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A new class of mammalian carboxylesterase CES6

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Abstract

Mammalian carboxylesterases (CES) exhibit broad substrate specificities, catalyse hydrolytic and transesterification reactions with a wide range of drugs and xenobiotics and are widely distributed in the body. Four CES classes have been previously described, namely CES1 (major liver form); CES2 (major intestinal form); CES3 (highest activity in the colon); and CES5, a secreted enzyme found in mammalian kidney and male reproductive fluids. *In silico* methods were used to predict the amino acid sequences, structures and gene locations for a new class of *CES* genes and proteins, designated as CES6. Mammalian CES6 amino acid sequence alignments and predicted secondary and tertiary structures enabled the identification of key CES sequences previously reported for human CES1, but with *CES6* specific sequences and properties: high isoelectric points (pI values of 8.8 – 9.4 compared with 5.4 – 6.2 for human CES1, CES2, CES3 and CES5); being predicted for secretion into body fluids compared with human CES1, human CES2 and CES3, which are membrane bound; and having Asn or Glu residues at the predicted CES1 Z-site for which a Gly residue plays a major role in cholesterol binding. Mammalian *CES6* genes are located in tandem with *CES2* and *CES3* genes, are transcribed on the positive DNA strand and contain 14 exons. Human and mouse *CES6*-like transcripts have been previously reported to be widely distributed in the body but are localized in specific regions of the brain, including the cerebellum. CES6 may play a role in the detoxification of drugs and xenobiotics in neural and other tissues of the body and in the cerebrospinal fluid.

Keywords

Mammals; amino acid sequence; genomics; carboxylesterase; CES6; drug detoxification

1. Introduction

Mammalian carboxylesterases (CES; E.C.3.1.1.1) comprise families of conserved enzymes involved in the biotransformation of many drugs and pro-drugs (He et al., 1999; Ahmad et al., 1999; Imai et al., 2003; Imai, 2006) and catalyse the hydrolysis or transesterification of xenobiotics, narcotics, carbamates and insecticides (Ahmad & Forgash, 1976; Leinweber,

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1987; Pindel et al., 1997; Satoh and Hosokawa, 1998; Satoh et al., 2002; Redinbo and Potter, 2005). CES also catalyses several lipid metabolic reactions (Tsujita and Okuda, 1993; Becker et al., 1994; Diczfalusy et al., 2001; Dolinsky et al, 2001), facilitates the conversion of lung alveolar surfactant (Krishnasamy et al., 1998; Ruppert et al., 2006) and is involved in the assembly of very-low density lipoproteins in liver (Wang et al., 2007).

Two families of mammalian CES have been extensively investigated, namely CES1 and CES2, the major liver and intestinal enzymes, respectively, that are both widely distributed in mammalian tissues (Shibata et al., 1993; Schewer et al., 1997; Pindel et al., 1997; Ghosh, 2000; Holmes et al., 2009). CES1 is predominantly responsible for the clearance of drugs and xenobiotics from the body whereas CES2 plays a major role in the first pass clearance of drugs and xenobiotics and the activation of pro-drugs in the intestine (Pindel et al., 1997; Imai, 2006; Imai et al., 2006). Two other mammalian CES families have also been described: CES3, expressed in human liver, colon and brain and capable of metabolising the anti-cancer drug, irinotecan and its oxidative metabolites (Sanghani et al., 2004); and CES5 (also called cauxin [for carboxylesterase-like urinary excreted protein] or CES7), which is a major urinary protein of the domestic cat (Miyazaki et al., 2003). The latter enzyme may serve two major roles in mammals, including regulating the production of a pheromone precursor in cat urine (Miyazaki et al., 2006) and participating in lipid and cholesterol transfer process in male reproductive fluids (Ecroyd et al., 2006). A review of the comparative biochemistry and genomics of mammalian CES5 has been recently published (Holmes et al., 2008a).

A limited amount of *in silico* data supports the presence of another CES gene family in mammals, which we have designated as CES6 (Holmes et al., 2008b,c). In this study, we have identified and characterized *in silico* new forms of mammalian CES6 and described predicted amino acid sequences, protein subunit structures, gene locations and exonic structures for *CES6* genes, as well as structural and phylogenetic relationships for *CES6* with other mammalian *CES* gene families. We have also observed the cellular distribution for *CES6*-like transcripts in mouse brain and proposed a role for this enzyme in neural and other tissues of the body in drug and xenobiotic metabolism.

2. Materials and Methods

2.1 In silico mammalian CES gene and protein identification

BLAST (Basic Local Alignment Search Tool) studies were undertaken using web tools from the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al*, 1997). Protein BLAST analyses used a human amino acid sequence, previously identified among gene products implicated in epidermal barrier function (GenBank **FLJ37464**) (Toulza et al., 2007) and designated as CES6 (Holmes et al., 2008b,c). Non-redundant protein sequence databases for several mammalian genomes were examined using the blastp algorithm, including the cow (*Bos taurus*) (Bovine Genome Project, 2008); mouse (*Mus musculus*) (Mouse Sequencing Consortium, 2002); rat (*Rattus norvegicus*) (Rat Genome Sequencing Project Consortium, 2004); and opossum (*Monodelphis domestica*) (Mikkelsen et al., 2007). This procedure produced multiple BLAST 'hits' for each of the protein databases which were individually examined and retained in FASTA format, and a record kept of the sequences for predicted mRNAs and encoded CES-like proteins. These records were derived from annotated genomic sequences using the gene prediction method: GNOMON and predicted sequences with high similarity scores for human CES6. With the exception of the rat, a predicted CES6-like protein sequence was obtained in each case and subjected to *in silico* analyses of predicted protein and gene structures. The rat genome was subjected to BLAT (BLAST-Like Alignment Tool) *in silico* analysis using the mouse CES6 protein sequence (Swiss-Prot TrEMBL primary accession number: Q8R0W5) (http://kr.expasy.org/uniprot) to interrogate rat genome sequences using the UC Santa Cruz

genome browser [http://genome.ucsc.edu/cgi-bin/hgBlat] (Kent *et al.* 2003) with the default settings to obtain an Ensembl generated protein sequence using the methods of Hubbard et al (2002) (http://www.ensembl.org/index.html).

