
<td>10</td>
<td>1.3</td>
<td>3.0</td>
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<tr>
<td>apoC-III-K21A</td>
<td>343</td>
<td>333</td>
<td>10</td>
<td>1.5</td>
<td>2.4</td>
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<tr>
<td>apoC-III-F64A/W65A</td>
<td>343</td>
<td>333</td>
<td>10</td>
<td>1.8</td>
<td>2.0</td>
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<tr>
<td>apoC-III-L9T/T20L</td>
<td>343</td>
<td>333</td>
<td>12</td>
<td>1.0</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Concentration of GdnHCl (M) required to obtain 50% reduction in the CD signal measured at 222 nm as a measure of protein denaturation.

* The "blue shift" in the Trp emission maximum.

summarized in Table 3. The midpoints of all the lipid-free apoC-III proteins (Figure 6A) were below 2 M; 1 M for apoC-III-L9T/T20L, 1.75 M for apoC-III-F64A/W65A, and 1.25 and 1.5 M for wild-type apoC-III and apoC-III-K21A, respectively. Once associated with DMPC (Figure 6B), the apoC-III proteins were stabilized and protected from denaturation by the phospholipid, resulting in a shift of each apoC-III:DMPC complex midpoint toward higher GdnHCl concentrations, as shown in the Table 3. The midpoint of the wild-type apoC-III:DMPC complex was 3 and 2.4 M for apoC-III-K21A:DMPC. ApoC-III-F64A/W65A:DMPC complex denatured more readily with the lowest midpoint of 2 M, suggesting a less stable lipid-associated complex, while the apoC-III-9L20T:DMPC complex showed the highest midpoint after DMPC binding, indicating the formation of the most stable apoC-III:DMPC complex. In addition, the fact that two-phase denaturation occurred for wild-type apoC-III:DMPC and apoC-III-L9T/T20L:DMPC complexes while one-phase denaturation occurred on apoC-III-F64A/W65A:DMPC and apoC-III-K21A:DMPC complexes is consistent with this result.

The maximum Trp emission wavelengths of the lipid-free and lipid-bound apoC-III proteins are also summarized in Table 3. The maximal wavelength of the Trp emission at 343 nm for all the lipid-free proteins shifted to 333 nm for the lipid-bound apoC-III proteins. In the case of the apoC-III-L9T/T20L, an even larger blue shift of 12 nm was observed. The observed blue shifts illustrate that the apoC-
Characterization of ApoC-III Wild Type and Mutants

Figure 6: Denaturation of native (A) and DMPC—apoC-III complexes (B) monitored by measuring the decrease in molar ellipticity measured at 222 nm at increasing GdnHCl (M) concentrations in the incubation mixture with (●) WT apoC-III, (▲) apoC-III-K21A, (■) apoC-III-F64A/W65A, and (•) apoC-III-L9T/T20L.

III tryptophans are buried in a more hydrophobic environment, due to the lipid binding.

Displacement of ApoE by ApoC-III Variants from Discoidal ApoE:DPPC Complexes. The capacity of apoC-III variants to displace apoE from the reconstituted apoE:DPPC complexes was followed by analyzing gel filtration profiles of apoE:DPPC:apoC-III mixtures (a) to check the integrity of the original apoE:DPPC complexes and (b) to identify the redistribution of apoE and apoC-III on the particles. The apoE:DPPC complex eluted at its original elution volume of 29 mL, and any displaced apoE or unbound apoC-III, not associated with the complex, eluted at higher elution volumes of 34 mL for apoE and 37 mL for apoC-III (data not shown).

Western blotting was used to determine the distributions of both apoE and apoC-III in the fractions. All the apoC-III variants bound to the apoE:DPPC complex, while a fraction of the apoE was displaced from its original complex and could be detected at higher elution volumes. These results illustrate that apoE had been displaced by apoC-III from the complex, and that apoC-III had been incorporated into the apoE:DPPC complex. A more accurate quantification by analysis of the distribution of both apoC-III and apoE in these gel filtration runs was determined by measuring by ELISA the masses of both apoC-III and apoE in the individual fractions (Table 4).

From this table, it is clear that for all apoC-III proteins bound to the complex, for the apoC-III-F64A/W65A variant alone, this amount was reduced as compared with wild-type apoC-III. However, the amount of apoE displaced from the complex (Table 4) was quite different among the variants.

Table 4: Mass (µg) of ApoC-III Bound to or Mass of ApoE Displaced from ApoE:DPPC Complexes

<table>
<thead>
<tr>
<th></th>
<th>apoC-III (µg) bound on complex</th>
<th>apoE (µg) displaced from complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoC-III wild type</td>
<td>62.2</td>
<td>10.2</td>
</tr>
<tr>
<td>apoC-III-K21A</td>
<td>65.3</td>
<td>0.1</td>
</tr>
<tr>
<td>apoC-III-F64A/W65A</td>
<td>35.0</td>
<td>0.1</td>
</tr>
<tr>
<td>apoC-III-L9T/T20L</td>
<td>46.2</td>
<td>21.9</td>
</tr>
</tbody>
</table>

* The mass of apoC-III or apoE was measured by ELISA in each fraction of the gel filtration of the respective apoE:DPPC:apoC-III mixtures.

Figure 7: Inhibition of LPL by different recombinant apoC-III variants using Intralipid as substrate. Bovine LPL (15 ng) was incubated for 30 min at 25 °C in a total volume of 200 µL with 3H-labeled Intralipid in the presence (A) and in the absence (B) of 40 ng of human apoC-III protein. (●) WT apoC-III; (▲) apoC-III-K21A; (■) apoC-III-F64A/W65A; and (•) apoC-III-L9T/T20L.

Western blotting was used to determine the distributions of both apoE and apoC-III in the fractions. All the apoC-III variants bound to the apoE:DPPC complex, while a fraction of the apoE was displaced from its original complex and could be detected at higher elution volumes. These results illustrate that apoE had been displaced by apoC-III from the complex, and that apoC-III had been incorporated into the apoE:DPPC complex. A more accurate quantification by analysis of the distribution of both apoC-III and apoE in these gel filtration runs was determined by measuring by ELISA the masses of both apoC-III and apoE in the individual fractions (Table 4).

From this table, it is clear that for all apoC-III proteins bound to the complex, for the apoC-III-F64A/W65A variant alone, this amount was reduced as compared with wild-type apoC-III. However, the amount of apoE displaced from the complex (Table 4) was quite different among the variants.

While wild-type apoC-III and apoC-III-K21A displaced between 6 and 10 µg of apoE from the complex, this increase was much more pronounced for the apoC-III-L9T/T20L variant (22 µg) and very much reduced for the apoC-III-F64A/W65A variant (0.3 µg), suggesting that the lipid affinity of the apoC-III protein determined the ability to displace apoE from its complex.

