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**Apolipoprotein M associates to lipoproteins through its retained signal peptide**

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## Abstract

Apolipoprotein M (apoM) is predominantly associated with HDL. In this study, it was investigated whether apoM's uncleaved signal peptide is necessary for the protein's ability to associate with lipoproteins. ApoM with a cleavable signal peptide, Q22A, was expressed, together with wild-type apoM, in HEK293 cells. On size-exclusion chromatography, the elution profile of wild-type apoM was similar to that of human HDL-associated plasma apoM. In contrast, the size of the Q22A mutant corresponded to free, unassociated apoM. This strongly indicates that the signal peptide is indeed necessary for apoM's ability to associate with lipid.

## Introduction

Apolipoprotein M (apoM) is composed of 188 amino acids and has an apparent molecular weight of 25 kilodalton [1]. Structural analysis and homology modelling have predicted that apoM belongs to the lipocalin protein superfamily, and a three-dimensional model has been constructed [2]. Although the protein is found in all major lipoprotein classes, the majority of apoM is found in high density lipoprotein, HDL [1]. In human plasma, the apoM concentration is approximately 0.9  $\mu\text{mol/l}$  [3].

In healthy humans, the level of ApoM is mainly correlated to plasma total cholesterol ( $r = 0.52$ ) [3]. The mechanism behind this relationship is not known. ApoM has been shown to be a target gene for the orphan nuclear receptor liver receptor homolog-1 (LRH-1) [4], a regulator of genes that decrease cholesterol levels in liver and intestine [5], and HNF-1 $\alpha$ , mutations in which gene are responsible for maturity onset of diabetes in the young type 3 (MODY3) [6].

The biological role of the protein is not fully understood. Two studies have demonstrated that overexpression of apoM reduces the development of atherosclerosis in LDLr<sup>-/-</sup> mice challenged with a cholesterol-rich diet [7,8]. However, a recent report, based on more than 2000 samples from two separate Nordic prospective case-control studies, found no association between plasma apoM concentration and the risk of a coronary heart disease event (Ahnström, Axler and Dahlbäck, manuscript in preparation).

A most unusual feature of apoM is that the signal peptide remains uncleaved in the circulating protein [1]. Normally, the signal peptide mediates import into the endoplasmic reticulum and is then cleaved by a type I signal peptidase after translocation [9]. To our knowledge, an uncleaved signal peptide has only been described for two other exported proteins,

paraoxonase 1 (PON-1) and haptoglobin-related protein (HPR) [10]. For PON-1, the signal peptide has been described to be necessary for lipoprotein association [11].

In this study, it was investigated whether the hydrophobic signal peptide is similarly responsible for apoM's ability to associate with lipoproteins and if a signal peptidase cleavage site could be generated with a single Q22A point mutation.

## Materials and methods

### **Transfection and expression of WT and Q22A apoM in HEK293 cells**

The amplification and cloning into the pcDNA3 vector (Invitrogen) of wild-type (WT) apoM has been described previously [1]. The Q22A mutation was introduced into pcDNA3 containing WT apoM by site-directed mutagenesis employing a QuikChange kit (Stratagene), according to the manufacturer's instructions. DNA sequencing was performed using a PE Applied Biosystems sequencing kit. Stable transfection of HEK293 cells was performed using Lipofectin (Invitrogen) according to a procedure described earlier [12] and colonies were screened for apoM expression by western blotting. For all experiments, WT and Q22A apoM-transfected cells, as well as cells transfected with empty pcDNA3 vector, were grown in 75 cm<sup>2</sup> TC Easy Flasks (Nunc), containing 25 ml Dulbecco's modified medium with 10% fetal calf serum, until ~90% confluent. Thereafter, fresh medium was applied and cells were incubated for another 48 hours, after which medium was collected and stored at 4° C.

### **ApoM sandwich ELISA**

A sandwich ELISA for apoM based on two monoclonal antibodies was used to quantify apoM as previously described [3]. The Q22A mutant was quantified against a standard curve prepared from recombinantly expressed signal peptide-free apoM.

## **Western blotting**

Western blotting analysis of plasma and cell medium from HEK293 cells was performed according to a procedure described previously [13] using polyclonal anti-human apoM antiserum [14] that had been affinity-purified on two 5 ml protein A and G-coupled HiTrap columns (GE Healthcare) according to the manufacturer's instructions.

## **ApoM particle size determination**

Gel filtration was performed on an ÄKTA fast performance liquid chromatography (FPLC) system (Amersham Pharmacia Biotech) to which was coupled an analytic size-exclusion column (Superose 12, Amersham Pharmacia Biotech) equilibrated with 20 mM Tris pH 7.5 containing 150 mM NaCl, 0.1% bovine serum albumin and 5 mM EDTA. Flow rate was 0.6 ml/min. The elution volume ( $V_e$ ) was monitored by absorbance at 280 nm. The partition coefficient,  $K_{av}$ , was calculated from the elution volume,  $V_e$ , and total bed volume,  $V_t$ , using the equation  $K_{av} = (V_e - V_0)/(V_t - V_0)$ , where  $V_0$  is the exclusion volume as determined by the elution volume of dextran blue. The total bed volume was determined by the exclusion volume and the elution volume of acetone. The Stokes radius (RST) of apoM-containing particles was calculated from a linear calibration plot of RST versus  $(-\log K_{av})^{1/2}$  obtained with thyroglobulin A (RST 85 Å), ferritin (RST 61 Å), aldolase (RST 48.1 Å), ovalbumin (RST 30.5 Å), and bovine pancreatic ribonuclease A (RST 16.4 Å, all Amersham Pharmacia Biotech).