BLAT analyses were subsequently undertaken for each of the predicted CES6 amino acid sequences using the UC Santa Cruz web browser [http://genome.ucsc.edu/cgi-bin/hgBlat] (Kent *et al.* 2003) with the default settings to obtain the predicted locations for each of the mammalian *CES6* genes, including predicted exon boundary locations and gene sizes. BLAT analyses were similarly undertaken of *CES1, CES2, CES3* and *CES5*-like human genes using previously reported sequences for encoded CES subunits in each case (see Table 1). Structures for human CES6 isoforms (splicing variants) were obtained using the AceView website (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html?human) to examine predicted gene and protein structures using the human CES6 cDNA sequence (GenBank FLJ37464) to interrogate this database of human mRNA sequences (Thierry-Mieg and Thierry-Mieg, 2006).

2.2 Predicted Structures and Properties for Mammalian CES Subunits

Predicted secondary and tertiary structures for human and other mammalian CES-like subunits were obtained using the PSIPRED v2.5 web site tools provided by Brunel University [http://bioinf.cs.ucl.ac.uk/psipred/psiform.html] (McGuffin *et al.* 2000) and the SWISS MODEL web tools [http://swissmodel.expasy.org/], respectively (Guex & Pietsch 1997; Kopp & Schwede 2004). The reported tertiary structure for the rabbit CES1 4-piperidino-piperidine complex (Bencharit et al., 2002) served as the reference for the predicted human CES6 tertiary structure, with a modeling range of residues 27 to 552. The predicted CES6 structure was compared with the previously described structure for the human CES1 Coenzyme A (Bencharit et al., 2003) for residues 21 to 553.

Theoretical isoelectric points and molecular weights for horse CES subunits were obtained using Expasy web tools (http://au.expasy.org/tools/pi_tool.html). SignalP 3.0 web tools were used to predict the presence and location of signal peptide cleavage sites (http://www.cbs.dtu.dk/services/SignalP/) for each of the predicted horse CES sequences (Emanuelsson et al 2007). The NetNGlyc 1.0 Server was used to predict potential N-lycosylation sites for human CES and mammalian CES6 subunits (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.3 Phylogenetic Studies and Sequence Divergence

Phylogenetic trees were constructed using an amino acid alignment from a ClustalW-derived alignment of CES protein sequences, obtained with default settings and corrected for multiple substitutions (Chenna *et al* 2003; Larkin *et al.* 2007) [http://www.ebi.ac.uk/clustalw/]. An alignment score was calculated for each aligned sequence by first calculating a pairwise score for every pair of sequences aligned. Alignment ambiguous regions, including the amino and carboxyl termini, were excluded prior to phylogenetic analysis yielding alignments of 473 residues for comparisons of mammalian CES6 sequences with human CES1, CES2, CES3 and CES5 sequences (Table 1). The extent of divergence for the mammalian CES6-like subunits, and the human CES1, CES2, CES3 and CES5 subunits were determined using the SIM-Alignment tool for Protein Sequences [http://au.expasy.org/tools/sim-prot.html] (Pietsch 1995; Schwede *et al.* 2003).

2.4 Mouse Brain CES6 Expression

The Allen Mouse Brain Atlas was interrogated for cellular transcription of the mouse *CES6* gene using the GenBank **BC026374** sequence (see Table 1) (Lein et al., 2007). Data for sagittal

sections of mouse brain were examined and recorded for mouse CES6 transcripts using web tools available on the Allen Brain Atlas web site (http://www.brain-map.org).

3. Results and Discussion

3.1 Alignments of mammalian CES6 and human CES1, CES2, CES3 and CES5 amino acid sequences

The amino acid sequences for five *in silico* derived mammalian CES6 subunits are shown in Figure 1 together with previously reported sequences for human CES1 (Shibata et al., 1993; Becker et al., 1994;Ghosh, 2000), CES2 (Schewer et al., 1997;Pindel et al., 1997), CES3 (Sanghani et al., 2004) and CES5 (Holmes et al., 2008a) (see Table 1). Alignments of human CES1 with human CES2, CES3, CES5 and CES6 showed 42–45% sequence identities, whereas the alignments of human, cow, mouse and rat CES6 subunits showed 70–87% sequence identities (Table 2). The predicted opossum CES6 amino acid sequence showed higher levels of identity with the other mammalian CES6-like sequences (52–56%) than with human CES1, CES2, CES3 and CES5 subunit sequences (38–41%). This comparison suggests that mammalian CES6 subunits are products of a separate *CES* gene family to those previously described for *CES1*, *CES2*, *CES3* and *CES5* gene families, which supports an earlier proposal (Holmes et al., 2008a).