LPL Inhibition by ApoC-III Variants. To identify the domains and residues of apoC-III that are responsible for LPL inhibition, the inhibitory effect of all the recombinant apoC-III variants was tested in the bovine LPL assay (Figure 7). Inhibition of the recombinant apoC-III was half that of the plasma human apoC-III in all cases (data not shown). All the recombinant apoC-III variants were able to inhibit LPL activity efficiently, both in the absence and in the presence of human apoC-III. However, in the presence of
apoC-II, the LPL inhibition was less pronounced at lower apoC-III concentrations (0–1.5 μM), with wild-type apoC-III showing an increase in LPL activity at these concentrations. From concentrations of 3 μM, the inhibition by all the apoC-III variants was well pronounced, and LPL activity was fully inhibited at apoC-III concentrations above 10 μM. In the absence of apoC-II, all the apoC-III variants showed effective inhibition at lower apoC-III concentrations. Comparing the $I_{50}$ of each apoC-III variant, in the absence of apoC-II, the $I_{50}$ of apoC-III-F64A/W65A was 1.3 μM while all the other apoC-III variants had a similar $I_{50}$ around 2 μM. In the presence of apoC-II, the $I_{50}$ for apoC-III-F64A/W65A was 2 μM, and for the other apoC-III variants 4.4 μM.

**DISCUSSION**

ApoC-III plays a key role in TG-rich lipoprotein metabolism, but the structure/function relationship of apoC-III has not been fully elucidated. We have therefore generated several apoC-III variants according to protein modeling predictions, designed to alter specific amino acid residues implicated in lipid binding and LPL inhibition of apoC-III, to investigate the structure/function relationship of human apoC-III. Sequence analysis and secondary structure predictions applied to apoC-III indicate the existence of two helical domains that are rather amphipathic, as previously suggested (11, 27). These segments were modeled, and mutants were designed to investigate further their role in the structure/function relationship of apoC-III. ApoC-III-K21A was designed to alter putative protein–protein binding properties in the N-terminus of apoC-III; apoC-III-L9T/T20L altered the angle of the tilted peptide located at residues 6–20 of apoC-III from 45° to 0°, and the apoC-III-F64A/W65A double mutation was designed to perturb the lipid binding properties of apoC-III by replacing two adjacent carboxyl-terminal aromatic amino acids with alanine.

**Lipid Binding.** Pownall and co-workers (44) have described that the rate of lipid–protein association was inversely correlated with the polypeptide molecular weight. In agreement with this, since apoC-III is a small protein, our DMPC clearing studies show a very efficient and fast clearing of the multilamellar DMPC solution.

Thrombin cleavage of apoC-III results in an N-terminal domain (residues 1–40) and a C-terminal domain (residues 41–79). Trieu et al. reported that the binding of apoC-III to surface phospholipid was mediated by the C-terminal helix between residues 50 and 62 (45). Indeed the association with DMPC indicated that the apoC-III-F64A/W65A showed severely diminished lipid binding properties, which confirms the original findings by Trieu et al. By analogy, it has been shown that the lipid binding domains in apoE and apoA-I are also localized in the C-terminus (38, 46).

Sparrow et al. concluded that the N-terminus of apoC-III (residues 1–40) was not involved in binding to lipid (11). Our results suggest otherwise; since the mutant apoC-III-L9T/T20L showed significantly increased lipid binding properties compared to wild-type apoC-III, the contribution of the N-terminus to overall lipid binding may be important. Our results indicate that the change in orientation of the N-terminal helix influences the lipid binding properties of the whole protein and the overall stability of the protein phospholipid complexes.

The size of the discoidal apoC-III:DMPC particles was dependent on the lipid binding properties of the variants. Whereas the WT apoC-III formed complexes with Stokes radii comparable to those reported by others (47), the size of the complexes formed with the poor lipid binder (apoC-III-F64A/W65A) was much larger than that of WT apoC-III particles, while the apoC-III mutant with enhanced lipid binding (apoC-III-L9T/T20L) formed disks with smaller radii as compared to WT. The relationship between size of discoidal complexes formed and lipid binding properties of apolipoprotein mutants was also observed in studies on apoA-I mutants (46).

A common observation is the increase in α-helical content of the apolipoprotein going from an aqueous solution to a lipid-bound state (47). Previous reports (47) show for apoC-III on average 26% α-helix in solution and 65–70% α-helix when lipid-bound. Careful analysis and deconvolution of our entire CD spectra (±50% TFE) showed a comparable increase in α-helicity in the presence of the helix-promoting solvent TFE for the WT and mutants K21A and L9T/T20L. In contrast, the double F64A/W65A mutant showed a larger increase in α-helical structure in TFE. This might be due to the fact that the mutation of those two bulky residues, located at the C-terminal extremity of the predicted second amphipathic helix, drastically improved the propensity of this segment to fold as an α-helix. Elimination of two consecutive bulky hydrophobic residues should favor α-helix formation. However, as a consequence of these mutations, the lipid binding affinity of this mutation was severely decreased, suggesting that these hydrophobic C-terminal residues in apoC-III have important functions in the initial interfacial recognition between the polypeptide and the phospholipid acyl chains during the initial phases of lipid binding.

In water, the maximum of Trp fluorescence emission for all apoC-III proteins tested was 343 nm, which is consistent with previous reports (46, 47). These data also suggest that (a) the Trp residues in the recombinant apoC-III are fully exposed to water, (b) the recombinant apoC-III proteins are not in a highly aggregated state, and (c) the shift of the fluorescence maximum emission to lower wavelengths (blue shift) in the presence of lipids indicates shielding of the Trp from the water and replacement of the local Trp environment by lipid molecules. In the case of the apoC-III proteins, this shift was quite large (10 nm) and even larger (12 nm) for one of our mutants tested (L9T/T20L), suggesting an even deeper insertion of this mutant in the lipid phase. These observations are consistent with the higher lipid binding affinity of this mutant and suggest a tight association with the phospholipids for this particular mutant.

