Exon 11 of the Rat Cholesterol Esterase Gene Encodes Domains Important for Intracellular Processing and Bile Salt-Modulated Activity of the Protein†

Linda P. DiPersio, Christopher P. Carter, and David Y. Hui*

Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0529

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ABSTRACT: The rat pancreatic cholesterol esterase is a 74 000 molecular weight protein encoded by a gene with 10 introns and 11 exons. The last exon of the cholesterol esterase gene is the largest and is also the least conserved exon among the cholesterol esterase genes of various species. The current study investigates the functional role of the exon 11 domain in rat cholesterol esterase. The transfection of native cholesterol esterase cDNA into COS cells resulted in an enzymatically active cholesterol esterase that was secreted by the cells. In contrast, transfection of cholesterol esterase cDNA with 88% of the exon 11 residues deleted from the sequence resulted in a protein that was not secreted by the cells. The cholesterol esterase with deletions in the exon 11 domain retained the ability to bind bile salt but was found to be enzymatically inactive. The inefficient secretion and the loss of enzyme activity for the truncated protein were not due to deletion of the proline-rich repeating units located in the exon 11 domain at the carboxyl terminus of the cholesterol esterase. The expression of rat cholesterol esterase with zero or one proline-rich units resulted in a truncated protein that was secreted by the transfected COS cells. The cholesterol esterases with reducing numbers of the proline-rich repeating units were also active in hydrolyzing *p*-nitrophenyl butyrate and cholesteryl oleate. The cholesterol esterase with fewer proline-rich repeating units were more active than the native enzyme in substrate hydrolysis at low bile salt concentrations. Taken together, these data indicated that the exon 11 domain, particularly residues 490–534, of rat cholesterol esterase is required for normal intracellular processing and secretion of the enzyme. The proline-rich repeating units of cholesterol esterase may also play a role in regulating substrate delivery to the protein.

The cholesterol esterase, also called carboxyl ester lipase or bile salt-stimulated lipase, is a 74 000 molecular weight protein that has been implicated to be important for dietary cholesterol absorption through the intestinal tract (Rudd & Brockman, 1984). The cholesterol esterase also acts in concert with pancreatic lipase and colipase to completely digest dietary triglycerides for intestinal absorption of the fatty acids (Lindstrom *et al.*, 1988; Chen *et al.*, 1989). The cholesterol esterase is synthesized in the acinar cells and stored in zymogen granules of the pancreas. The enzyme can be released from storage as a component of the pancreatic juice after food ingestion (Treadwell & Vahouny, 1968), in response to elevated levels of gastric hormones such as cholecystokinin and secretin (Huang & Hui, 1991). The bile salts present in the intestinal lumen bind to the cholesterol esterase, inducing changes in protein conformation (Jacobson *et al.*, 1990), and resulting in oligomerization and activation of the enzyme (Treadwell & Vahouny, 1968).

The mechanism of cholesterol esterase-catalyzed hydrolysis of substrates was shown to proceed through an acylenzyme intermediate between alcoholysis and hydrolysis (Lombardo & Guy, 1981; Stout *et al.*, 1985). Subsequent chemical modification studies revealed the requirement of a serine, a histidine, and an acidic amino acid residue in catalytic activity of the protein (Lombardo, 1982). These results suggested a serine-hydrolase mechanism for cholesterol esterase catalysis similar to that described for other serine esterases, such as the acetylcholinesterase and serum butyrylcholinesterase (Quinn, 1987; Chatonnet & Lockridge, 1989; Taylor, 1991). Recently, the specific amino acid residues responsible for the cholesterol esterase activity have been identified using the site-specific mutagenesis approach. These experiments demonstrated that Ser^{194}, His^{435}, and Asp^{320} form the catalytic triad of the cholesterol esterase (DiPersio *et al.*, 1990, 1991; DiPersio & Hui, 1993). The amino acid sequences encompassing these active-site residues were similar to sequences surrounding the catalytic triad residues of acetylcholinesterase and cholinesterase (MacPhee-Quigley *et al.*, 1985; Gibney *et al.*, 1990; Lockridge *et al.*, 1987), suggesting that these serine esterases were derived from a common primordial gene.

The structure of both the rat and the human cholesterol esterase gene has been determined (Fontaine *et al.*, 1991; Kumar *et al.*, 1992). The nucleotide sequence of the cholesterol esterase gene revealed a structure containing 11 exons interrupted by 10 introns. Analysis of the intron–exon positions in the cholesterol esterase gene suggested that each exon may signal an unique structural or functional domain of the protein (Fontaine *et al.*, 1991). For example, exon 1 of the cholesterol esterase gene encodes the 5′-untranslated region of the mRNA and the signal peptide region of the protein. Exon 3 was identified as the domain containing the heparin-binding site of cholesterol esterase (Kissel *et al.*, 1989; Kumar *et al.*, 1992). The active-site triad residues of Ser^{194}, Asp^{320}, and His^{435} were found to be encoded by exons 5, 8, and 10, respectively (Fontaine *et al.*, 1991). In addition, the seventh exon of the cholesterol esterase gene encompasses a domain containing a cysteine disulfide bond, suggesting the importance

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* To whom correspondence should be addressed at the Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, 231 Bethesda Ave., Cincinnati, OH 45267-0529. Phone: (513) 558-9152. Fax: (513) 558-2289.

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of this domain in maintaining the structural integrity of the protein.

