Transthyretin (TTR) is a plasma homotetrameric protein that acts physiologically as a transporter of thyroxine (T4) and retinol, in the latter case through binding to retinol-binding protein (RBP). A fraction of plasma TTR is carried in high density lipoproteins by binding to apolipoprotein AI (apoA-I). We further investigated the nature of the TTR-apoA-I interaction and found that TTR from a variety of sources (recombinant and plasmid) is able to process proteolytically apoA-I, cleaving its C terminus after Phe-225. TTR-mediated proteolysis was inhibited by serine protease inhibitors (phenylmethylsulfonyl fluoride, Pefabloc, diisopropyl fluorophosphate, chymostatin, and N-α-tosyl-L-phenylalanine-chloromethyl ketone), suggesting a chymotrypsin-like activity. A fluorogenic substrate corresponding to L-lysine-chloromethyl ketone; wt, wild-type.

Previously, apolipoprotein AI (apoA-I) is also a TTR ligand; physiologically, a fraction of plasma TTR circulates in high density lipoproteins (HDLs) through binding to apoA-I (4). Its major protein component. The physiological meaning of this interaction remains to be explained, but it first suggested that it might be relevant in physiological conditions, namely in lipid and/or TTR metabolism. Previous evidence suggested that TTR and lipoprotein biology might be related. In the kidney, megalin, a member of the lipoprotein receptor family, is responsible for TTR tubular reabsorption (5). Furthermore, TTR uptake by the liver (its major site of degradation) is sensitive to the receptor-associated protein, an inhibitor of uptake of all the ligands of the lipoprotein receptor family (6).

Several point mutations in TTR have been linked to the occurrence of TTR-related amyloidosis (ATTR), a disorder that is characterized by the extracellular systemic deposition of mutated or wild-type (wt) TTR as amyloid fibrils (7, 8), leading to organ dysfunction and death. The mechanisms by which soluble proteins self-assemble into a fibrillar structure are unknown. Proteolysis has been thought to play an important role in most types of amyloidoses; in several cases, an amyloid peptide is generated by proteolysis of the corresponding protein precursor (9, 10). C-terminal TTR peptides with proteolysis occurring between amino acid residues 42 and 59, in addition to intact protein, have been found in amyloid fibrils of some ATTR patients (11). This raised the hypothesis that proteolysis could trigger fibril formation.

Amyloidogenic variants of apoA-I have also been reported (12). ApoA-I, a 243-amino acid protein, is synthesized by the liver and plays a key role in the formation, metabolism and catabolism of HDL cholesterol esters. Numerous attempts have been made to determine the arrangement of the amphipathic helices of apoA-I in nascent HDL discs, as they appear to be a critical intermediate in reverse cholesterol transport; however, the tertiary arrangement of apoA-I molecules on HDL particles is not defined yet. In apoA-I-related amyloidosis, amyloid fibrils are characterized by deposition of N-terminal fragments of variable length in the mutated protein (12). Interestingly we identified an amyloidogenic variant of apoA-I, L178H, where fibrils present both mutated apoA-I and wt TTR that colocalized in amyloid deposits as seen by immunohistochemistry and analysis of extracted fibrils (13). This data further suggested that the interaction between apoA-I and TTR might be relevant not only physiologically but also in pathological conditions. In this report we further investigated the apoA1-TTR interaction and verified that TTR is able to process apoA-I proteolytically.