The [GdnHCl] at which both free and lipid-bound proteins denatured is comparable to the values reported for other apolipoproteins such as apoE and apoA-I (38). The difference in denaturation behavior between the WT and some of the mutants suggests differences in the overall stability of the protein folds. The WT, K21A, and L9T/T20L lipid-bound mutants denatured in a biphasic manner as reported for apoE (38), suggesting that the unfolding of the lipid-bound apoC-III might also occur through stable intermediates. The results for the L9T/T20L mutant suggest an overall higher stability when lipid-bound, due to its deeper insertion into the lipid phase as evidenced by the Trp emission behavior of this mutant. In contrast, the F64A/W65A mutant contains a high
The mechanism by which apoC-III influences plasma Tg levels has been the subject of debate. There is considerable evidence that this is due, in part, to the effects on apoE-mediated clearance of Tg-rich particles. Cross-breeding of apoC-III transgenic mice with apoE transgenic mice normalizes plasma Tg levels. From these studies, it was concluded that delayed clearance of VLDL Tg in the apoC-III overexpressors was due to the low amount of apoE relative to apoC-III on the VLDL particle. Other studies suggest, however, that the hypertriglycerideremia in the apoC-III/apoE transgenic crosses is due to excess apoC-III on VLDL rather than apoE displacement (14). We chose to analyze the apoE displacement by apoC-III on discoidal apoE complexes because (a) these particles are more homogeneous than VLDL or HDL isolated from individual donors, (b) endogenous apoC-III would affect the experiments, and (c) apoC-III-deficient plasma was not available. Moreover, it is known that variable exchangeable pools of apoE are present in plasma VLDL and HDL (15). The displacement of apoE by apoC-III depends on the lipid binding affinity of the apoC-III, as reported previously for displacement of apoA-I by apoA-II (40). ApoC-III-L9T/T20L, the variant with the strongest lipid binding properties, replaced 31.2% of apoE from the apoE:DMPC complex (WT apoC-III displaced 17%), while only 8.4% of apoE was replaced by apoC-III-F64A/W65A, the variant with diminished lipid binding properties. LPL Inhibition. The results obtained from the study by McDonathy et al. examining the LPL inhibition by synthetic apoC-III peptides, suggested that the N-terminal domains of apoC-III, in particular the segments containing amino acids 1-7 and amino acids 17-25, are important in the modulation of LPL activity (72). In the current study, mutation of K21A and permutation of L9T/T20L in the N-terminus of apoC-III showed LPL inhibition comparable to recombinant wild-type apoC-III, suggesting that these changes do not affect LPL inhibition. These data therefore do not provide any further insight as to whether the N-terminus of apoC-III is involved in LPL inhibition, and additional mutations might be necessary to examine this. However, the fact that all the recombinant apoC-III variants inhibited LPL more efficiently than plasma apoC-III suggests that the N-glycosylation of plasma apoC-III may in fact modulate LPL inhibition. ApoC-III-F64A/W65A, which bound least well to DMPC particles, displayed the most inhibitory effect on LPL, and this strongly suggests that LPL inhibition by apoC-III is unlikely to depend entirely on lipid binding leading to the displacement of apoC-II or LPL from the lipid substrate. Furthermore, the enhanced lipid binding of apoC-III-L9T/T20L did not appear to alter LPL inhibition, suggesting that these two functions are independent. The fact that apoC-III with altered lipid binding capacity can inhibit LPL activity is consistent with the notion that apoC-III inhibition of LPL lipolysis is due to protein:protein interactions rather than protein:lipid interactions (48). The involvement of the C-terminus of apoC-III in LPL inhibition supports the findings of Sparrow et al., who showed that the inhibitory effect of apoC-III could be localized to the C-terminal domain between residues 41 and 79 (11). This current study allows us to speculate on the mechanism of LPL inhibition by apoC-III. ApoC-III variants were able to inhibit LPL activity in the presence or absence of the LPL activator apoC-II, although with different $k_i$. In the presence of apoC-II, low concentrations of apoC-III inhibit less efficiently LPL, and in fact at low concentrations of wild-type apoC-III, LPL activity increased. Since apoC-III-F64A/W65A bound poorly to lipid, increased amounts of free apoC-III-F64A/W65A may directly interact with LPL. We therefore suggest that apoC-III inhibition of LPL may result from the balance of direct protein:protein interaction. Whether apoC-III interacts directly with apoC-II cannot be resolved by these results, and additional experiments would be needed to resolve this.

Taken together, these data identify that the hydrophobic residues F64 and W65 are crucial for the lipid binding properties of apoC-III and redistribution of the N-terminal helix of apoC-III (L9T/T20L) enhances the stability of the lipid-bound protein. Thus our structure/function relationship studies of wild type and mutant apoC-III suggest that the C-terminal hydrophobic residues in the sequence have important functions in the initiation of lipid binding, while redistribution of the hydrophobicity of the N-terminal helix improves the lipid binding and stability of the apoC-III polypeptide. This suggests that the N-terminal domain of apoC-III is necessary for the efficient functioning of WT apoC-III and/or normal lipoprotein metabolism. Indeed, this imperfect amphipathic domain could serve, for example, to balance apolipoprotein exchange. This is consistent with our results on the displacement of apoE from preformed complexes which is dependent on the lipid affinity of the apoC-III mutant. Our results further suggest that the inhibition of LPL activity by apoC-III is independent of the ability to bind lipids and by doing so to displace the LPL activator apoC-II, but may be due to direct protein:protein interactions with LPL and/or apoC-II. Further studies will be required to clarify these points.

REFERENCES


Characterization of the lipid-binding properties and lipoprotein lipase inhibition of a novel apolipoprotein C-III variant Ala23Thr

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Abstract We have identified a G-to-A transition in exon 3 of the APOC3 gene resulting in a novel Ala23Thr apolipoprotein (apo) C-III variant, associated with apoC-III deficiency in three unrelated Yucatan Indians. The Ala23Thr substitution modifies the hydrophobic/hydrophilic repartition of the helical N-terminal peptide and hence could disturb the lipid association. In vitro expression in Escherichia coli of wild-type and mutant apoC-III enabled the characterization of the variant. Compared with wild-type apoC-III-Ala23, the mutant apoC-III-Thr23 showed reduced affinity for dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles with higher amounts of free apoC-III. Displacement of apoE from discoidal apoEdipalmitoylphosphatidylcholine (DPPC) complex by apoC-III-Thr23 was comparable to wild type but the less efficient binding of the apoC-III-Thr23 to the discoidal complex resulted in a higher apoE/apoC-III (mol/mol) ratio (34%) than with wild-type/apoE:DPPC mixtures. The inhibition of lipoprotein lipase (LPL) by apoC-III-Thr23 was comparable to that of wild type, and therefore effects on LPL activity could not explain the reduced triglyceride (Tg) levels in Thr-23 carriers. Although the roles of apolipoprotein (apo) B, apoE, and apoA-I and apoC-II in lipid metabolism are well defined, this is not true for apoC-III. apoC-III is a 79-amino acid glycoprotein, accounting for 26% of the protein in very low density lipoprotein (VLDL) and 2% in high density lipoprotein (HDL) (1). Plasma apoC-III levels are positively correlated with plasma triglyceride (Tg) and cholesterol levels (2–4), and elevated apoC-III levels have been found in hypertriglyceridemic individuals (5) and in patients with coronary artery disease (CAD) (6). Furthermore, apoC-III acts as a marker of Tg-rich lipoprotein (TGRL) metabolism and the apoC-III HDL:VLDL ratio has been found to be negatively associated with the progression of atherosclerosis in a number of studies (7, 8).

The mechanisms by which apoC-III regulates the catabolism of TGRL is complex, involving both the inhibition of lipoprotein lipase (LPL), thus reducing Tg hydrolysis (9–11), and the displacement of apoE on the VLDL particles, thus retarding apoE-mediated uptake of TGRL by hepatocytes. This is supported by in vivo studies showing a rapid conversion of VLDL to LDL in individuals with combined apoA-I and apoC-III

Supplementary key words genetic variation • LPL inhibition • lipid binding • DMPC • apoE displacement

The major function of apolipoproteins is the transport and distribution of lipids among various tissues in the body and the control of enzyme activity. Although the roles of apolipoprotein (apo) B, apoE, and apoA-I and apoC-II in lipid metabolism are well defined, this is not true for apoC-III. apoC-III is a 79-amino acid glycoprotein, accounting for 26% of the protein in very low density lipoprotein (VLDL) and 2% in high density lipoprotein (HDL) (1). Plasma apoC-III levels are positively correlated with plasma triglyceride (Tg) and cholesterol levels (2–4), and elevated apoC-III levels have been found in hypertriglyceridemic individuals (5) and in patients with coronary artery disease (CAD) (6). Furthermore, apoC-III acts as a marker of Tg-rich lipoprotein (TGRL) metabolism and the apoC-III HDL:VLDL ratio has been found to be negatively associated with the progression of atherosclerosis in a number of studies (7, 8).