The largest exon in the cholesterol esterase gene is exon 11. Interestingly, the four proline-rich repeating units of rat cholesterol esterase (Kissel *et al.*, 1989), as well as the 16 proline-rich repeats of the human enzyme (Hui & Kissel, 1990; Reue *et al.*, 1991), are encoded by this last exon. Although the functional role of this domain remains unknown, this exon was found to be the least conserved exon among the cholesterol esterase genes of various species (Colwell *et al.*, 1993). In the present study, we have continued our previous approach and used site-specific mutagenesis techniques to address the role of exon 11-encoded domains in the structure and function of cholesterol esterase.

EXPERIMENTAL PROCEDURES

Materials. Reagents for mutagenesis, the corresponding bacterial cells, nitrocellulose paper, and electrophoresis reagents were obtained from BioRad. Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs. The COS-1 cells were obtained from American Type Culture Collection (no. CRL 1650). The expression plasmid vector pCMV-5 (Andersson *et al.*, 1989) was a generous gift from Dr. David Russell, University of Texas Southwestern Medical School, Dallas TX. Pharmacia Biotech Inc. was the source of the DEAE-dextran. Cholesterol [^{14}C]oleate, ^{125}I-labeled anti-rabbit IgG, and [^{35}S]cysteine were obtained from Amersham Corp. Tissue culture media were purchased from GIBCO-BRL. All other reagents were of the highest grade obtainable from either Fisher or from Sigma.

Design of Mutagenic Oligonucleotide Primers. A mutagenic oligonucleotide primer, corresponding to the cholesterol esterase mRNA sequence coding for residues 533–540, was synthesized such that the CAC codon for His^{536} was replaced by the stop codon TAG. A unique *Bam*HI restriction site was also introduced into the primer immediately after the new stop codon at residues 536–538. The latter mutation did not alter the coding sequence preceding the stop codon and was designed to facilitate identification and subcloning of the mutagenized cDNA into expression vector. Mutants produced by this mutagenic primer were designated as CEH-Δ4P.[1]

A second 28-nucleotide primer, corresponding to the cholesterol esterase mRNA sequence coding for residues 544–552, was synthesized such that a TAG stop codon was substituted for the GTC codon which coded for the valine residue at position 548. A second mutation was also introduced into this primer such that an *Xba*I restriction site was inserted at the same time within residues 547–549. This second mutation altered only the last codon (Pro^{547}) before the stop codon, and one codon after the stop codon (Pro^{549}). Mutants produced by this primer were designated as CEH-Δ3P.

Site-Specific Mutagenesis. Mutagenesis was performed using the oligonucleotide-directed method of Zoller and Smith (1984), with modifications as described by Kunkel *et al.* (1987). The full-length rat cholesterol esterase cDNA (Kissel *et al.*, 1989), containing *Sma*I restriction sites at both ends, was subcloned into the *Sma*I cloning site of M13mp18. The recombinant DNA was transfected into dut$^-$ and ung$^-$ *Escherichia coli* CJ236 cells. These cells were then grown in the presence of uracil, which was incorporated into the DNA in place of thymidine. Single-stranded DNA was isolated from these cells and used as template for mutagenesis.

The mutagenic oligonucleotide primers were used to hybridize with the uracil-containing cholesterol esterase cDNA in single-stranded M13mp18 template. All four deoxynucleotides, the Klenow fragments of DNA polymerase I, and T4 DNA ligase were then added to produce heteroduplex DNA. The mixture was incubated for 1.5 h at 37 °C. Competent *E. coli* DH5αF′ cells were transfected with the reaction mixture. The presence of uracil in the parent strain resulted in its hydrolysis, while the non-uracil-containing mutagenized daughter strand survived endogenous hydrolysis. The resulting clones were screened initially by digestion of the M13 replicative form DNA with either *Bam*HI or *Xba*I restriction enzymes. Candidate clones containing the mutagenized cholesterol esterase cDNA were sequenced completely to rule out undesirable spontaneous mutations and rearrangements.

Transfection and Expression of Cholesterol Esterase cDNA. The native form of rat cholesterol esterase cDNA or the CEH-Δ4P- and CEH-Δ3P-mutagenized cDNA was isolated from the M13 recombinant vectors by digestion with *Eco*RI plus *Sac*I, *Eco*RI plus *Bam*HI, and *Eco*RI plus *Xba*I, respectively, and then subcloned into the expression vector pCMV (Andersson *et al.*, 1989). In addition, another truncated form of cholesterol esterase was obtained by digesting the native cDNA with *Eco*RI and *Kpn*I, producing a 1550 bp fragment of DNA, and then subcloned into similarly digested pCMV vector. The protein expressed from this truncated cDNA was designated as CEH-X11. The recombinant plasmids containing native or truncated cholesterol esterase cDNA in the proper orientation were purified by CsCl centrifugation and used to transfect COS-1 cells in T75 flasks using the DEAE-dextran method. The cells were transferred to 5 mL of serum-free medium 48 h after transfection and were incubated for an additional 24 h at 37 °C before analysis.