**Experimental Procedures**

Proteins and Protease Inhibitors—Recombinant TTR was produced in Escherichia coli D1210 transformed with pNTR plasmid carrying TTR cDNA (14). The protein was isolated and purified as described previously (15). Briefly, after osmotic shock of bacteria, protein extracts...
were run on diethylaminomethyl (DEAE)-cellulose (Whatman) ion exchange chromatography, dialyzed, lyophilized, and isolated in native preparative Prosieve agarse (FMC Corp.) gel electrophoresis following the supplier’s instructions. After electrophoresis, the TTR band was excised and electroeluted in an Elutrap system (Schleicher & Schuell) in 38 mM glycine and 5 mM Tris, pH 8.5, overnight at 50 °C (44). A final purification by high pressure liquid chromatography (HPLC) was performed on a Protein Pak 125 column (Waters) coupled to a high precision pump (P-302) and a halocrome-280 UV detector (Gilion), using as eluent 200 mM sodium phosphate buffer, pH 7, at a flow rate of 0.4 ml/min, and 400-µl fractions were collected. Protein standards used for calibration were bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and lysozyme (14 kDa). Serum TTR was purified as described previously (15). Briefly, plasma TTR was washed against 77 mM NaCl and 50 mM phosphate buffer, pH 7.6, and run on a DEAE-cellulose ion exchange column, after which protein fractions were dialyzed, lyophilized, and chromatographed on a blue Sepharose column (Amersham Biosciences). Plasma TTR was isolated by preparative electrophoresis, followed by electroelution and HPLC as described above for recombinant TTR. Serum RBP was isolated by affinity in a TTR column and saturated with 3.3 mg/ml all-trans retinol (Sigma) in ethanol as follows: (i) 25 µl of all-trans-retinol were incubated with 800 µl of RBP (1 mg/ml) at 37 °C in the dark for 1 h; and (ii) excess retinol was separated from RBP by gel filtration in 10-ml Biogel P-6 DG columns (Bio-Rad). Recombinant Apo-A-I, α2-macroglobulin, and diisopropylfluorophosphate (DFP) were from Roche Applied Science. Mercaptoethanol was from Promega. E-64, pepstatin, and leupeptin were from Roche Applied Science.

**Analysis of Apo-A-I Cleavage Products—**For N-terminal sequencing of cleavage products, the apoA-I-TTR reaction was run on a 15% SDS-PAGE gel, transferred to a polyvinylidene fluoride membrane (Amersham Biosciences), and stained with Coomassie Blue for 5 min. Pieces of membrane containing apoA-I fragments were excised, and N-terminal sequencing was performed on an Applied Biosystems model 494 protein sequencer (Applied Biosystems) using a Microsequencer matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). apoA-I cleavage products were cut from a 15% SDS-PAGE gel stained with Coomassie Blue. Gel bands were reduced by incubation with 0.01 M diithiothreitol and 0.1 M Tris (pH 8.5) at 60 °C for 30 min and subsequently overnight at 37 °C in 50 mM Tris, 200 mM NaCl, 5 mM CaCl2, and 0.02% Brij 35 (development buffer; Bio-Rad). Gels were then stained with 0.5% Coomassie Blue in 40% methanol and 10% acetic acid for 2 h and destained in 40% methanol and 10% acetic acid overnight at 4 °C. For Western blot analysis, the protein precipitate hydrolyzed to a nitrocellulose membrane (Amersham Biosciences), blocked with 5% blocking buffer (5% nonfat dried milk in phosphate buffered saline), incubated for 1 h at room temperature with rabbit polyclonal anti-TTR (Dako; 1:2000 in blocking buffer), and incubated for 1 h at room temperature with anti-rabbit IgG conjugated with horseradish peroxidase (The Binding Site; 1:5000 in blocking buffer). The immunoblot was developed using 3,3′-diaminobenzidine (Sigma) as substrate.

**Fluorometric Assay of Proteolysis—** TTR proteolytic activity was tested with a fluorogenic peptide (Abz-ESFKVSE-DDnp, Global Peptide) encompassing the sequence of apoA-I cleaved by TTR. Abz-ESFKVSE-DDnp is an internally quenched fluorescent peptide in which Abz (α-amino-ε-caproic acid) is the fluorescent donor and E64tosyl-L-phenylalanine-chloromethyl ketone (TPCK), Nα-tosyl-L-lysine-chloromethyl ketone (TLCK), antipain, aprotinin, phosphoramidon, E64, EDTA, bestatin, pepstatin, and leupeptin were from Roche Applied Science.