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Abbreviations: apo, apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoprotein; IPTG, isopropyl-β-D-thiogalactopyranoside; LCAT, lecithincholesterol acyltransferase; LPL, lipoprotein lipase; MHP, molecular hydrophobicity potential; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tg, triglycerides; TGRL, triglyceride-rich lipoprotein; TRP, tryptophan; VLDL, very low density lipoprotein.

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deficiency (10). Liver perfusion studies have suggested that apoC-III directly inhibits the hepatic uptake of chylomicrons and VLDL (12, 15), possibly by impairing the interaction of apoE on the lipoproteins, with LDL receptors (14). Confirmation of this role of apoC-III in the clearance of TGRL comes from transgenic mouse studies. Disruption of the apoC3 gene in mice results in protection from postprandial hypertriglyceridemia (15), while the overexpression of human APOC3 results in hypertriglyceridemia, with a positive correlation between apoC-III levels and Tg concentration (16). This elevated Tg in human APOC3 transgenic mice is due to an increased number of VLDL particles in the circulation, which contain more Tg and apoC-III and less apoE, thus diminishing apoE-mediated lipoprotein uptake (17). ApoC-III overexpression also reduces the ability of VLDL to bind to glycosaminoglycans, thus decreasing efficient lipolysis at the cell surface (18).

The APOC3 gene is located on chromosome 11q23, in a cluster with APOAI and APOA4 (19). Genetic variation at the APOAI-C3-A4 gene cluster has been found to be associated with differences in plasma lipid, lipoprotein, and apo levels (20, 21). Several common variants of the APOC3 gene have been shown to be associated with high plasma Tg levels in some samples (17, 22–24), with elevated levels of apoC-III in healthy individuals (2), and with premature CAD (25, 26). However, deficiency of apoC-III is rare. A 6.5-kilobase inversion in the APOAI-C3-A4 gene cluster and a complete deletion of the APOAI-C3-A4 gene cluster have been reported to cause combined deficiency of apoA-I and apoC-III (27) and familial apoA-I-C-III-A-IV deficiency, respectively (28). In both cases, plasma HDL levels were undetectable, Tg levels were low, and homozygous individuals had premature atherosclerosis. To date, four rare structural variants of apoC-III have been identified: Gln38Lys, Asp45Asn, Lys58Glu, and Thr74Ala (29–32).

In a previous study of the Mayan population of the Yucatan peninsula, an APOC3 allele was reported to be associated with low plasma apoC-III levels (33). Molecular analysis revealed no gross DNA rearrangements or deletions in the APOAI-C3-A4 locus. In the current study, we report the identification of a novel apoC-III mutation in these Mayan individuals and the use of recombinant technology to express wild-type and mutant apoC-III for structure-function analysis.

### Subjects and Methods

**Subjects**

The lowland Mayan Indians inhabit the Yucatan peninsula and other parts of Central America. Samples were collected in 1985 and 1987 from a series of local villages, as part of a study of chronic disease risk factors in Amerindians (33). All known first- and second-degree relatives were excluded. Ten to 20 ml of ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood were drawn in vacutainers, maintained on wet ice, and returned to the laboratory within 3 to 5 days. Sixteen individuals from one remote village of the original sample were included in this study. The 192 Caucasian control males (aged 51–60 years), free from CAD, were recruited from a general practitioner practice in Camberley, south London (21).

**Lipoprotein measurements**

Plasma apoC-III was quantified in triplicate by single radial immunodiffusion (34), using commercial plates and control standards from Daiichi Pharmaceuticals (Fort Lee, NJ). Total plasma cholesterol and triglyceride levels were determined manually by enzymatic methods (35).

**DNA amplification by polymerase chain reaction**

Genomic DNA was isolated from frozen buffy coats. The APOC3 gene was amplified with primer pairs 1 and 5, 2 and 5, and 6 and 8 covering the 5′ promoter from −345 to 3′ bp 3545, which included all exons and the 3′ untranslated region (Table 1). All the primers, W-1 detergent, and DNA Taq polymerase were obtained from Gibco-BRL (Gaithersburg, MD). The polymerase chain reaction (PCR) was performed in a reaction mixture (total volume, 50 μl) including 250 ng of primers, one of which was biotinylated, 200 ng of genomic DNA, and 1 U of Taq polymerase. The reactions were performed on an Intelligent Heating Block (Cambio, Cambridge, UK) at 95°C for one cycle of 5 min, 55°C for 1 min, and 72°C for 2 min, and subsequently for 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, in the reaction buffer provided by the manufacturer, with MgCl₂ ranging from 0.10 to 0.15 mM.

**Direct sequencing for mutation detection**

PCR products were purified with streptavidin-coated beads (Dyna1, Bromborough, UK) to separate the double-stranded DNA. Sequencing was carried out according to the Sequenase version 2 protocol (United States Biochemical, Cleveland, OH).

**AcI digestion**

Fifteen microliters of the PCR products amplified with primers 4 and 5 was incubated at 37°C with 10 units of AcI (New England Biolabs, Hitchin, UK) for 16 h in a total volume of 20 μl, using the buffer recommended by the manufacturer. DNA fragments were separated by electrophoresis on a 10% acrylamide gel, stained with ethidium bromide, and viewed under ultraviolet light.

### Table 1. Oligonucleotides used for PCR and direct sequencing for mutation detection

<table>
<thead>
<tr>
<th>5' Oligonucleotide</th>
<th>Sequence</th>
<th>3' Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>5'-CCAGGGTGAAGGCGATGG</td>
<td>5'-CTACCTTTAGGGGCCACGCCAC</td>
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<tr>
<td>5'-GGTCCGAGCTGACACCATGTT</td>
<td>5'-CAATGGGTGGTCAAGCAGAAGC</td>
<td></td>
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<tr>
<td>5'-GGATGGTTGCCTACAGAGGATG</td>
<td>5'-CAATGGGTGGTCAAGCAGAAGC</td>
<td></td>
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<tr>
<td>5'-CTACCITAGGGGCCACGCCAC</td>
<td>5'-CAATGGGTGGTCAAGCAGAAGC</td>
<td></td>
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<tr>
<td>5'-ATGAAAAAGCAGGCTGACCTG</td>
<td>5'-CAATGGGTGGTCAAGCAGAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-GAGAAATTTTAVGAGTTGTTG</td>
<td>5'-CAATGGGTGGTCAAGCAGAAGC</td>
<td></td>
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* Indicating biotinylated primers.

2 Journal of Lipid Research Volume 41, 2000
DNA polymorphisms

Briefly, the genotypes of the Sst and PvuII polymorphisms of the APOC3 gene were determined as reported previously (36), the −75C→A substitution of the apoAI promoter region and the 1100C→T transition of the APOC3 gene were genotyped by PCR in combination with allele-specific oligonucleotide melting (21).