The predicted amino acid sequence for human CES6 was six residues shorter (561 amino acids) than for human CES1 (567), but two residues longer than for human CES2 (559 amino acids) (Figure 1). A comparison of the mammalian CES6 sequences for key residues of human CES1 involved in catalytic, subcellular localization, oligomeric and regulatory functions revealed that some of these have been strictly conserved, whereas others have undergone changes (sequence numbers used refer to human CES1). Those retained for the CES families examined included the active site catalytic triad (Ser228; Glu345; His458) (Cygler et al., 1993); and cysteine residues forming two disulfide bonds (Cys95/Cys123 and Cys280/Cys291) (Lockridge et al., 1987). The hydrophobic N-terminus signal peptide (residues 1–18) which is involved in the microlocalization of mammalian liver CES1 (von Heijne 1983;Zhen et al., 1995; Potter et al., 1998) has been retained for all CES sequences examined with the exception of the opossum CES6 sequence (Figure 1). In contrast, the CES1 C-terminal endoplasmic reticulum (ER) retention sequence (His-Ile-Glu-Leu) (Robbi & Beaufay 1983;Munro & Pelham 1987;Zhen et al., 1995) has been retained for the human CES2 and CES3 subunits, whereas mammalian CES6 and human CES5 C-terminal sequences have other C-terminal sequences. The steroid binding site for human CES1 (‘Z-site’ Gly358) has been substituted for all of the mammalian CES6 sequences, and the human CES1 ‘side door’ (Val424-Met425-Phe426) and product release ‘gate’ (Phe551) residues (Redinbo and Potter, 2005) have also undergone major changes. However, the mammalian CES6 sequences have retained an Ala-Thr-Phe tripeptide at the aligned position to that of the human CES1 ‘side door’ (Figure 1) which may reflect an important structural change for the CES6 family of enzymes. Other key residues included the ‘charge clamp’ residues contributing to the oligomeric subunit structures for human CES1 (Lys78/Glu183 and Glu72/Arg193). One of the CES1 ‘charge clamps’ supporting oligomeric structures was retained for the aligned residues of human, cow and opossum CES6 (Glu73/Arg186) (human CES6 residue numbers used) whereas mouse and rat CES6 sequences do not support potential ‘charge clamps’ in aligned positions to those observed for human CES1 (Figure 1). Human and baboon CES2 have been shown to be monomeric enzymes (Pindel et al., 1997;Holmes et al., 2009) (see Figure 1 for human CES2) and it is likely that mouse and rat CES6 may also be monomeric due to the absence of charge clamps reported for human CES1 (Fleming et al, 2005). In contrast, human, cow and opossum CES6 may support an oligomeric structure due to the presence of at least one potential charge clamp for these enzymes (Figure 1). The N-glycosylation site for human CES1 (Asn79-Ala80-Thr81)

(Ozols, 1989;Kroetz et al, 1993;Bencharit et al., 2003;2006;Fleming et al., 2005) has been previously shown to contribute to enzyme stability and contributing to maximal catalytic for this enzyme (Kroetz et al., 1993;Fleming et al., 2005). This site was not retained for any of the predicted mammalian CES6 sequences, however, two alternate potential N-glycosylation sites were observed (Asn276-Ser277-Thr278; and Asn388-Ile389-Thr390 [human CES6 sequence]) and similar roles for these CES6 sites are proposed (Figure 1; Table 3). Human CES6 also showed a third potential N-glycosylation site (Asn214-Val215-Thr216) that may contribute further to enzyme stability and catalytic efficiency for this enzyme. Major differences for the theoretical isoelectric points (pI) of the mammalian CES6 subunits were observed with each showing much higher pI values (8.8–9.4) than for the human CES1, CES2, CES3 and CES5 subunits examined (5.4–6.2). The higher pI values were also observed for the five human CES6 isoforms examined.

3.2 Comparative Mammalian CES6 Genomics

The NCBI AceView web browser defines the human CES6 gene (GenBank FLJ37464) (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html?human) by 86 GenBank accessions from 79 cDNA clones, some from brain, lung, melanocyte and other tissues (Thierry-Mieg and Thierry-Mieg, 2006). Human CES6 transcripts included 10 alternatively spliced variants (CES6 isoforms) which apparently differed by truncation of the 5' end, presence or absence of exons, overlapping exons with different boundaries, alternative splicing or retention of introns (Figure 2). Isoforms CES6a (**FLJ37464**.aApr07), CES6b, CES6c, CES6d and CES6e encoded proteins with variable content of key residues in comparison with the CES6 mRNA (GenBank **FLJ37464**) sequence (Figure 3). These five CES6 isoforms contained the serine (human CES6 Ser221) and glutamate (Glu239) catalytic triad residues however only CES6c and CES6d contained the third active site residue (His350). CES6a, CES6b and CES6d isoforms contained both disulfide bridges reported for human CES1 as compared with CES6c and CES6e isoforms which retained cysteine residues suitable for one of the bridges reported for human CES1. The functions for these CES6 isoforms remain to be determined.

Fig. 1 shows the locations of the intron-exon boundaries for the mammalian CES6 gene products examined, and compares them with human CES1, CES2, CES3 and CES5 subunits and their positioning within the aligned amino acid sequences. Exon 1 corresponded to the encoded signal peptide in each case (with the exception of opossum CES6 which lacked a predicted signal peptide), with the last exon encoding the endoplasmic reticulum targeting sequence (for human CES1, CES2 and CES3) or the C-terminal sequence involved in a proposed role in the secretion of human CES5 (Holmes et al., 2008a). There is identity or near identity for the intron-exon boundaries for each of the mammalian *CES6* genes and for the human *CES1*, *CES2*, *CES3* and *CES5* genes, with the exception of an additional human CES1 exon boundary (forming exon 9), and the absence of an intron-exon boundary for human CES2 within exon 5 (Figure 1). The mammalian *CES6* genes were usually located within a *CES2-CES3-CES6* gene cluster on the same chromosome as a second *CES1-CES5* gene cluster, with the exception of the opossum CES2-CES6 cluster on chromosome 1, for which a homologue *CES3* gene has not as yet been described (Holmes et al., 2008b) (Table 1).