Characterization of Native and Truncated Cholesterol Esterase. One milliliter of culture media containing proteins secreted by the transfected cells was dialyzed against deionized water, lyophilized, and then reconstituted in 50 μL of buffer containing 62.5 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, and 5% β-mercaptoethanol. The samples were heated to 90 °C for 10 min, electrophoresed on 10% SDS–polyacrylamide gels, and then transferred to nitrocellulose paper for immunoblotting with antibodies against rat cholesterol esterase (DiPersio *et al.*, 1992). The amount of cholesterol esterase in each sample was quantitated on the basis of the amount of immunoreactive proteins as described (DiPersio *et al.*, 1990, 1991; DiPersio & Hui, 1993). Control experiments established that the immunoassay was in the linear range from 20 ng to 5 μg of purified rat cholesterol esterase.

In experiments comparing the amount and activity of native and truncated cholesterol esterase, the transfected COS cells were incubated for 24 h at 37 °C in cysteine-free media supplemented with [^{35}S]cysteine at 20 μCi/mL. At the end of the incubation period, the media were collected and dialyzed against phosphate-buffered saline. Four milliliters of the dialyzed media was used for immunoprecipitation with anti-cholesterol esterase as described previously (Huang & Hui, 1991). The amount of cholesterol esterase in each sample was determined based on the amount of radioactivity in the immunoprecipitated ^{35}S-labeled protein. Both the native and the truncated forms of cholesterol esterase contained four

[1] Abbreviations: CEH-X11, truncated form of cholesterol esterase with essential complete deletion of the exon 11 domain and contains only residues 1–489 of the protein; CEH-Δ4P, truncated form of cholesterol esterase with deletion of all 4 proline-rich repeating units; CEH-Δ3P, truncated form of cholesterol esterase with deletion of 3 proline-rich repeating units; bp, base pair(s).

Table 1: Exon 11-Encoded Domains of Native and CEH-X11 Truncated Cholesterol Esterase[a]

Native Cholesterol Esterase		
1503	GAC CCC AAC ATG GGC AAC TCA CCC GTG CCC ACA CAC TGG TAC CCT TAT ACC ACG GAG AAT	1562
476	Asp Pro Asn Met Gly Asn Ser Pro Val Pro Thr His Trp Tyr Pro Tyr Thr Thr Glu Asn	495
CEH-X11 Truncated Cholesterol Esterase		
1503	GAC CCC AAC ATG GGC AAC TCA CCC GTG CCC ACA CAC TGG TAC *CAC GCG TAT CGA TAA GCT*	1562
476	Asp Pro Asn Met Gly Asn Ser Pro Val Pro Thr His Trp Tyr *His Ala Tyr Arg* *	493

[a] The nucleotide sequences coding for the initial 20 residues of the exon 11-encoded domains are shown on the top line. The derived amino acid sequences are indicated on the bottom line. The residue numbers were assigned according to the sequence of the full-length cDNA and the native mature protein, according to Kissel *et al.* (1989). The sequence highlighted by italics was sequenced from the pCMV expression vector immediately following the cDNA insert. The recombinant form of the native cholesterol esterase terminates at residue 592. The CEH-X11 truncated cholesterol esterase terminates at the stop codon as indicated, thus coding for a protein with 493 residues.

cysteines per molecule (Kissel *et al.*, 1989). This value was used to determined the concentration of cholesterol esterase in each sample.

Cholesterol esterase activity in the lysate and cultured media of transfected cells was measured by using either *p*-nitrophenyl butyrate or cholesteryl [^{14}C]oleate as the substrate, as previously described (Kissel *et al.*, 1989; DiPersio *et al.*, 1990). In selected experiments, the transfected cells were harvested by scraping into a buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM glutathione, and 250 mM sucrose, and then sonicated with a Branson sonifier. Cell particulate fraction was separated from the cytosol by centrifugation for 1 h at 100000*g*. The pellet was resuspended in sonication buffer, and both the supernatant and resuspended pellet were assayed for cholesterol esterase activity as described above.

The bile salt-binding activity of the cholesterol esterase was analyzed by resuspending the 100000*g* sonicated cell pellet in 1.5 mL of sonication buffer containing 0.2% Tween-20. A 0.75-mL aliquot of cell culture medium containing the native cholesterol esterase was also mixed with 0.75 mL of 0.4% Tween-20 for comparison. The samples were mixed for 30 min at ambient temperature and then centrifuged at 100000*g*. The insoluble debris was discarded, and 0.5 mL of each supernatant was mixed with 0.5 mL of cholic acid–agarose (Sigma). The samples were mixed in an end-to-end fashion overnight at room temperature. At the end of the incubation period, the agarose beads were removed by centrifugation, and the supernatants were analyzed for cholesterol esterase protein by electrophoresis and immunoblotting.

RESULTS

Initial experiments to determine the role of the exon 11 domain in cholesterol esterase function were performed by taking advantage of a unique *Kpn*I restriction site located within the exon 11 domain of the cholesterol esterase gene. The subcloning of the 1545 bp *Eco*RI- and *Kpn*I-digested cholesterol esterase cDNA into pCMV vector, and its expression in transfected COS cells, resulted in a truncated protein (CEH-X11) that contains residues 1–489 of rat cholesterol esterase and four additional residues at the carboxyl terminus of the protein (Table 1). Thus, the CEH-X11 protein contains only 14 of the 117 residues encoded by exon 11 of the cholesterol esterase gene. The expression of the truncated CEH-X11 in transfected COS cells was compared with that of the native cholesterol esterase. In agreement with results of previous studies (DiPersio *et al.*, 1990), expression of the cholesterol esterase cDNA in transfected COS cells resulted in secretion of the native cholesterol esterase into the cultured medium (Figure 1, lane 3, left panel), with very little amount of the protein found in the cell extract (Figure 1, lane 3, right panel). The amount of intracellular cholesterol esterase could be increased by inclusion of brefeldin A in the cultured medium (Figure 1, comparing lane 2 in left and right panels). In contrast, cells transfected with the CEH-X11 cDNA did not secrete significant amounts of anti-cholesterol esterase immunoreactive protein into the medium (Figure 1, lane 1, left panel). Most of the truncated cholesterol esterase was found in the cell extract (Figure 1, lane 1, right panel). The total amount of truncated cholesterol esterase found in the cell extract and in the medium was similar to that of the native cholesterol esterase (Figure 2), suggesting that the truncation did not affect synthesis and/or degradation of the protein.