Molecular hydrophobicity potential calculations; three-dimensional construction of the peptide

Construction of the peptides was carried out as previously described (37). The method accounted for the contribution of the lipid-water interface, the concomitant variation of the dielectric constant, and the transfer energy of atoms from a hydrophobic to a hydrophilic environment (38). The calculation of the molecular hydrophobicity potential (MHP) along the peptide was carried out by assuming that the hydrophobic interaction between an atom and a point M decreases exponentially with the distance according to the equation (39):

\[
\text{MHP} = \text{E} \cdot \text{r} \cdot \exp[\text{r}_i - \text{d}_i]
\]

where E \text{r}_i is the energy of transfer of atom i, r_i is the radius of the atom i, and d_i is the distance between atom i and a point M.

Calculations were made in a plane perpendicular to the main orientation of the long axis of the helix, which was moved every 2 Å along this axis.

Secondary structure prediction

The secondary structure prediction was carried out at the NPS@ (Network Protein Sequence @nalysis) web site http://npsa.pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_seccons.html. Various predictive methods are used to obtain the consensus: SOPMA (40), PHD (41, 42), GORIV (44), DPM (45), DSC (46), SIMPMA (47, 48), and HNNC (49).

Generation of pET23b/APOC3 construct

The human APOC3 cDNA (a kind gift from J. Taylor, San Francisco, CA) in the pBSSK vector (Stratagene, La Jolla, CA) was used as a template for the amplification of the APOC3 gene by PCR. This was performed in a 50-μl reaction containing 50 ng of plasmid DNA pBSSK/APOC3 as template, 250 ng of each primer, and 1 unit of Taq DNA polymerase (Stratagene) with an internal NddI (underlined) (5′-GGGAATTCCATATGTCAAGGGCCAGAGATG-3′) and a reverse primer designated with XhoI restriction site (underlined) (5′-GTGGCTCTCTGCACGGCCAGCCACTGCTTGACG-3′), were used for PCR, under the following conditions: the initial cycle of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Reactions were performed on an automated Omigene PCR machine (Hybaid, Middlesex, UK). The resulting 264-bp human APOC3 fragment was digested with NddI and XhoI, and subcloned into a similarly digested pET23b vector (Novagen, Cambridge, UK) according to standard methodology. The ligation mixtures were transformed into competent cells of the XL1-Blue strain of E. coli B834 and BL21 (Novagen), respectively, for optimum expression and used to inoculate an overnight culture in SOC medium containing ampicillin at 75 μg/ml (Sigma, Irvine, UK). Thirty milliliters of the overnight culture was used to inoculate 1 liter of SOC medium (ampicillin at 75 μg/ml) and shaken (220 rpm/min) at 37°C. Cells were induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM; Sigma) when the optical density at 600 nm (OD600) of the culture reached 0.6. The cells were harvested (3,000 rpm for 15 min at 4°C) after 2 h of induction for wild-type apoC-III and after 1 h for mutant apoC-III. Cells were lysed in 15 ml of 8 M urea, 50 mM NaH2PO4, 10 mM Tris-HCl, 500 mM NaCl (pH 8.0) buffer (sonication on ice; three times, 1 min each), and then centrifuged at 18,000 rpm (30 min at 4°C), and the supernatant was then incubated with 1 ml of Talon™ cobalt metal affinity resin (2 h at 4°C) (Clontech, Hampshire, UK). The resin was pelleted by centrifugation (3,000 rpm for 5 min), and batch washed (four times). The recombinant human apoC-III fusion proteins were eluted from the column with a buffer consisting of 8 M urea, 50 mM NaH2PO4, 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid), and 500 mM NaCl (pH 6.0–6.5). The fractions containing recombinant apoC-III were identified by electrophoresis on Tricine-sodium dodecyl sulfate (SDS) polyacrylamide gels (17.5% precast gels; Bio-Rad, Hemel Hempstead, UK), and dialyzed overnight at 4°C against 4 M urea, 5 mM NH4HCO3 (pH 8.0). This solution was loaded on an anion-exchange column, Mono Q® (Pharmacia, Upplands, Sweden) and eluted with a linear NaCl gradient (from 0.10 to 0.25 M) in 4 M urea, 5 mM NH4HCO3 (pH 8.0) buffer. The fractions containing the pure protein (elution at an NaCl concentration of approximately 0.15 M) were pooled and the final purity of the product was verified as described above. Both apoC-III variants were at least 95% pure. The protein content in the samples was determined by OD measurement at 280 nm, using a molar extinction coefficient of 19,630 (M−1 cm−1).

Lipid-binding properties of apoC-III

Association of recombinant apoC-III variants with lipid was followed by monitoring the turbidity decrease of dimyristoylphosphatidylcholine (DMPC) multimellar vesicles (Sigma) at 325 nm as a function of the temperature. The DMPC vesicles were obtained as previously described (50) and 40 μg of apoC-III protein was added to 80 μg of DMPC vesicles in a 0.01 M Tris-HCl buffer containing 150 mM NaCl (pH 8.0), 8.5% KBr, 0.01% NaN3, and 0.01% EDTA, in a Uvikon 931 spectrophotometer (Kontron, Milan, Italy). The kinetics study of lipid/apoC-III association was followed by monitoring the rate of clearance of the turbidity of DMPC liposomes at 325 nm, as a function of time at 20 and 30°C. The rate constant $k = -\ln(0.5)/t_{1/2}$, where $t_{1/2}$ is...
the time needed to obtain a 50% reduction of the optical density signal at 325 nm.

Preparation and isolation of phospholipid: apoC-III complexes

ApoC-III:DMPC complexes were prepared by incubation of the recombinant apoC-III with DMPC vesicles, at DMPC-to-protein ratio of 2.1 (w/w) at 25°C for 16 h. The complexes were isolated by gel filtration on a Superose 6HR (Amersham Pharmacia Biotech UK, Little Chalfont, UK) column in 0.01 M Tris-HCl buffer containing 150 mM NaCl (pH 7.4) and NaHCO

Circular dichroism measurements

Circular dichroism (CD) spectra of each recombinant apoC-III protein and their complexes with DMPC are measured on a Jasco (Easton, MD) 600 spectropolarimeter at room temperature. Measurements were carried out at a protein concentration of 200 μg/ml in 5 mM NH

Displacement of apoE from the apoE:DMPC complexes

The ApoE: DMPC complexes were prepared by the sodium cholate dialysis method as described (51), using DMPC (Sigma) at a 2:1 (w/w) ratio and using recombinant apoE purified from E. coli as described (52). The protein-lipid mixtures were incubated overnight at 4°C and then extensively dialyzed against 10 mM Tris-HCl buffer, pH 8.0.

Detection of apoC-III and apoE proteins by Western blotting

Samples were separated on a Tricine-SDS polyacrylamide gel (17.5%), or on a 15% SDS polyacrylamide gel. Proteins were transferred onto a wet blot system (Bio-Rad) in a Tris (5 mM)-glycine (192 mM) buffer, pH 8.3, with 20% methanol. Proteins were visualized with rabbit anti-apoC-III or rabbit anti-apoE polyclonal antibodies and a secondary peroxidase-labeled goat antirabbit antibody (Sigma). The bound peroxidase was visualized with BM chemiluminescence luminal substrate (Boehringer-Mannheim, Mannheim, Germany).