3.3 Secondary and Tertiary Structures for Mammalian CES6 Sequences

Figure 1 shows the secondary structures previously reported for human CES1 (Bencharit et al., 2003;2006;Fleming et al, 2005) or predicted for mammalian CES6 sequences and for human CES2, CES3 and CES5 sequences. Similar α-helix β-sheet structures were observed for the four human CES gene products and the five mammalian CES6 gene products examined, particularly near key residues or functional domains, including the α-helix within the N-terminal signal peptide and the β-sheet and α-helix structures surrounding the active site Ser228

(human CES1). However, other sites showed differences in predicted secondary structures for the mammalian CES6 sequences, including in particular, two additional α-helices (human CES6 residues 173–176 and 235–247). Moreover, the predicted N-glycosylation sites for mammalian CES6 sequences were each located between α-helices which may assist with N-glycoside binding. In comparison with the human CES1 'side door' (human CES1 Val424-Met425-Phe426), which has been reported to facilitate acyl product release following hydrolysis (Bencharit et al., 2003;2006;Fleming et al, 2005), the mammalian CES6 sequences showed an extended α-helix extending across this region and a conserved sequence (human CES6 424Ala-425Thr-426), which may play a significant role in acyl product release for this family of enzymes. However, predictions of CES6 secondary structures may not fully reflect structures *in vivo* and may serve only as a guide as to the comparative structures for mammalian CES6 subunits. The predicted tertiary structure for human CES6 was sufficiently similar to the mammalian CES1 structure to be based on a previously reported rabbit CES1-4-piperidino-piperidine complex 3-D structure (Bencharit et al., 2002) (Figure 4). However, the predicted human CES6 structure was based on an incomplete sequence for this enzyme (residues 27–552) as was the reported 3-D structure for the human CES1-Coenzyme A complex (residues 21–553) (Bencharit et al., 2003).

3.4 Phylogeny of Human CES Gene Products and of Mammalian CES5

A phylogenetic tree (Figure 5) was constructed by the progressive alignment of human CES1, CES2, CES3 and CES5 amino acid sequences and five mammalian CES6 sequences. A cluster of the CES6 'like' gene products was observed distinct from the other five CES families which supported a proposal that the mammalian CES6 sequences examined form part of a single enzyme class. Table 2 summarizes the percentages of identity for these enzymes and shows that mammalian CES6 sequences are ≥ 55% identical which is in contrast with the 39–45% identities observed comparing sequence identities between CES families. In addition, more closely related species showed higher levels of sequence identity for CES6, such as the rodent species (mouse and rat) which were 87% identical, as compared with marsupial (opossum) and eutherian mammal (human, cow, mouse and rat) CES6 sequences, with 52–56% identical sequences. Holmes et al. (2008b) have previously described evidence for a rapid early series of gene duplication events into at least five *CES* gene family clusters (*CES1*, *CES2*, *CES3*, *CES5* and *CES6*) at around 328–378 million years ago. Based on this report, we have concluded that the *CES6* primordial gene predated the eutherian mammalian common ancestor (estimated at 88–99 million years ago) (Murphy et al., 2001;Woodburne et al., 2003) by > 200 million years. In addition, it is also apparent that the mammalian CES6 gene has been derived from a common ancestor shared by all mammals (both eutherian and marsupial) ~ 200 million years ago (Woodburne et al., 2003).

3.5 CES6 Tissue Distribution and Likely Functions

Mammalian CES6 is a member of the *CES* super-family of genes encoding enzymes of broad substrate specificity and responsible for the hydrolysis or transesterification of a wide range of naturally occurring compounds, including xenobiotics, narcotics and clinical drugs, and which have the capacity to catalyze several lipid metabolic reactions (Becker et al., 1994; Satoh and Hosokawa, 1998; Satoh et al., 2002; Redinbo and Potter, 2005; Tsujita and Okuda, 1993; Diczfalusy et al., 2001; Dolinsky et al, 2001). More specific roles for mammalian CES have also been described, including the activation of several prodrugs used in treating cancer (Humerickhouse et al., 2000; Ohtsuka et al., 2003; Tabata et al., 2004), influenza (He et al., 1999) and high blood pressure (Takai et al., 1997).

The differential tissue distribution and microlocalization of CES family members may provide an important clue as to their roles within mammalian organisms. In contrast with mammalian CES1 and CES2, which are predominantly localized within the liver and intestine endoplasmic

reticulum, are strongly membrane bound and responsible for drug clearance from the body and first pass metabolism of drugs in the digestive system, respectively, mammalian *CES6* is predominantly expressed in peripheral tissues, including brain, lung and melanocytes (Thierry-Mieg and Thierry-Mieg, 2006) and is likely a secreted form of CES. Figure 6 shows a sagittal section of mouse brain examining the distribution of CES6 transcripts (GenBank BC026374 mRNA) within different regions of the brain (provided by the Allen Institute for Brain Science) (Lein et al., 2007). The cerebellum folds exhibited highest staining levels although transcripts were observed throughout the brain, including the hippocampus, amygdalar nuclei, the olfactory bulb, the cortex, the pons and the medulla regions. The expression of CES6 transcripts in the brain and its likely distribution in neural fluids may provide a guide as to its role, such as protecting the brain and other neural tissues from drugs via the blood brain barrier or the cerebrospinal fluid. It is proposed that mammalian CES6 (at least in the mouse) plays a distinctly different role to that of the major liver (CES1) and intestine (CES2) enzymes, and may serve as a secreted form of neural CES with specialized functions, which remain to be fully determined.