## Results and discussion

In order to examine whether the retained signal peptide of apoM is necessary for the protein's ability to associate with lipoproteins, apoM with a cleavable signal peptide, expressed in a eukaryotic cell system, was considered the preferred model. A central feature of signal peptides cleavable by a type I signal peptidase is that the central hydrophobic region is followed by small and neutral residues at positions -3 and -1 relative to the cleavage site [15]. The amino acid sequence for human apoM was analysed by SignalP 3.0, a neural networks-based signal peptide prediction tool [16,17], which identified a likely cleavage site between positions 22 and 23. Substituting the comparatively bulky glutamine in position 22, (i.e. -1 relative to the cleavage site) for the residue most common at this position, alanine, predictably increased the maximum c-score ("cleavage site score") from 0.17 to 0.64. This is well above the cut-off value of 0.32, indicating a high probability that this construct would be cleaved in eukaryotic cells.

This mutation, Q22A, was introduced into a pcDNA3 vector containing WT apoM by site-directed mutagenesis and confirmed by DNA sequencing. The WT and Q22A apoM constructs, as well as empty pcDNA3 vector, were introduced into HEK293 cells by transfection. ApoM levels in medium from both WT and Q22A transfected cells were approximately 0.5 mg/L in medium collected after 48 hours as determined by ELISA. No apoM signal was detected in medium from cells transfected with empty vector. In an earlier study, Q22A apoM displayed similar immunoreactivity in a monoclonal antibody-based ELISA to plasma-derived apoM and a similar partial glycosylation pattern, indicating that it was correctly folded [12]. Successful cleavage of the signal peptide was verified by western blotting (fig 1). It was additionally confirmed by western blotting using polyclonal rabbit

antiserum against the signal peptide of apoM. No signal peptide could be detected for Q22A (picture not shown).

To investigate whether signal peptide-free apoM retained the ability to associate with apolipoproteins, WT and Q22A apoM cell medium, together with pooled human plasma, was subjected to size-exclusion chromatography employing a calibrated Superose 12 column (fig 2). Fractions were analysed by ELISA. The elution profile achieved from WT apoM medium corresponded well to that of pooled human plasma, with peak fractions giving a calculated Stokes' radius of respectively 62.9 and 64.5 Å. This corresponds to the size of apoM-containing HDL [14], indicating that WT apoM mainly associated with bovine HDL. In contrast, apoM in Q22A medium eluted considerably later, with a calculated Stokes' radius in the peak fraction of 31.8 Å, a size corresponding to free, unassociated apoM. Compared to the narrow peak achieved for Q22A medium, the peak achieved for plasma and WT apoM was broader. This is in line with earlier observations [14] and reflects the heterogeneity of apoM-containing HDL particles.

In conclusion, apoM with a cleavable signal peptide was constructed and expressed in HEK293 cells. In contrast to wild-type apoM which associated to HDL as predicted, signal peptide-free apoM did not associate with lipoproteins, strongly indicating that the signal peptide is necessary for apoM's ability to associate with lipid.

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## Figure legends

Figure 1. Results from western blotting of plasma (P) and medium from HEK293 cells transfected with pcDNA3 vector containing WT or Q22A apoM or empty vector ( $\emptyset$ ). 0.5  $\mu$ l of plasma and 10  $\mu$ l of cell medium were loaded onto a 12% SDS-PAGE gel, transferred to a PVDF membrane and blotted with rabbit polyclonal anti-human apoM antiserum. The markers represent apparent molecular weight in kilodaltons derived from a molecular weight standard run in an adjacent lane.

Figure 2. Results from size-exclusion chromatography. Human pooled plasma (black circles) and medium from HEK293 cells expressing WT apoM (open boxes) and Q22A apoM (open triangles) was run on a Superose 12 column. The fraction size was 0.25 ml and apoM was quantified by ELISA. The minor peak at elution volume 8.5 ml observed for human plasma represents apoM in apoB-containing lipoproteins.

Figure 1

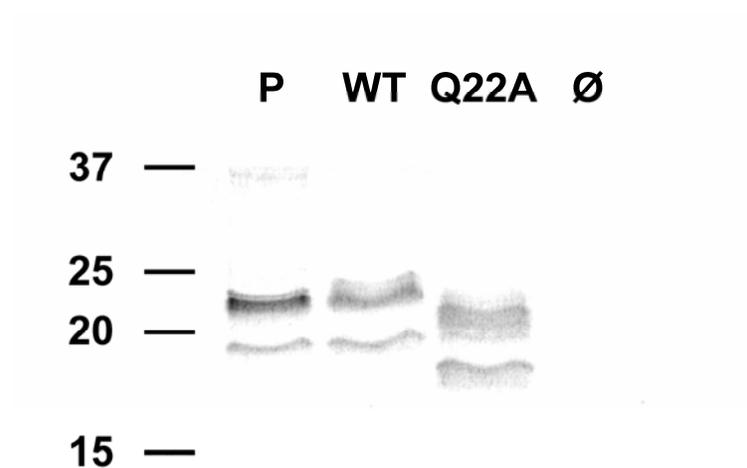


Figure 2