LPL inhibition by apoC-III variants

Bovine LPL (EC 3.1.1.34) (Sigma) catalytic activity, in the presence of apoC-III wild type and mutant, was measured with an emulsified [3H]triolein substrate, Intralipid™ (100 mg/ml; Pharmacia Laboratories, Milton Keynes, UK) (55). The incubation medium contained 2% (v/v) of the emulsion, 6% (w/v) bovine serum albumin (BSA; Sigma), 5% (v/v), and 3 IU of heparin (Sigma). As a source of apoC-II, hophylized human plasma apoCII was dissolved in 5 M urea, 10 mM Tris-HCl (pH 8.5) at 2 mg/ml. The experiments were optimized for both apoCII and LPL, to give optimal hydrolysis with minimum apoC-II concentration. The emulsion was preincubated with each apoC-III sample with or without 40 ng of apoCII for at least 15 min, after which time the bovine LPL (10 μl of a 1.5-μg/ml solution) was added, in a final volume of 200 μl. The mixture was shaken in a water bath at 25°C for 30 min. The reaction was stopped, the fatty acids were extracted, and the radioactivity was counted (55).

Statistical analysis

To test whether there was a statistically significant difference in the kinetic measures, a Wilcoxon signed rank test was used. Time was used to pair the variables. A P value of <0.05 was taken to be significant.

RESULTS

Identification of the novel Ala23Thr-APOC3 mutation

The nucleotide sequences of the APOC3 gene including the 5'-flanking region, all exons, and the 3'-flanking region (~345 to 3545), were examined in four DNA samples from Mayan Indians with plasma apoC-III levels of 1.6, 1.9, 8.4, and 18.4 mg/dl, respectively, using PCR and direct sequencing. In the two individuals with apoC-III deficiency (ID57 and ID67; Table 2), a G1125-to-A transition in exon 3 of the APOC3 gene, changing amino acid 23 from alanine to threonine, was identified (results not shown). Both subjects were heterozygous for the sequence change. In addition, a number of other sequence differences were detected among the four individuals accounting for known polymorphisms of the APOC3 gene (56). No other novel sequence difference was detected. The G1125-to-A sequence change abolishes an AcI cutting site (CCGC) and this enabled confirmation of the sequence change by PCR and restriction enzyme digestion. This method was used to screen the additional 12 Mayan Indian DNA samples and a sample of healthy men from south London (21). None of the 192 Caucasians were carriers of the sequence change, but Mayan sample ID105 was a carrier of the mutation. This individual's plasma apoC-III levels (5.5 mg/dl) were lower than the sample
TABLE 2. Plasma apoC-III and lipid levels in Mayan Indian sample

<table>
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<th>ID</th>
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<th>BMI</th>
<th>ApoC-III</th>
<th>Tg</th>
<th>Chol</th>
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* Mean (±SD) from the other 13 Mayans (5 males and 8 females) included in the study.

Mean (7.7 mg/dl) but was not among those with the lowest apoC-III levels. The available lipid and lipoprotein data for the three APOC-Thr23 carriers and 13 noncarriers are presented in Table 2. The two individuals with the lowest plasma apoC-III had levels that were 20 and 25% of the sample mean, with Tg and total cholesterol levels that were correspondingly low (reduced to 25 and 48% and 56 and 78%, respectively). Levels of apoC-III in the third carrier were 71% of the sample mean and plasma lipid levels were slightly above that of the noncarriers. This subject was male, older than the female carriers, and had a low body mass index (BMI) of 19.1 kg/m².

To identify the haplotype on which the A1125 allele (APOC-Thr23) occurred, DNA polymorphisms of the APOAI-C3-A4 gene cluster (the −75G→A substitution of the gene encoding apoA-I, the SstI polymorphism, the 1100C→T transition, and the PvuII polymorphism of the APOC3 gene) were used. The data are compatible with the mutation for Thr-23 being present on a rare A—75, S—, T1100, V— haplotype in all three individuals (Table 2).

Molecular modeling

Molecular modeling was used to predict the significance of the Ala23Thr substitution on the secondary structure and thus its effect on the function of the protein. The N-terminal domain of apoC-III is helical, as suggested by the secondary structure predictions (Fig. 1A and B). The region from residue 15 to 33, corresponding to the core of the predicted α-helical N-terminal domain, was examined in detail. The distribution of the hydrophobic and hydrophilic envelopes suggests that this segment is amphipathic, because there is a good segregation between the hydrophobic and hydrophilic faces of the α helix (Fig. 2A). The Ala23Thr substitution induces a modification of the hydrophobic distribution around the α helix (Fig. 2B and C). Analysis of the isopotential surfaces showed that Thr-23 induces a hydrophilic domain into the hydrophobic potential that is likely to influence the affinity of apoC-III-Thr23 for a lipid surface.

Expression of recombinant apoC-III

To obtain large quantities of apoC-III proteins to investigate the structure-function relationship, an E. coli expression system was developed that yielded milligram quantities of the recombinant C-terminus histidine-tagged apoC-III proteins. For optimal expression wild-type apoC-III was expressed in E. coli B834 and apoC-III-Thr23 was expressed in E. coli BL21. For reasons of stability different induction times were used, 2 h for wild type and 1 h for apoC-III-Thr23. After induction by IPTG at 37°C the fusion proteins reached about 5% of the total cellular protein (as determined by Coomassie blue staining of SDS-polyacrylamide gels). Because the fusion proteins were contained in inclusion bodies they were purified in the presence of urea, using a combination of Talon affinity agarose and anion-exchange chromatography. The resulting apoC-III fusion proteins were shown to be more than 95% pure and approximately 2 mg of the pure recombinant fusion protein was obtained from 1 liter of culture medium.

Lipid-binding properties of the recombinant apoC-III proteins

DMPC-binding experiments were performed on three separate occasions to assess the effect of wild-type and mutant apoC-III on the kinetics of the interaction with DMPC multilamellar vesicles. Both wild-type and mutant apoC-III-Thr23 bound to DMPC rapidly and maximally around the transition temperature of the lipid (23°C), as indicated by the dramatic decrease in turbidity of the DMPC dispersions (data not shown). The rate of interaction between the apoC-III proteins and DMPC was monitored by monitoring the decrease in turbidity at 325 nm. Experiments were performed below the transition temperature at 20°C and above the transition temperature at 30°C. At both temperatures tested, the rate constant of the binding
Fig. 1. Secondary structure predictions of apoC-III. (A) apoC-III-Ala23; (B) apoC-III-Thr23; (C) consensus prediction around the Ala23Thr mutation (region 10–30). Top: apoC-III-Ala23. Bottom: apoC-III-Thr23. Different predictive methods were used (see Subjects and Methods) and a consensus was obtained. The first column presents the names of the various predictive methods. The first line presents the apoC-III sequence; the last line presents the consensus prediction, h, α helix; e, β sheet; c, coil; t, turn; ?, no consensus secondary structure.