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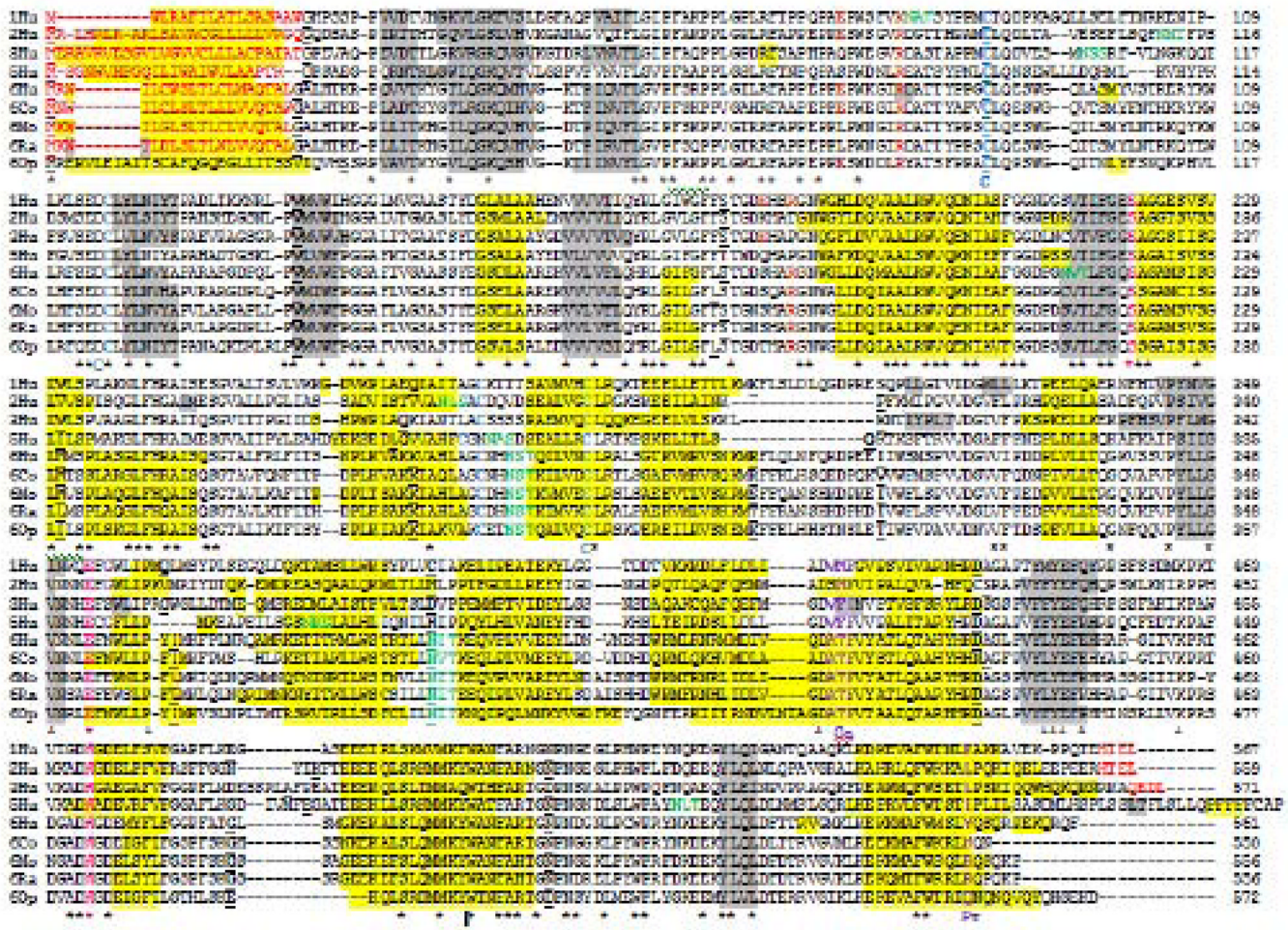

Figure 1. Amino acid sequence alignments for human CES1, CES2, CES3 and CES5 and mammalian CES6 sequences

See Table 1 for sources of CES sequences; * shows identical residues for human CES1, CES2 and CES5; Residues involved in processing at N- (Signal peptide) and C- termini (MTS-microsomal (endoplasmic reticulum) targeting sequence); **N-glycosylation residues** at 79NAT (Human CES1) and potential N-glycosylation sites; active site triad residues Ser; Glu; and His. **'Side door'**, **'Gate'** (Ga) residues; Product releasing (Pr) and Cholesterol binding Gly residue (**Z site**) for human CES1 Disulfide bond C residues for human CES1. **Charge clamp residues identified for human CES1**; Helix (Human CES1 or predicted helix; Sheet (Human CES1) or predicted sheet. **Bold underlined font shows known or predicted exon junctions. Hu-human; Co-cow; Mo-mouse; Ra-rat; and Op-opossum.**