The effects of exon 11 deletion on cholesterol esterase hydrolytic activity were determined by comparing the hydrolytic activity of the CEH-X11 truncated protein with native cholesterol esterase expressed in COS cells. Results showed that the truncated cholesterol esterase in the transfected cell extract was not capable of converting the water-soluble substrate *p*-nitrophenyl butyrate to *p*-nitrophenol (Figure 3). The hydrolytic activity was similar to those observed for background substrate autohydrolysis, even when an equivalent of 750 ng of the truncated cholesterol esterase was used in the assay. In contrast, extracts containing 15–100 ng of native cholesterol esterase displayed significant esterase hydrolytic activity (Figure 3). Furthermore, when normalized to the amount of immunoreactive cholesterol esterase present in each sample, the bile salt-activated esterase activity of the normal cholesterol esterase found in cell extracts was similar between samples incubated with or without brefeldin A, a reagent that blocks protein processing to post-Golgi compartments (Misumi *et al.*, 1986). Additionally, the small amounts of CEH-X11 truncated protein in the medium were also incapable of hydrolyzing *p*-nitrophenyl butyrate (data not shown). Thus, the inability of CEH-X11 to hydrolyze *p*-nitrophenyl butyrate was also unrelated to its localization in the endoplasmic reticulum of the cells.

The inability of CEH-X11 truncated cholesterol esterase to hydrolyze *p*-nitrophenyl butyrate may be due to deletion

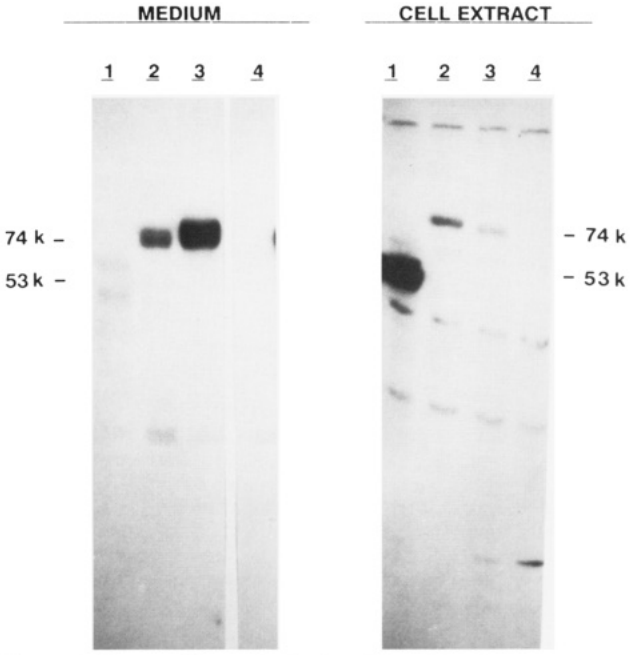

FIGURE 1: Immunoblot analysis of conditioned medium and extract from COS cells transfected with cholesterol esterase cDNA. The COS cells transfected with various forms of cholesterol esterase cDNA were cultured in T75 flasks with 5 mL of serum-free medium for 24 h at 37 °C. At the end of the incubation period, 1 mL of media was dialyzed against water and then lyophilized. The cells were lysed by sonication in buffer containing 250 mM sucrose and 5 mM glutathione. Cell particulate fractions were separated from the cytosol by centrifugation for 1 h at 100000*g*. The samples were then electrophoresed in 10% SDS–polyacrylamide gels, transferred to nitrocellulose paper, and incubated with anti-cholesterol esterase antiserum. The immunoreactive proteins were determined by further incubation with ^{125}I-labeled anti-rabbit IgG and then exposing the nitrocellulose to X-ray films. Samples in *lane 1* were obtained from cells transfected with the CEH-X11 cDNA; *lane 2* contained samples transfected with the native cholesterol esterase cDNA and expressed in the presence of brefeldin A; *lane 3* contained samples transfected with native cholesterol esterase cDNA and expressed in the absence of brefeldin A; and *lane 4* contained samples from COS cells transfected with control vector DNA without cDNA insert. The immunoreactive bands at 74 kDa represented the native cholesterol esterase while the bands marked at 53 kDa were the truncated CEH-X11 protein.

of the bile salt-binding domain important for enzyme activation. This possibility was addressed directly by examining the interaction of the recombinant proteins with cholic acid immobilized on agarose beads. As shown in Figure 4, significant amounts of anti-cholesterol esterase immunoreactive proteins were detected in cell extract or conditioned medium from COS cells expressing native cholesterol esterase in the presence or absence of brefeldin A, respectively. Anti-cholesterol esterase reactive protein was also evident in Tween-20-solubilized extracts from cells expressing the CEH-X11 truncated cholesterol esterase. Incubation of the samples with cholate–agarose followed by precipitation of the cholate–agarose-bound fraction resulted in nearly complete removal of both the native and the truncated cholesterol esterase from the samples (lanes 1, 4, and 7 in Figure 4). These experiments documented that deletion of residues 490–592 has no effect on the ability of cholesterol esterase to bind bile salt, suggesting that the inability of the truncated cholesterol esterase CEH-X11 to hydrolyze *p*-nitrophenyl butyrate was not due to deletion of the bile salt-activating domain of cholesterol esterase.