**TTR Proteolysis Assay Using Apo-A-I as Substrate—** TTR and apoA-I at an equimolar ratio were incubated in 50 mM Tris, pH 6.8, at 37 °C for 3 h unless stated otherwise. After incubation, reactions were run on 12% SDS-PAGE gels containing 200 mg of recombinant TTR were run per lane. After electrophoresis, gels were fixed, stained with Coomassie Blue. Pieces of membrane containing apoA-I fragments were excised, and C-terminal sequencing was performed on an Applied Biosystems model 494 protein sequencer (Applied Biosystems) using a Microsequencer matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). apoA-I cleavage products were cut from a 15% SDS-PAGE gel stained with Coomassie Blue. Gel bands were reduced by incubation with 0.01 M diithiothreitol and 0.1 M Tris (pH 8.5) at 60 °C for 30 min and subsequently overnight at 37 °C in 50 mM Tris, 200 mM NaCl, 5 mM CaCl2, and 0.02% Brij 35 (development buffer; Bio-Rad). Gels were then stained with 0.5% Coomassie Blue in 40% methanol and 10% acetic acid for 2 h and destained in 40% methanol and 10% acetic acid overnight at 4 °C. For Western blot analysis, the protein precipitate hydrolyzed to a nitrocellulose membrane (Amersham Biosciences), blocked with 5% blocking buffer (5% nonfat dried milk in phosphate buffered saline), incubated for 1 h at room temperature with rabbit polyclonal anti-TTR (Dako; 1:2000 in blocking buffer), and incubated for 1 h at room temperature with anti-rabbit IgG conjugated with horseradish peroxidase (The Binding Site; 1:5000 in blocking buffer). The immunoblot was developed using 3,3′-diaminobenzidine (Sigma) as substrate.

**Fluorometric Assay of Proteolysis—** TTR proteolytic activity was tested with a fluorogenic peptide (Abz-ESFKVSE-DDnp, Global Peptide) encompassing the sequence of apoA-I cleaved by TTR. Abz-ESFKVSE-DDnp is an internally quenched fluorescent peptide in which Abz (α-amino-ε-caproic acid) is the fluorescent donor and E64...
Identification of TTR Proteolytic Activity—We have established previously that a fraction of plasma TTR circulates in HDLs through binding to apoA-I (4). To further address the nature of the interaction between TTR and apoA-I, we tried to obtain a complex of the two proteins by incubation at 37 °C and physiological pH. By SDS-PAGE analysis, we observed that apoA-I was cleaved in the presence of recombinant TTR (Fig. 1A, lane 4). A major apoA-I fragment of ~26 kDa was seen; however, with higher incubation times, two extra apoA-I fragments could be observed (data not shown). This was the first evidence suggesting that TTR might be a cryptic protease able to use apoA-I as a substrate. To confirm this hypothesis, incubation of apoA-I with plasma TTR was performed, and the same pattern of SDS-PAGE migration was obtained (Fig. 1A, lane 5). The fact that incubation of apoA-I with either recombinant TTR or plasma TTR from different sources generated the same pattern of apoA-I degradation (Fig. 1A) pointed to a TTR-intrinsic proteolytic activity and argued against the presence of a contaminant protease in the preparation. TTR preparations presented decreased proteolytic activity or completely lost activity with repeated freeze-thawing cycles. LCQ/MS/MS, a powerful tool for identifying protein contaminants in preparations, was performed in one of the recombinant TTR preparations and only TTR-related peaks were observed (data not shown), further suggesting that TTR itself is a novel cryptic protease. To further verify the ability of TTR to act as a protease, we used casein zymography because casein is well known to act as a universal protease substrate (20). When TTR was run on casein zymogram gels, one major band of degraded casein was visualized (Fig. 1B, arrow). To ascertain whether TTR was responsible for the appearance of the unstained, degraded casein band, we performed anti-TTR Western analysis of zymograms, which showed that the band with caseinolytic activity corresponded in fact to TTR (Fig. 1B).