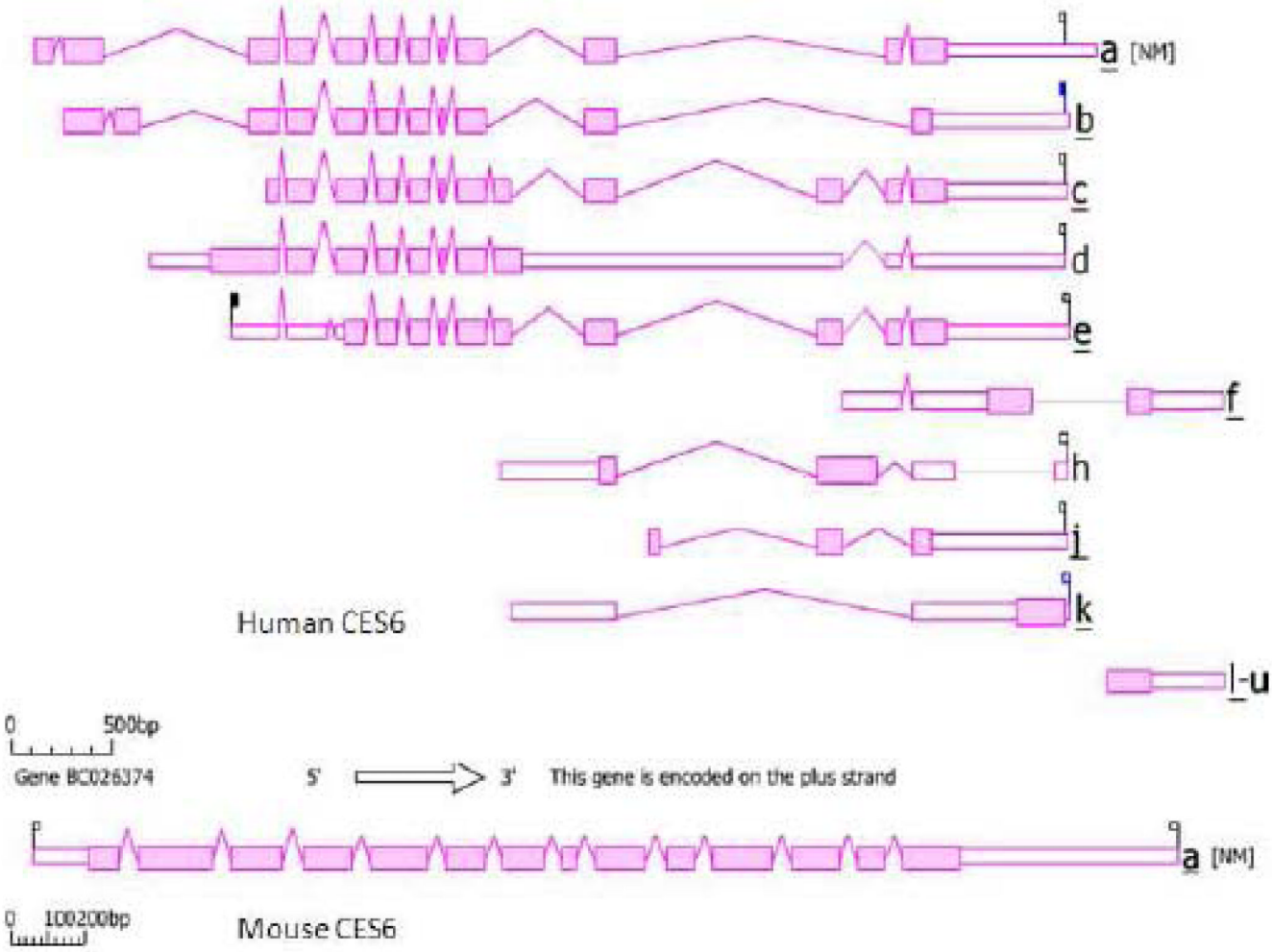

Figure 2. Gene Structures and Splicing Variants for the Human *CES6* (FLJ37463) Gene and for the Mouse *CES6* (BC026374) Gene

Derived from the AceView website (Thierry-Mieg and Thierry-Mieg, 2006). http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/ Mature isoform variants (a, b, c etc) are shown with capped 5'- and validated 3'-ends for the predicted mRNA sequences. NM numbers refer to annotated RefSeq sequences for human and mouse *CES6* genes. Scale refers to base pairs of nucleotide sequences.

Figure 3. Amino acid sequence alignments for human CES6 and for CES6 isoform variants

See Table 1 for sources of CES6 sequences. Residues involved in processing at N- (Signal peptide); **N-glycosylation residues** at potential N-glycosylation sites; active site (AS) triad residues Ser (S); Glu (E); and His (H). **'Side door'** residues (ATF for human CES6) Disulfide bond Cys (C)residues. **Charge clamp residues (CC)previously identified for human CES1**; Predicted helix; Predicted Sheet. **Large font underlined shows predicted exon junctions.**