of the mutant apoC-III (the k value) was slower as compared with the wild type (Fig. 3A and B and Table 3), suggesting a less efficient binding of the mutant apoC-III. These values at 20°C were 0.43 ± 0.03 and 0.29 ± 0.03 min⁻¹ for wild-type and mutant apoC-III, respectively (P < 0.0001) (Fig. 3A) and at 30°C the values were 0.17 ± 0.02 and 0.07 ± 0.01 min⁻¹, respectively (P < 0.0001) (Fig. 3B). The resulting DMPC:apoC-III discoidal particles (formed at 23.5°C) were fractionated on a Superose 6 PG gel-filtration column and the apoC-III content of these fractions was monitored by comparing the Trp emission intensity at 330 nm. These results were reproducible, and results from a representative experiment are presented in Fig. 4. Although both apoC-III proteins were able to clarify the DMPC solution, the amount of unbound apoC-III and the size of the discoidal complexes formed were quite different for each protein. In the gel-filtration run no free lipid, which would typically elute within the void volume of the column (below elution volume of 20 ml), could be detected. Both complexes eluted as homogeneous peaks although at different maxima; wild-type apoC-III eluted at 29.0 ml or with a size corresponding to a Stokes radius of 61 Å, while the mutant C-III eluted at 27.3 ml, corresponding to larger particles with a Stokes radius of 72 Å. Moreover, the amount of unbound apoC-III eluting at 38 ml is higher for the mutant apoC-III than for the wild-type apoC-III despite the fact that identical amounts of protein and incubation ratios of protein:lipid were used for the preparation of the complexes. Although the differences are small, in terms of molecular weight these represent a large difference.

Physicochemical characteristics of the native and lipid-bound apoC-III

The secondary structure of the native and lipid-bound apoC-III was determined by CD measurements and the results are presented in Table 3. Both proteins showed a similar α-helical content both in native and lipid-bound form.
The percentage of α-helical structure increased by 17% for the wild type and by 15% for the mutant apoC-III on binding to DMPC.

Displacement of apoE by apoC-III variants from discoidal apoE:DPPC complexes

The capacity of apoC-III to displace apoE from the reconstituted apoE:DPPC complexes was monitored by analyzing gel-filtration profiles of apoE:DPPC-apoC-III mixtures. Briefly, as described in Subjects and Methods, the apoE:DPPC complexes were incubated in vitro with the apoC-III proteins at a 1:1 (w/w) ratio for 2 h. The mixture was then gel filtrated on a Superose 6 PG column to check the integrity of the original apoE:DPPC complexes and to identify the distribution of apoE and apoC-III on the particles. The apoE:DPPC complex eluted at its original elution volume and any displaced apoE or unbound apoC-III, not associated with the complex, eluted at higher elution vol-

Fig. 2. MHP surfaces around segment 15–33. (A) CPK representation of peptide 15–33. The N terminus is on the left, and the C terminus is on the right. Ala-23 is highlighted in blue. (B) apoC-III-Ala23 and (C) apoC-III-Thr23. MHP surfaces around segment 15–33 in the same orientation. Green surfaces represent hydrophilic domains whereas orange surfaces represent hydrophobic domains. The surfaces are cut with a plane to visualize the mutated residue (in blue), identified with an arrow.
Fig. 3. Kinetics of clarification of DMPC liposomes by apoC-III. Decrease in turbidity of DMPC multilamellar vesicles mixed with 40 μg of recombinant protein of wild-type apoC-III-Ala23 (solid squares) and mutant apoC-III-Thr23 (solid triangles), at a lipid-to-protein ratio of 2:1 (w/w) over 10 min at (A) 20°C and (B) 30°C. Results are expressed as a percentage of the original optical density signal of DMPC multilamellar vesicles measured at 325 nm. These results are representative of three repeat experiments.

The 2.5-fold difference in LPL activity in the presence and absence of apoC-II is low and reflects the fact that the experiment was optimized to be performed at the lowest concentration of apoC-II that activated LPL. The ability of increasing concentrations of recombinant apoC-III's (range, 1.5–15 μM) to inhibit bovine LPL was estimated in the presence (Fig. 5A) or absence (Fig. 5B) of apoC-II. Both wild type and the apoC-III variant showed a similar ability to inhibit LPL and were more potent inhibitors than human plasma apoC-III (results not shown). These results represent the means of three experiments.

DISCUSSION

We have identified a novel apoC-III Thr-23 for alanine substitution associated with apoC-III deficiency, identified in three Mayan Indians from the Yucatan Peninsula, with two of the three carriers having low plasma Tg levels. Because samples from relatives were not available, cosegregation of the phenotype with Thr-23 could not be confirmed. The 1125G→A transition, creating the amino acid substitution, occurs at a CpG dinucleotide, a "hot spot" for mutational events, raising the possibility that the mutation could have occurred more than once. However, this is unlikely in view of the rarity of apoC-III deficiency and APOC3 gene mutations. Using DNA polymorphisms of the APOA1-C3-A4 gene cluster, all three individuals with the mutation were carriers of a haplotype defined by -75A, S-, HOOT, V-. Although samples were not available from sufficient unrelated Yucatan subjects to determine the frequency of this haplotype, the haplotype common to all three carriers is defined by the rare alleles of the ~75G→A, 1100C→T and Ssd in studies of Caucasians (57, 58), sug-

<table>
<thead>
<tr>
<th>TABLE 3. Physicochemical characteristics of recombinant wild type and apoC-III-Thr23 and the interaction with apoEDPPC complexes</th>
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<tbody>
<tr>
<td>apoC-III-Ala23</td>
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<tr>
<td>% Increase in a Helix Molar Ratio in DPPC Complex (of Wild Type)</td>
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<td>apoC-III-Thr23</td>
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* The kinetics study of DMPC:apoC-III association was performed by monitoring the rate of clearance of the turbidity of DMPC liposomes at 325 nm, as a function of time at different temperatures. The rate constant k was determined as described in the Subjects and Methods section.

* The ratio of bound apoC-III on the complex/unbound apoC-III, estimated from the relative tryptophan fluorescence emission intensity of the gel-filtered samples.
ggesting that in these individuals the mutation, occurring on this rare allele, is identical by descent. By comparison with the C-terminal domain, the N terminus, and particularly residues 12–40 of apoC-III, are well conserved among species (59), increasing the probability that this mutation is functionally important. We therefore carried out molecular modeling and expression studies to explore the possible mechanisms by which the Ala23Thr mutation leads to apoC-III deficiency.