Serine Protease Inhibitors Block ApoA-I Cleavage by TTR—To characterize the nature of the proteolytic activity of TTR, we used a spectrum of protease inhibitors and tested their ability to block apoA-I cleavage by TTR. α2-macroglobulin (α2M), an inhibitor of all classes of proteases, completely inhibited proteolysis of apoA-I (data not shown); the serine protease inhibitors Pefabloc, PMSF, and DFP abolished apoA-I processing by TTR, whereas inhibitors of other protease classes were without effect (Fig. 2). Chymostatin and TPCK, inhibitors of chymotrypsin-like serine proteases, were also able to block TTR activity (Fig. 2). These data suggested that TTR has a chymotrypsin-like serine protease activity.

Identification of the Cleavage Site on ApoA-I—To establish the cleavage site in apoA-I, we started by performing N-terminal sequencing of apoA-I fragments generated upon incubation with TTR. As the identified N terminus of the major proteolytic fragment of apoA-I was intact, we concluded that cleavage was occurring in the C terminus of the protein, and we subse-
Transthyretin, a New Cryptic Protease

TABLE I

| Position | Sequence | Full-length apoA-I | TTR-digested apoA-I |
|----------|----------|--------------------|---------------------|
| 1–10     | DEPPQSFWDR | +                  | +                   |
| 1–12     | DEPPQSFWDRVK | +                | +                   |
| 13–23    | DLATYVDVNLK  | +                  | +                   |
| 13–40    | DLATYVDVNLKEGDSYDYSQFEGSALGK | +         | +                   |
| 24–40    | DSGRDYSQFEGSALGK | +        | +                   |
| 46–59    | DYSQFEGSALGK  | +                  | +                   |
| 50–77    | LREQLGTVQFEPWNLK  | +                | +                   |
| 60–83    | LREQLGTVQFEPWNLKEGETLR  | +         | +                   |
| 62–77    | EQLGTVQFEPWNLK  | +                  | +                   |
| 62–83    | EQLGTVQFEPWNLKEGETLR  | +        | +                   |
| 97–106   | VQYLDPDFQR  | +                  | +                   |
| 97–107   | VQYLDPDFQRK  | +                  | +                   |
| 107–116  | KWQEMELRYQRK | +                | +                   |
| 108–116  | KWQEMELRYQRK  | +                | +                   |
| 117–131  | QKVEFLAEQLQEGAR | +        | +                   |
| 119–131  | VEFLARELQEGAR  | +                  | +                   |
| 119–133  | VEFLARELQEGARQK  | +        | +                   |
| 132–140  | QKHLHEQKR  | +                  | +                   |
| 141–149  | LSP1GEEMR  | +                  | +                   |
| 141–151  | LSP1GEEMRDR  | +                 | +                   |
| 154–160  | ARAVDQAL  | +                  | +                   |
| 154–173  | ARAHVDDLRTTHLAPYSDLRQR | + | +                   |
| 161–171  | THLYSPD6LR  | +                  | +                   |
| 189–195  | LAEYHAK  | +                  | +                   |
| 207–215  | AKPAEDLRL  | +                  | +                   |
| 216–226  | QGGLPVLESFK  | +                  | +                   |
| 216–239  | QGGLPVLESFKSFSLALEEYTKK | −     | +                   |
| 227–238  | VSFLSALEEYT    | +                  | +                   |
| 227–239  | VSFLSALEEYTKK  | +                 | +                   |

a, present; −, absent.

quently analyzed the major apoA-I proteolytic fragment by MALDI-MS. A C-terminally deleted apoA-I was identified, presenting MS peaks spanning from amino acid residues 1 to 215 of the protein (Table I). As can be seen by comparison of the MALDI-MS of tryptic peptides derived from full-length apoA-I and apoA-I digested by TTR (Table I), cleavage by TTR occurred between amino acid residues 216 and 226 of apoA-I, because the 216–226 peptide peak, corresponding to the sequence VSFLSALEEYTK, was missing in the tryptic MS analysis of apoA-I digested by TTR (Table I). To determine the exact cleavage site, the highest molecular weight fragment of apoA-I digested by TTR was subjected to C-terminal sequencing. This analysis identified the cleavage site in apoA-I as being after Phe-225. For subsequent proteolytic assays, a fluorogenic peptide corresponding to amino acid residues 223–228 of apoA-I (Abz-ESFKVS-EDDnp), which encompasses the sequence that is being cleaved by TTR, was used.