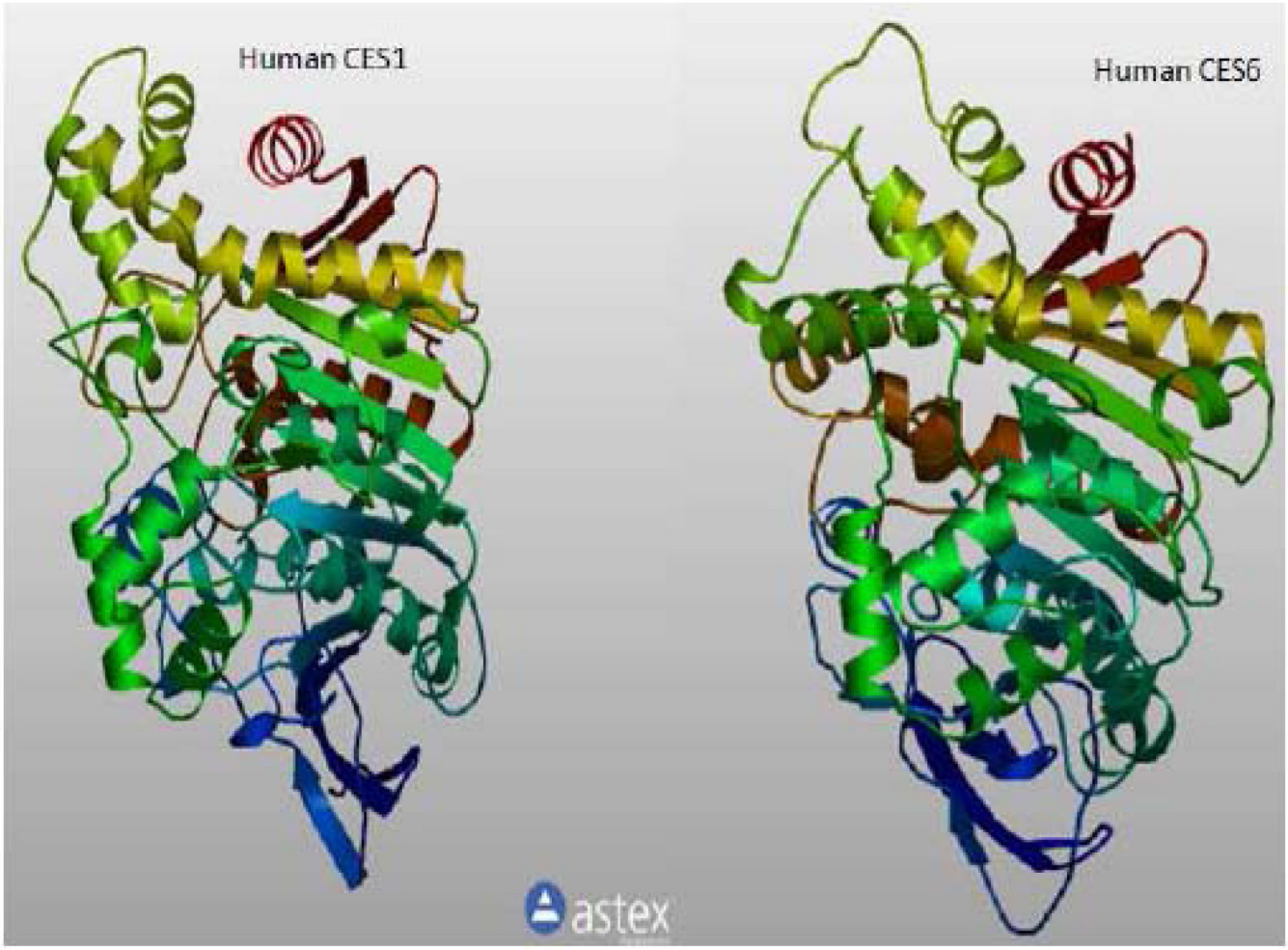

Figure 4. Three Dimensional Structures for Human CES1 and CES6 Subunits

Predicted human CES6 3-D structure was obtained using the SWISS MODEL web site http://swissmodel.expasy.org/workspace/index.php? and the predicted amino acid sequence for this enzyme (see Table 1). The rainbow color code describes the 3-D structures from the N- (blue) to C-termini (red color). The structures are based on the known 3-D structures for rabbit CES1 complexed with 4-piperidino-piperidine (Bencharit et al., 2002) (with a modeling range of residues 27 to 552) and the previously reported structure for the human CES1 Coenzyme A complex (Bencharit et al., 2003).

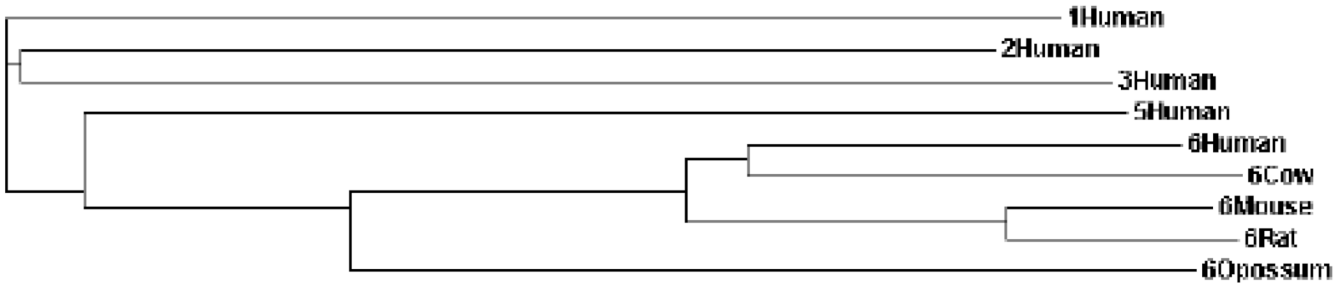

Figure 5. Phylogenetic tree of mammalian CES6 and of human CES1, CES2, CES3 and CES5 sequences

The tree is labeled with the CES gene family number and the species name: human (*Homo sapiens*); cow (*Bos taurus*); mouse (*Mus musculus*); rat (*Rattus norvegicus*); and opossum (*Monodelphis domestica*). Note the separation of the human CES1, CES2, CES3 and CES5 genes from the mammalian CES6 family cluster. The gene duplication events generating five distinct gene families (CES1, CES2, CES3, CES5 and CES6) have been previously estimated to have occurred ~ 328–378 million years ago (Holmes et al., 2008c).

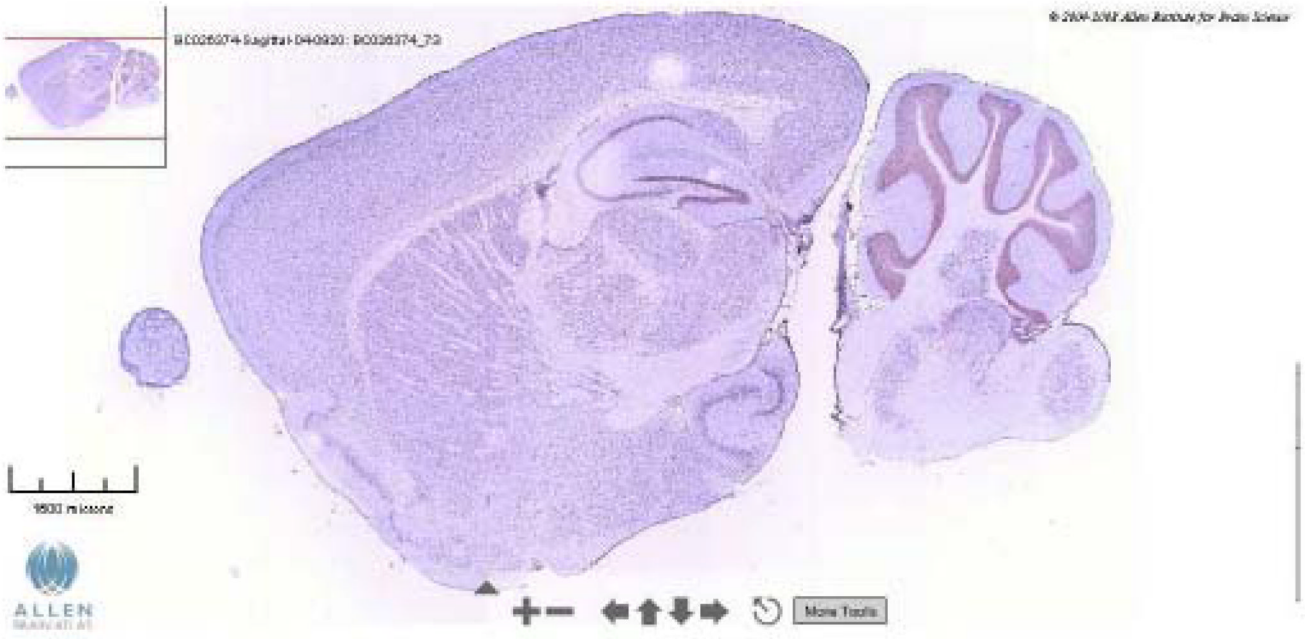

Figure 6. Sagittal section of mouse brain showing the distribution of CES6 transcripts

The three distinct regions of the mouse brain (C57BL6/J strain) included the cerebellum (right), cerebrum (middle) and olfactory lobe (left). Note major staining of mouse CES6 transcripts in the cerebellum folds; the hippocampus and amygdalar nuclei regions of the cerebrum; and the olfactory lobe. The section was obtained from the Allen Brain Atlas web site (http://www.brain-map.org) (Lein et al., 2007) using the GenBank **BC026374** sequence for mouse CES6 (see Table 1).