Consideration was given to the possibility that defects in the CEH-X11 truncated cholesterol esterase are due to deletion of the proline-rich repeating units at the carboxyl terminus

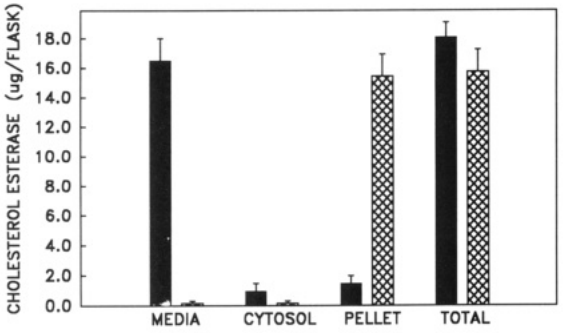

FIGURE 2: Quantitation of recombinant cholesterol esterase expression in transfected COS cells. The COS cells were transfected with cDNA for native cholesterol esterase (solid bars) or the truncated CEH-X11 cDNA (stripped bars). Cell conditioned media were isolated for SDS–polyacrylamide gel electrophoresis and immunoblotting with anti-cholesterol esterase antibodies as described in the legend to Figure 1. The cell lysate was centrifuged for 1 h at 100000*g*. The supernatant fraction, representing cell cytosol, and the pellet, containing the particulate fraction, were resuspended in gel sample application buffer before analysis. The amount of cholesterol esterase present in each sample was quantitated by immunoreactivity of the protein, based on comparison with standard purified cholesterol esterase. The total fraction represented the sum of the cholesterol esterase present in cell media, cytosol, and pellet fractions. The data were obtained from duplicate assays of three experiments. The error bars represent the standard error of the means.

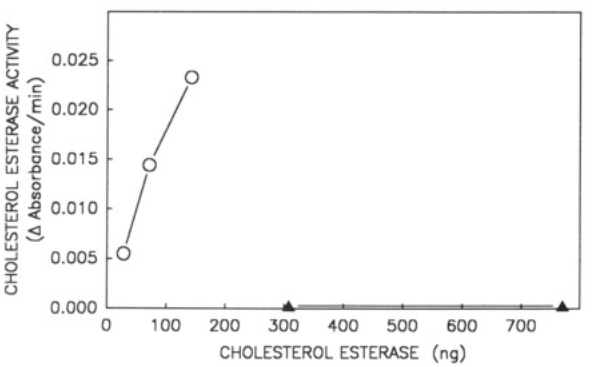

FIGURE 3: Bile salt-stimulated cholesterol esterase activity in extracts from transfected COS cells. Extracts were prepared by sonication of cells transfected with native cholesterol esterase cDNA and expressed in the presence of brefeldin A (○), or from cells transfected with the CEH-X11 cDNA (▲). The cholesterol esterase activity in each sample was determined on the basis of the hydrolysis of *p*-nitrophenyl butyrate. The reaction was monitored by changes in absorbance at 405 nm due to the production of *p*-nitrophenol. The amount of cholesterol esterase present in each sample was determined by the quantitative immunoblotting assay as described in the legend to Figure 2.

of the protein. Alternatively, the sequence between residues 490 and 538, immediately preceding the proline-rich repeating units, may serve a specific role in dictating proper protein conformation for secretion and enzymatic activity. To test these possibilities, site-specific mutagenesis was performed to introduce a termination codon into the coding sequence such that expression of the cDNA will result in cholesterol esterase with zero (CEH-Δ4P) or one (CEH-Δ3P) of the proline-rich repeats (Table 2). Examination of cultured medium from cells transfected with these mutagenized cDNA revealed that expression and secretion of CEH-Δ4P and CEH-Δ3P were similar to those of control cholesterol esterase (data not shown). Additionally, when the cultured medium containing equivalent amounts of cholesterol esterase was tested for enzyme activity, CEH-Δ4P and CEH-Δ3P were found to be as active as the native cholesterol esterase in hydrolysis of *p*-nitrophenyl butyrate (Figure 5) and cholesteryl [^{14}C]oleate (Figure 6).