Hydrolysis of the Fluorogenic Peptide Abz-ESFKVS-EDDnp by TTR—Different TTR preparations available in our laboratory were used to test the proteolytic cleavage of the Abz-ESFKVS-EDDnp fluorogenic peptide. All of the recombinant batches of protein, as well as the TTR isolated from plasma, retained the ability to cleave the fluorogenic peptide. In an attempt to establish a possible correlation between proteolysis and pathological or physiological processes, the preparations used included both wt or different TTR mutations related either to amyloidogenesis or to structural mutants with biological implications such as T4 binding. The following TTR mutants were used: (i) V30M, the most frequent mutation related to ATTR; (ii) L65P, which is associated with the most aggressive form of ATTR; (iii) T119M, a nonpathogenic variant shown to have a protective effect on the clinical evolution of the disease; and (iv) E92C and S117C, mutants designed with the purpose of stabilizing the dimer/tetramer interactions. TTR activity was apparently independent from these constraints (data not shown), suggesting that the conformational changes occurring either on amyloidogenic variants of the protein or in stabilized dimer/tetramer interactions do not compromise proteolytic activity. The optimum pH determined for TTR cleavage was 6.8; the same optimum pH was determined for cleavage of apoA-I (data not shown). The cleavage reaction of the fluorogenic peptide yielded a K_m of 29 μM, as determined by Michaelis-Menten kinetics (Fig. 3A). The proteolytic activity of TTR preparations purified by HPLC was tested in each of the eluted fractions in activity assays using Abz-ESFKVS-EDDnp as substrate. The fractions corresponding to TTR elution clearly overlapped with the fractions having proteolytic activity toward Abz-ESFKVS-EDDnp (Fig. 3B), further demonstrating TTR-specific cleavage of the fluorogenic peptide. The putative influence of the major TTR ligands (T4 and RBP) on its proteolytic activity was tested; we observed that, whereas RBP completely inhibited TTR activity, T4 only produced an ∼15% decrease of TTR mediated proteolysis (Fig. 3C). As a control for the effect of TTR ligands in its proteolytic activity, the activity of chymotrypsin in the presence of either RBP or T4 was tested. The activity of chymotrypsin was unaltered by the presence of RBP or T4 (Fig. 3C), therefore showing that the inhibition produced by these ligands is TTR-specific.