Table 1

Mammalian carboxylesterase (CES) genes and enzymes examined

Mammal	CES	GenBank ID [1]Ensembl	UNIPROT ID	Amino acids	Chromosome location	Strand	CES gene order	Gene size kbs	Exons	pI	Subunit MW
Human	CES6	**FLJ37464**	Q5XG92	561	16: 65,580,134-65,602,403	Positive	CES2-CES3-**CES6**	22,269	14	9.4	63,529
Cow	CES6	**BC149217**	POC6R3	550	18: 33,691,872-33,705,665	Positive	CES2-CES3-**CES6**	13,794	14	8.9	61,777
Mouse	CES6	**BC026374**	Q8ROW5	556	8: 107,655,873-107,673,417	Positive	[2]CES2-[3]CES3-**CES6**	17,545	14	8.8	62,123
Rat	CES6	[1]Ens.19169		556	19: 34,948,600-34,965,647	Positive	[2]CES2-CES3-**CES6**	17,048	14	8.8	62,684
Opossum	CES6	[4]**XP1372421**		572	1: 677,585,523-677,672,078	Negative	[2]CES2-**CES6**	86,556	14	8.9	64,502
Human	CES1	**L07765**	P23141	567	16: 54,394,265-54,424,576	Negative	CES4-**CES1**-CES5	30,311	14	6.2	62,521
	CES2	**BX538086**	O00348	559	16: 65,525,828-65,536,493	Positive	**CES2**-CES3-CES6	10,665	12	5.7	61,807
	CES3	**NM024922**	Q6UWW8	571	16: 65,552,639-65,566,552	Positive	CES2-**CES3**-CES6	13,913	13	5.4	62,282
	CES5	**BC069501**	Q6NT32	575	16: 54,437,867-54,466,634	Negative	CES4-CES1-**CES5**	28,767	13	6	63,936
Human	CES6a	**FLJ37464a**		505	16: 65,580,135-65,600,543	Positive	CES2-CES3-**CES6**	20,409	12	9.4	56,780
	CES6b	**FLJ37464b**		461	16: 65,587,034-65,600,466	Positive	CES2-CES3-**CES6**	13,433	10	9.2	52,225
	CES6c	**FLJ37464c**		442	16: 65,592,261-65,600,543	Positive	CES2-CES3-**CES6**	8,282	11	9.2	49,908
	CES6d	**FLJ37464d**		376	16: 65,591,860-65,596,319	Positive	CES2-CES3-**CES6**	4,460	8	10	41,677
	CES6e	**FLJ37464e**		367	16: 65,592,758-65,600,543	Positive	CES2-CES3-**CES6**	7,786	10	9.4	41,868
		[4]RefSeq mRNA									

GenBank mRNA (or cDNA) IDs identify previously reported sequences (see http://www.ncbi.nlm.nih.gov/Genbank/)

[1]N-scan ID identifies a gene prediction using the N-SCAN gene structure prediction software provided by the Computational Genomics Lab at Washington University in St. Louis, MO, USA (see http://genome.ucsc.edu); UNIPROT refers to UniprotKB/Swiss-Prot IDs for individual CES subunits (see http://kr.expasy.org). Sources for CES sequences were provided by the above sources (see Table 1).

The opossum CES6 protein sequence (XP1372421) was obtained from a blast using the known mouse CES6 sequence (Q8R0W5) and web tools of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

[2]There are multiple *CES2* genes in mouse, rat and opossum.

[3]The mouse genome contains 2 closely localized *CES3*-like genes.

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Table 2

Percentage identities for mammalian CES6 and human CES1, CES2, CES3 and CES5 amino acid sequences.

CES Gene	Human CES1	Human CES2	Human CES3	Human CES5	Human CES6	Cow CES6	Mouse CES6	Rat CES6	Opossum CES6
Human CES1	100	45	42	42	43	41	43	41	41
Human CES2	45	100	46	43	39	39	41	39	41
Human CES3	42	46	100	41	40	40	41	40	38
Human CES5	42	43	41	100	42	42	42	42	41
Human CES6	43	39	40	42	100	**75**	**71**	**71**	56
Cow CES6	41	39	40	42	**75**	100	**70**	**70**	55
Mouse CES6	43	41	41	42	**71**	**70**	100	**87**	52
Rat CES6	41	39	40	42	**71**	**70**	**87**	100	52
Opossum CES6	41	41	38	41	56	55	52	52	100

Numbers show the percentage of amino acid sequence identities. Numbers in **bold** show higher sequence identities for eutherian mammalian CES6 sequences.

Table 3

Predicted N-glycosylation sites for mammalian CES6 and human CES1, CES2, CES3 and CES5 subunits.

Human CES1	79NAT										1
Human CES2			111NMT		276NLS						2
Human CES3		103NSS									1
Human CES5						281NAS		363NKS		513NLT	3
Human CES6				213NVT			276NST		388NIT		3
Cow CES6							276NST		386NVT		2
Mouse CES6							276NST		388NIT		2
Rat CES6							276NST		388NIT		2
Opossum CES6							283NST		397NIT		2

Numbers refer to amino acids in the CES sequences, including N-asparagine; A-alanine; I-isoleucine; K-lysine; L-leucine; M-methionine; Sserine; T-threonine; and V-valine. Note that the mammalian CES6 sequences examined contained at least two potential N-glycosylation sites.