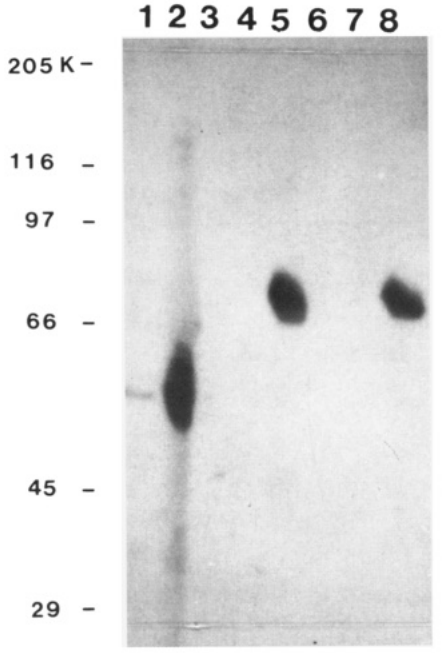

FIGURE 4: Interactions of native cholesterol esterase and the truncated CEH-X11 with bile salt. The cell particulate fraction or conditioned media from transfected COS cells were solubilized with 0.2% Tween-20 and then incubated with equal volumes of cholate–agarose at 23 °C overnight. The agarose beads were removed by centrifugation, and the supernatants were analyzed for cholesterol esterase protein by electrophoresis on 10% SDS–polyacrylamide gels followed by immunoblotting with anti-cholesterol esterase and ^{125}I-labeled anti-rabbit IgG. *Lane 1* contained cholate–agarose supernatant of extracts from COS cells transfected with the CEH-X11 cDNA; *lane 2* contained the CEH-X11 transfected cell extract before cholate–agarose precipitaton; *lanes 4 and 5* contained supernatants after and before cholate–agarose precipitation, respectively, of solubilized extracts from cells transfected with native cholesterol esterase and expressed under brefeldin A treatment. *Lanes 7 and 8* contained supernatants after and before cholate–agarose precipitation of conditioned media from cells transfected with native cholesterol esterase and expressed in the absence of brefeldin A.

Table 2: Mutagenesis To Delete Proline-Rich Repeating Units of Cholesterol Esterase[a]

CEH-Δ4P

Residues 533-540		Val Gly Asp His Thr Pro Pro Glu
Coding Strand	5′-	G GTT GGT GAC CAC ACT CCC CCT GAG G - 3′
Complement	3′-	C CAA CCA CTG GTG TGA GGG GGA CTC C - 5′
Oligo-1	3′-	C CAA CCA CTG ATC CTA GGG GGA CTC C - 5′
Coding Strand	5′-	G GTT GGT GAC TAG GAT CCC CCT GAG G - 3′
Amino acids		Val Gly Asp STOP

CEH-Δ3P

Residues 544-552		Glu Ala Ala Pro Val Pro Pro Thr Asp
Coding strand	5′-	GAG GCT GCC CCC GTC CCA CCT ACA GAC G -3′
Complement	3′-	CTC CGA CGG GGG CAG GGT GGA TGT CTG C -5′
Oligo-2	3′-	CTC CGA CGG GAG ATC TGT GGA TGT CTG C -5′
Coding Strand	5′-	GAG GCT GCC CTC TAG ACA CCT ACA GAC G -3′
Amino acids		Glu Ala Ala Leu STOP

[a] Oligonucleotide primers were designed for site-specific mutagenesis to produce truncated forms of cholesterol esterase with deletions of all four proline-rich repeating units (CEH-Δ4P) or with deletion of three proline-rich repeats (CEH-Δ3P). The primers were synthesized on the basis of the sequence coding for the amino acid residues of rat cholesterol esterase as indicated.

The role of the proline-rich repeating units on the bile salt sensitivity of cholesterol esterase was evaluated by examining the bile salt-dependent activation of enzyme activity in hydrolysis of *p*-nitrophenyl butyrate. The water-soluble substrate was chosen for these assays instead of using cholesteryl [^{14}C]oleate because the effects of bile salt on cholesteryl esters will complicate interpretation of the data.

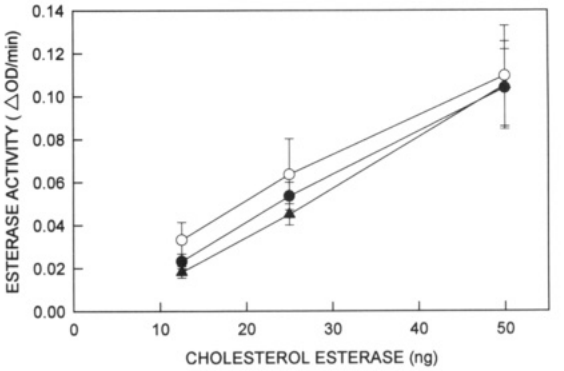

FIGURE 5: Esterase activity in media of cells expressing recombinant forms of native cholesterol esterase cDNA (●), CEH-Δ4P cholesterol esterase with deletions of all four proline-rich repeating units (○), or CEH-Δ3P cholesterol esterase with deletions of three proline-rich repeating units (▲). The amount of cholesterol esterase used in each assay was calculated based on the amount of [^{35}S]cysteine incorporated into immunoprecipitable cholesterol esterase in each sample. The enzymatic activity was determined based on hydrolysis of *p*-nitrophenyl butyrate as measured by changes in optical density at 405 nm.