All the soluble apolipoproteins, including apoC-III, have a similar gene arrangement of repeated blocks of functionally identical amino acids, which have the characteristics of amphipathic α helices, with hydrophobic residues segregated on one side of the helix and polar residues on the other (60, 61). These amphipathic helices are involved in the lipid-binding properties of the apolipoproteins, and the amphipathicity of apolipoprotein is correlated with the ability of the peptide to interact with phospholipid. On the basis of the consensus secondary structure predictions the N-terminal domain of apoC-III is predicted to fold as a typical amphipathic lipid-binding α helix, especially between residues 5 and 40 (37, 62). Calculations of the hydrophobicity potentials around segment 15–33 of this N-terminal helix suggest that the hydrophobic isopotential envelope, which is undisturbed and prominent for the wild-type protein, is disturbed in the Thr-23 mutant. The Thr-23 for alanine substitution introduces a hydrophilic patch in the center of the hydrophobic isopotential envelope (Fig. 2B and C). This substitution would be predicted to decrease the energy of interaction with lipids at the N terminus. The secondary structure prediction shows that the mutation induces a local structural variability that would be predicted to reduce the stability of the first helical domain (Fig. 1A and B). Because the amphipathic helical structure appears to be the lipid-interacting unit in apolipoproteins (37, 63), the two modeling methods suggest a decreased lipid-binding affinity for the Thr-23 mutant. These predictions are borne out by the in vitro experiments, which were carried out with recombinant wild-type and mutant apoC-III. Both recombinant proteins have a terminal histidine tag, but all the physicochemical data point to the similar behavior of the histidine-tagged and natural wild-type apoC-III, confirming that function is not affected by its presence. Functional characterization of uncleaved N-terminal histidine-tagged recombinant apoA-I (64, 65) and C-terminal recombinant histidine-tagged lecithin: cholesterol acyltransferase (LCAT) (57) has been performed, and the tagged proteins are reported to be no different when compared with plasma apoA-I or LCAT, respectively. In addition, the extra six histidine residues are unlikely to be well structured and therefore are unlikely to affect function. For the lipid moiety, DMPC was used. DMPC is commonly used in in vitro experiments examining, for example, apoA-I and E lipid-binding properties (66, 67). We chose to analyze the apoE displacement by apoC-III on discoidal apoE complexes because these particles are more homogeneous than VLDL or HDL isolated from individual donors. Furthermore, apoC-III-deficient plasma was not available and endogenous apoC-III would affect the experiments.

With respect to the lipid-binding properties, data from both the kinetics of DMPC binding and the analysis of the gel filtration of the DMPC complexes demonstrate that, although apoC-III-Thr23 does bind to phospholipids, the affinity of this mutant apoC-III is statistically significantly lower than that of the wild-type apoC-III-Ala23, although these differences are modest. Wild-type apoC-III formed a smaller complex with DMPC compared with the apoC-III-
Thr23:DMPC complex, which had a reduced bound/free apoC-III ratio, suggesting that apoC-III-Thr23 has a lower affinity for the phospholipid than the wild-type apoC-III. The secondary structure analyses by CD revealed that for both proteins the α-helical content of apoC-III after binding to DMPC increased by about 15-17%, illustrating that as a result of the binding to the phospholipid the secondary structure of both proteins was stabilized.

In vivo, APOC3 transgenic mice display severe hypertri-

glyceridemia as a result of a dramatic reduction in hepatic uptake of TGRL (68, 69). The mechanism for this has been suggested to be due to the inhibition of VLDL binding to the LDL receptor due to the high plasma levels of apoC-III, and could be corrected by breeding these mice with apoE transgenics (70). These counteracting effects of apoC-III and apoE are confirmed by in vitro studies showing that apoC-III decreases and apoE increases lipoprotein binding to low density lipoprotein receptor-related protein (71). Taken together these data suggest that apoC-III interferes with the apoE-mediated uptake of lipoproteins by the displacement of apoE, thus reducing TGRL clearance.

Cardin, Jackson, and Johnson (72) reported that a common binding site existed on DMPC vesicles for various apo-
lipoproteins and thus apoE and apoC-III may share common binding sites on such lipid particles. Their binding to lipid particles is competitive, reversible, and in equilibrium. In the present study displacement among the apolipoproteins occurred without the concomitant dissociation of lipid from the lipid particles, because no free lipid was seen when apoC-III displacement of apoE from the complex was analyzed by gel filtration. The displacement of apoE by apoC-III depends on the lipid-binding affinity of apoC-III; in other words, it is dependent on the amphipathicity of the α helix of each protein. The displacement of apoE from the apoE:DMPC complex by apoC-III-Thr23 was comparable with wild-type apoC-III, but binding of apoC-III on the complex was decreased for the mutant apoC-III. This is probably due to the reduced lipid-binding ability of the mutant apoC-III, resulting in a higher apoE/apoC-III-Thr23 complex, compared with wild-type apoC-III.

In the current study, in vitro effects on LPL hydrolysis by recombinant apoC-III-Thr23 protein were comparable to that of wild-type recombinant apoC-III, suggesting that this amino acid change, located in the N terminus of apoC-III, does not affect LPL inhibition.

To date, four rare naturally occurring amino acid vari-
ants have been reported (29-32). A Gln38Lys substitution was associated with higher apoC-III levels and Tg levels (32). This amino acid substitution results in an additional charge on the protein that might enhance lipid binding and/or alter the effect on LPL, which could explain the raised plasma apoC-III and Tg levels; however, no in vitro studies were carried out to confirm this. A Lys58Glu change has been reported, associated with 30-40% lower apoC-III levels and with reduced apoC-III-Glu58 on VLDL and HDL resulting in apoE-enrichment of HDL, creating atypically large HDL particles (31). Neither the Asp45Asn variant (29), nor the Thr74Ala variant, which disrupts a glycosylation site, is associated with dyslipidemia (30). Both Lys58 and Ala23 are conserved among several mammalian species (59, 62).

Extrapolation of in vitro data to the in vivo situation must be carried out with caution. The molecular modeling and the in vitro data, using recombinant apoC-III, demonstrate that residue 23 lies in an amphipathic helix that is important in lipid binding but not for the direct inhibition of LPL. We can only speculate that in vivo, in Thr23 carriers, apoC-III-Thr23, with its lower lipid-binding affinity, might be catabolized more rapidly in plasma, resulting in low plasma apoC-III levels. This could result in TGRL particles with a higher apoE/apoC-III ratio that might be cleared faster by receptors and thus result in low Tg levels. The lower plasma Tg levels seen in two of the three carriers of the variant might reflect this increased cellular uptake of the TGRL particles mediated by apoE because the apoC-III-Thr23 competes less well for lipid binding. The third Thr23 carrier had raised plasma Tg and cholesterol levels when compared with the mean values for the Mayan control sample and a low BMI. Difference in lipid levels could not be explained by apoE genotype because all three carriers had the same apoE3 genotype. This suggests that other unidentified genetic and/or environmental factors (e.g., diet or smoking) may be modulating the effect of the Ala23Thr variant.

It is surprising that in the heterozygous state this apoC-

III-Thr23 variant appears to act in a dominant manner. This might reflect the coinheritance of other unidentified mutations affecting the clearance of TGRL. In vitro studies using short peptides of apoE suggest that apoE may function as a dimer when acting as a ligand for lipoprotein binding to the LDL receptor (73). Thus, by analogy, an alternative hypothesis is that apoC-III normally acts as a multimer and heteromultimers containing both apoC-III-Ala23 and apoC-III-Thr23 may be unstable or their associations with lipids may be weak, which would result in a dramatic increase in apoC-III catabolism and cause apoC-III deficiency in heterozygous individuals. Unfortunately, because of the geographic location of the only identified carriers of this variant, it is not possible to confirm these predictions in vivo by detailed turnover studies and lipid profiles. However, the findings shed light on the functional domains of this important apolipoprotein and the modeling and E. coli expression system will allow other functional regions to be examined.  

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