Assessment of Proteolysis Using Cellular Assays—To assess the ability of TTR to exert its proteolytic activity in cellular assays, we started by comparing the degradation of DQ gelatin by hepatomas without TTR expression (SAHep) with the degradation caused by hepatomas with high levels of TTR expression (Huh7). DQ gelatin is an intramolecularly quenched substrate of gelatinases and several other proteinases (including serine proteinases) that allows a rapid and highly sensitive assessment of proteolytic activity. Whereas Huh7 presented high degradation of DQ gelatin (Fig. 4A, Huh7, second set of vertical panels from the left), SAHep displayed lower fluorescence (Fig. 4A, SAHep, far left set of vertical panels), indicative of a decrease in the capacity to degrade this substrate. Furthermore, in the presence of Pefabloc, a protease inhibitor of TTR activity, Huh7 hepatomas lost their ability to degrade DQ gelatin, as observed by the lack of fluorescence (Fig. 4A, Huh7, third set of
vertical panels from the left); in contrast, the addition of aprotinin, a protease inhibitor that we previously showed to be unable to block TTR-mediated proteolysis, did not affect the ability of Huh7 to degrade DQ-gelatin (Fig. 4A, Huh7, far right set of vertical panels). These results suggested that, in cellular assays, TTR was proteolytically active. We followed by performing additional experiments in which gelatin was replaced by the specific TTR substrate; to quantify TTR-related proteolysis in cellular assays, hydrolysis of Abz-ESFKVS-EDDnp in different cell lines expressing TTR was assessed and compared with the activity of control cell lines lacking TTR expression. Each of the cell lines expressing TTR presented an ~2-fold increased level of degradation of the fluorogenic peptide when compared with that of the same cell line lacking TTR expression (Fig. 4B). We therefore show that TTR produced in cells of different sources is able to cleave a peptide corresponding to the C terminus of apoA-I. To demonstrate that this increase in cleavage of Abz-ESFKVS-EDDnp is TTR-specific, we tested the
Fig. 4. Analysis of TTR proteolytic activity in cellular assays. A, assessment of proteolysis in cellular assays using DQ gelatin (40 μg/ml) as substrate. Comparison of the proteolytic activity of SAHep (hepatomas without TTR expression, far left set of vertical panels) and Huh7 (hepatomas with TTR expression; second set of vertical panels from the left). Effect of Pefabloc (1 mg/ml) and aprotinin (2 μg/ml) on Huh7 proteolytic activity (third and fourth sets of vertical panels from the left, respectively). First and second sets of vertical panels, magnification 20×; third and fourth sets of vertical panels, magnification 40×. Upper panels, transmission; lower panels: fluorescein isothiocyanate (FITC) channel. B, hydrolysis of the fluorogenic peptide Abz-ESFKVS-EDDnp (40 μg/ml) by cell lines either lacking (−) or expressing (+) TTR in the presence (+) or absence (−) of RBP (molar excess) in the cell culture media.
influence of RBP, a TTR ligand that inhibits its proteolytic activity, using the RN22 cell line. In the presence of RBP, RN22 expressing TTR lost the ability to degrade Abz-ESFKVS-EDNp, displaying degradation levels similar to the ones of RN22 cells without TTR expression (Fig. 4B, left). This result further demonstrates that TTR is a novel cryptic protease with potential physiological relevance.

**DISCUSSION**

In this report we describe TTR as a novel cryptic protease and identify apoA-I as one of its possible natural substrates. At this point we cannot exclude the hypothesis that under physiological conditions TTR might have other substrates; these putative new substrates should be the subject of future investigation to further elucidate the role of TTR activity. The presence of a non-identified proteinase contaminant in our preparations, which would be co-purified with TTR, is highly unlikely given the data reported here, namely the similar enzymatic properties of TTRs from different sources, the high degree of purity of our preparations as demonstrated by LCQ/MS/MS, the co-elution of the enzymatic activity with the TTR peak isolated by HPLC, and the specific inhibition of activity by TTR ligands. In relation to the different sources of TTR used in our assays, it is interesting to note that the sole known post-translational modification occurring in TTR is the oxidation of the Cys-10 residue that is able to form S-sulfonated and S-thiolated adducts (i.e. to be conjugated to cysteine, cysteinyl-lycine, and glutathione). Mass spectrometry of TTR preparations from both eukaryotically expressed protein and bacterially derived recombinant TTR generates a major peak corresponding to the unmodified form of the protein; a minor peak corresponding to oxidised Cys-10 TTR, which is lost by incubation with reducing agents, may also be present (21).

Given the lack of further post-translational modifications, recombinant and eukaryotic TTR can be regarded as identical.

Other cryptic plasma proteases have been reported, including human plasma fibronectin, a multifunctional glycoprotein that has been shown to have in its structure a cryptic degradative serine proteinase activity, fibronectinase (22). Recombinant fibronectinase and isolated human fibronectinase were subsequently used to define substrate specificity and roles in physiological and pathological processes (23). Based on the inhibition of TTR proteolytic activity by the general serine proteinase inhibitors TPCK and chymostatin as well as its cleavage preference for a Phe residue on P1 and an optimum pH of 6.8, we propose a chymotrypsin-like serine protease activity for TTR. The specificity of TTR cleavage on full-length apoA-I (Phe-Lys) has similarity with other well characterized chymotrypsin-like proteases, namely endopeptidase H2, a human urinary chymotrypsin-like serine proteinase that hydrolyzes Phe-Lys bonds in neuropeptides (24), and mammalian chymotrypsin-like enzymes, including rat mast cell proteases I and II and human skin chymases that have preference for a Phe residue on P1 and hydrophobic amino acid residues in P2 and P3 (25).