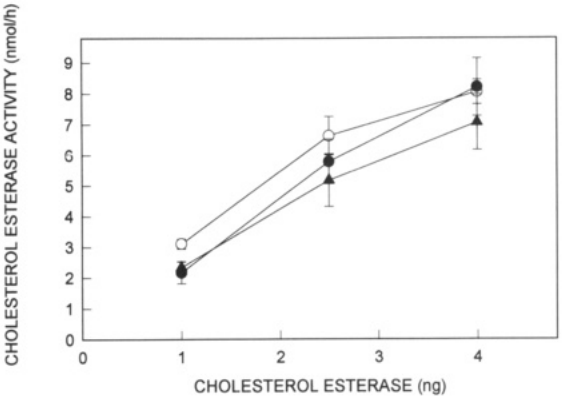

FIGURE 6: Cholesterol esterase activity in media of cells expressing recombinant forms of native cholesterol esterase cDNA (●), CEH-Δ4P cholesterol esterase with deletions of all four proline-rich repeating units (○), or CEH-Δ3P cholesterol esterase with deletions of three proline-rich repeating units (▲). The amount of cholesterol esterase used in each assay was calculated based on the amount of [^{35}S]cysteine incorporated into immunoprecipitable cholesterol esterase in each sample. The enzymatic activity was determined based on hydrolysis of cholesteryl [^{14}C]oleate to [^{14}C]oleate.

The results showed that cholesterol esterases without any proline-rich repeats (CEH-Δ4P) or with one proline-rich domain (CEH-Δ3P) were equally active as the native cholesterol esterase in hydrolyzing *p*-nitrophenyl butyrate when tested as high concentrations of taurocholate (Figure 7). Surprisingly, both CEH-Δ4P and CEH-Δ3P were more active than native cholesterol esterase, containing all four proline-rich repeating units, in hydrolyzing the water-soluble substrate at low concentrations of bile salt (Figure 7). The data revealed that the cholesterol esterase devoid of proline-rich repeating units displayed a 10-fold higher rate of *p*-nitrophenyl butyrate hydrolysis than the native enzyme at 0.4–0.8 mM taurocholate. A 3-fold difference in esterase activity was observed at 1.2 mM taurocholate. However, the rates of substrate hydrolysis for the native cholesterol esterase and enzymes devoided of the proline-rich repeating units were similar above 2 mM taurocholate.

The effects of bile salt on the activity of native and truncated cholesterol esterases were further investigated by varying the concentration of *p*-nitrophenyl butyrate used in the assay. Analysis of the data by Lineweaver–Burk plot revealed that the apparent K_m for native cholesterol esterase in hydrolyzing

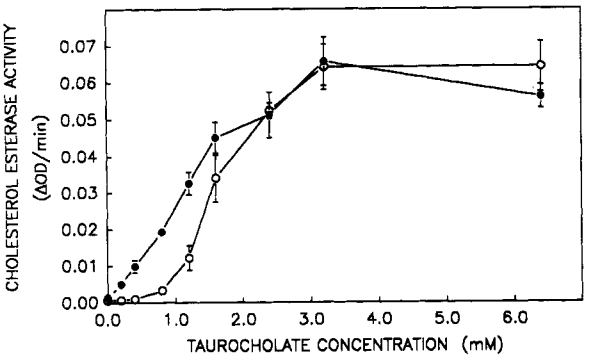

FIGURE 7: Bile salt-dependent cholesterol esterase activity of recombinant forms of native cholesterol esterase (○) or the CEH-Δ4P cholesterol esterase without any proline-rich repeating units (●). Each assay contained 2.5 ng of the cholesterol esterase, and the hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenol was monitored by changes in optical density at 405 nm.

Table 3: Kinetic Data for Native and Truncated Cholesterol Esterase Hydrolysis of *p*-Nitrophenyl Butyrate[a]

[TOC] (mM)	K_m (mM)			V_{max} (ΔOD/min)		
	native	CEH-Δ4P	CEH-Δ3P	native	CEH-Δ4P	CEH-Δ3P
1.2	23.7	11.4	11.4	0.013	0.035	0.038
3.0	3.8	3.8	3.8	0.068	0.070	0.065

[a] Twenty nanograms of native cholesterol esterase, CEH-Δ4P, or CEH-Δ3P was incubated in 400 μL of buffer containing 0.5 M Tris-HCl, pH 7.4, and taurocholate (TOC) at the concentration indicated. The reaction was initiated by the addition of 150 μL of freshly prepared *p*-nitrophenyl butyrate (0–250 μg/mL in sodium acetate, pH 5.0). Enzyme activity was determined by monitoring the appearance of the product, *p*-nitrophenol, with a spectrophotometer set at 405 nm.

p-nitrophenyl butyrate was 3.8 mM in the presence of 3 mM taurocholate (Table 3). The K_m increased 6.2-fold by decreasing the concentration of taurocholate from 3 to 1.2 mM (Table 3). Although these K_m values appeared higher than those reported by others for the human enzyme (Hernell & Olivecrona, 1974; Lombardo *et al.*, 1978; Wang, 1981), our results were obtained by expression of the rat cholesterol esterase, and the K_m values reported here were similar to those reported for the cholesterol esterase of the rat pancreas (Erlanson, 1970). Interestingly, at 1.2 mM taurocholate, the K_m values for *p*-nitrophenyl butyrate were 2-fold lower for CEH-Δ4P and CEH-Δ3P than for the native enzyme (Table 3). However, the K_m was similar for all three enzymes when the assay was performed at 3.0 mM taurocholate (Table 3). The V_{max} for the *p*-nitrophenyl butyrate hydrolytic reaction was also similar for the three forms of cholesterol esterase at 3.0 mM taurocholate, but the native enzyme was found to be more sensitive to low taurocholate concentration than the cholesterol esterases with one or zero proline-rich repeating units (Table 3).

DISCUSSION

The results of this study showed that deletion of 88% of the residues in the exon 11 domain of cholesterol esterase (CEH-X11) resulted in a protein that was not secreted by transfected COS cells. The truncated CEH-X11 protein was found in particulate fractions, indicative of its endoplasmic reticulum or Golgi apparatus localization in the cells. One possible explanation for the impaired secretion of CEH-X11 is the requirement of the exon 11 domain in maintenance of the protein conformation necessary for secretion [reviewed in Lodish (1988)]. This hypothesis was supported by recent observations of impairment in the intracellular transport of naturally occurring mutant proteins, such as the α_2-plasmin inhibitor (Miura & Aoki, 1990) and the C9 complement factor (Dupuis *et al.*, 1993). The importance of the exon 11 domain in proper folding of the cholesterol esterase was also supported by the observations that, despite the retention of the three domains forming the catalytic triad of cholesterol esterase and the ability to interact with the bile salt activator (Figure 4), the CEH-X11 did not display any esterase activity and failed to catalyze *p*-nitrophenyl butyrate hydrolysis. However, it must be noted that the truncated CEH-X11 was found to accumulate intracellularly to the same extent as the amount of nonmutagenized cholesterol esterase secreted by similarly-transfected COS cells. The latter result suggested that the truncation did not result in a more rapidly degraded protein, similar to that observed for other improperly folded nascent polypeptide chains [reviewed in Lodish (1988)]. Thus, it is also possible that the exon 11 domain may be required for interaction with other intracellular proteins that are important for folding, assembly, and secretion of the cholesterol esterase [reviewed in Gething and Sambrook (1992)]. Alternatively, the additional four residues at the C-terminus of the truncated protein may also have affected protein processing and transport out of the cell.

Regardless of the precise mechanism by which the exon 11 domain may function to dictate intracellular processing and transport of cholesterol esterase, the results of this study showed that this requirement was independent of the proline-rich repeating units at the carboxyl terminus of the protein. The cholesterol esterase containing only one proline-rich repeating unit (CEH-Δ3P), or cholesterol esterase without any proline-rich repeats (CEH-Δ4P), was secreted normally by the transfected COS cells. More importantly, both CEH-Δ3P and CEH-Δ4P were equally active as native cholesterol esterase in hydrolysis of the water-soluble substrate *p*-nitrophenyl butyrate and the lipid substrate cholesteryl oleate. On the basis of these observations, we concluded that a domain between residues 490 and 534 encoded by exon 11 of the cholesterol esterase gene is important for intracellular transport.

The results of the current study also showed a bile salt concentration-dependent enzymatic activity for both native cholesterol esterase and cholesterol esterases with reduced numbers of proline-rich repeating units. The bile salt concentration-dependent cholesterol esterase activity was consistent with results reported previously for bile concentration-dependent changes in the conformation of rat cholesterol esterase (Jacobson *et al.*, 1990) and porcine cholesterol esterase (Tsujita *et al.*, 1987). The differences observed in the amount of bile salt required to maximally stimulate cholesterol esterase activity reported here and that required for conformational changes reported by Jacobson *et al.* (1990) may be attributed to differences in the type of bile salt and the pH used in the respective studies. In any event, these results indicated that bile salt activation of cholesterol esterase is mediated *via* induction of conformational changes of the enzyme. These changes in protein conformation may be necessary for substrate delivery to the active site of the cholesterol esterase. If this hypothesis is correct, then the increased enzymatic reactivity at low bile salt concentrations observed when the proline-rich repeating units were removed would suggest that the repeating units at the carboxyl terminus may pose a steric constraint in substrate delivery to the active site of the cholesterol esterase. The activation of bile salt, or the deletion of these proline-rich repeating units, would remove the steric hindrance and facilitate active-site interaction with

substrates. The decrease in the apparent K_m value at low taurocholate concentrations when the proline-rich repeating units were removed from the protein is consistent with this hypothesis.

Since the submission of this paper, Hansson *et al.* (1993) published a paper reporting that the catalytic activity of human bile salt-stimulated lipase (cholesterol esterase) was retained in the absence of any proline-rich repeating units. Although our results with the rat cholesterol esterase were consistent with this observation, notable differences exist between the two systems. Hansson and his co-workers showed that the truncated human enzyme displayed identical bile salt dependency as the native enzyme in hydrolysis of emulsified lipid substrates (Hansson *et al.*, 1993), while our study indicated that the truncated rat enzyme was more active than native cholesterol esterase in hydrolyzing *p*-nitrophenyl butyrate at low taurocholate concentrations. The differences between the two studies may be due to differences between the human and the rat enzyme: The human cholesterol esterase contains 16 and the rat contains 4 proline-rich repeating units (Kissel *et al.*, 1989; Reue *et al.*, 1991; Hui & Kissel, 1990). However, it is more likely that the difference of the two studies is due to different substrates used in the assay. The use of lipid emulsions, such as triolein emulsified with gum arabic used by Hansson *et al.* (1993), precluded the testing of enzyme activity at bile salt concentrations below the critical micellar value of the bile salt. Thus, the inability of the truncated human enzyme to hydrolyze triolein at low taurocholate concentrations may be related to the requirement of high bile salt in solubilization of the substrate. Our study used the water-soluble substrate *p*-nitrophenyl butyrate and demonstrated differences in catalytic activity between native and truncated forms of the cholesterol esterase.

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