Analysis of the primary structure of TTR to identify a possible canonical catalytic triad (His-Asp-Ser) is difficult to accomplish; TTR has eleven Ser residues, four Asp residues, and four His residues. By analysis of the tridimensional structure of the TTR tetramer, only Ser-46 seemed to accommodate a possible arrangement compatible with a canonical catalytic triad. However, when site-directed mutagenesis was performed at Ser-46, TTR retained the ability to cleave Abz-ESFKVS-EDNp, therefore showing that Ser-46 is not the catalytic residue. One should not exclude the possibility that TTR might be a non-canonical serine protease, as several others have been identified recently (26); for instance, the Birnaviridae viral protein (VP4), a protein with a region related to the protease domain of bacterial and organelle ATP-dependent Lon protease, employs a Ser-Lys catalytic dyad (27) in which the Lys residue plays the role of a general base and activates the hydroxyl group of Ser for catalysis. Moreover, TTR is a tetramer of four identical subunits, and it is possible that residues from different monomers can contribute to the catalytic triad. It is interesting to note that tryptase, a mast cell-specific protease that has been recently implicated as a mediator of inflammation, is a proteinase that is only active as a heparin-stabilized tetramer (28).

Given the possibility that in vivo the proteolytic activity of TTR might be modulated by a third factor, we evaluated the effect of TTR main ligands in its activity. Our results suggest that the binding site of TTR (a central hydrophobic channel in the TTR tetramer) does not correspond to the proteolytically active site of the protein, because when TTR is complexed to T4 its activity is well retained; however, binding of RBP to TTR abolishes proteolysis. This fact can be explained otherwise, that is, by steric hindrance caused by RBP (which would cause inaccessibility of the substrate) or by the fact that the amino acid residues on TTR that bind RBP may play a role in catalysis.

The C-terminal domain of apoA-I is important in both lipid binding and self-association (29); furthermore, it has been reported previously that the deletion of residues 221–243 (helix 10) abolishes cholesterol efflux in cultured cells (30). It is therefore possible that the regulation of the C-terminal truncation of apoA-I performed by TTR has an impact on lipid metabolism. The susceptibility of the C terminus of apoA-I to proteolysis is well documented in the literature (31) and agrees with the fact that the N terminus of this protein is fully structured when compared with the unstructured C terminus. It has been reported recently that mast cell human chymase, a chymotrypsin-like neutral protease, cleaves the C terminus of apoA-I in discoidal pre-β-migrating reconstituted HDL particles at position Phe-225 (32), the same position that is cleaved by TTR. In this study it was concluded that chymase reduces the ability of apoA-I in discoidal reconstituted HDL particles to induce cholesterol efflux, therefore supporting the concept that extracellular proteolysis of apoA-I is one pathophysiologic mechanism leading to the generation and maintenance of foam cells in atherosclerotic lesions. The same reasoning is possible for TTR cleavage of apoA-I after Phe-225; therefore the ability of TTR to cleave apoA-I present in HDL or discoidal pre-β-migrating reconstituted HDL should be addressed further.

We have previously identified an amyloidogenic variant of apoA-I in which analysis of fibrils revealed co-deposition of N-terminal fragments of mutated apoA-I and wt TTR (13). Because we identified C-terminal cleavage of apoA-I by TTR here, one can speculate that this event may trigger apoA-I fibril formation. Understanding the role of TTR proteolysis in amyloidoses and under physiological conditions, namely the relevance of apoA-I as a natural substrate of TTR, might have an impact not only in amyloidoses but also in disorders related to lipid metabolism. The evidence that TTR contains a cryptic protease in its structure is new and, together with recent findings describing similar activity in fibronectin (23), constitutes an important model for studying the physiological relevance of cryptic proteases.

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