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The Unique N-Terminal Sequence of Testis Angiotensin-Converting Enzyme Is Heavily O-Glycosylated and Unessential for Activity or Stability

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SUMMARY: The testis-specific isozyme of angiotensin-converting enzyme (ACE) is identical, from residue 68 to the C terminus, to the second half or C-terminal domain of somatic ACE. However, the first 67 residues, comprising the signal peptide and a Ser-/Thr-rich 36-residue sequence that constitutes the N terminus of mature testis ACE, are unique. We have expressed a mutant human testis ACE lacking this 36-residue N-terminal sequence and find that compared to the wild-type protein the mutant is 15 kDa smaller due to the loss of >90% of all O-linked sugars, but that it retains full enzymatic activity and is stable in culture. Heavy O-glycosylation is a property of testis ACE that is not shared by the somatic enzyme and is attributable to this unique sequence.

Angiotensin-converting enzyme (ACE; EC 3.4.15.1), a zinc-metallopeptidase involved in blood pressure regulation, exists as two isozymes: Somatic ACE, a ubiquitous ectoenzyme found in most tissues, and testis ACE, which is apparently limited to developing spermatozoa (1). The somatic isozyme consists of two homologous domains (2,3), whereas the testis isozyme is identical, except for the first 67 residues, to the C-terminal half of somatic ACE (4-6).

It has now been established that the two isozymes arise by transcription of the same gene at tissue-specific initiation sites: the start site for the testis ACE mRNA is located within an intron of the somatic ACE pre-mRNA (7-9). As a consequence, testis ACE contains a unique N-terminal region that comprises a leader peptide distinct from that used by somatic ACE and a 36-residue, Ser- and Thr-rich sequence that constitutes the N terminus of the mature protein (4-6). The significance of this N-terminal sequence is unknown. To address this question, we expressed recombinant human testis ACE lacking this sequence in Chinese hamster ovary (CHO) cells. We find that this sequence is extensively O-glycosylated but that it is not required for the enzyme's activity or stability.

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Abbreviations: ACE, angiotensin-converting enzyme; hTACE, recombinant wild-type human testis ACE; CHO, Chinese hamster ovary; nt, nucleotide(s).

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MATERIALS AND METHODS

Construction of expression vector. The plasmid pLEN-ACEΔ36N, which encodes human testis ACE lacking the first 36 N-terminal residues of the mature protein, is based on pLEN-ACE 6/5 (10,11) and was constructed by deletion mutagenesis using the polymerase chain reaction (12), as follows. Nucleotides (nt) 3-118 and 224-638 of the full-length testis ACE cDNA (4) were amplified with pairs of primers designed to generate overlapping hybrid sequences, using pΩ-ACE as the template (11). After removal of the inside primers by electrophoresis in low-melt agarose (NuSieve GTG, FMC Bioproducts), the excised products were amplified with the outside primers to generate a recombinant sequence from which nt 119-223 [encoding Gln-2 to Asn-36 (4)] has been deleted, but preserving nt 23-118 that encode the signal peptide (4) (Fig. 1). Using suitable restriction sites, the recombinant fragment was then inserted into pLEN-ACE6/5 from which the corresponding sequence had been removed, to give pLEN-ACEΔ36N. In common with pLEN-ACE6/5, pLEN-ACEΔ36N also lacks the 3′ end of the testis ACE cDNA that encodes the transmembrane and cytoplasmic domains. This results in direct secretion of the recombinant protein but does not alter its activity or stability (10,11). In the ensuing discussion, "wild-type" hTACE will refer to the anchor-minus recombinant protein encoded by pLEN-ACE6/5.

Expression of pLEN-ACEΔ36N. The mutant protein was expressed in CHO cells stably transfected with pLEN-ACEΔ36N, and purified from the conditioned media by methods described previously (10,11).

RESULTS AND DISCUSSION

Expression of hTACEΔ36N in CHO cells. The pattern of expression of hTACEΔ36N was virtually identical to that observed previously for wild-type hTACE (10,11). When cells were grown in continuous culture for 2-3 weeks with media being harvested every 2-3 days, no differences were noted in the levels of ACE activity in the media of CHO cells stably transfected with pLEN-ACEΔ36N versus pLEN-ACE6/5. Moreover, hTACEΔ36N could be obtained in electrophoretically homogeneous form (Fig. 2A) following single-step affinity purification of the conditioned media in a yield comparable to that reported for hTACE (1.5-2 mg/L) (11).

Amino acid composition. Compared to wild-type hTACE, the composition of hTACEΔ36N (Table 1) is notable for significant decreases in Glx, Ser, and Thr residues, which are prominent residues in the deleted N-terminal sequence (Fig. 1).

N-terminal sequence analysis. hTACEΔ36N (0.5 nmol) was subjected to 10 cycles of automated Edman degradation (13), and gave the sequence:

(ala,ser)-X-(leu,val)-Val-thr-Asp-Glu-Ala-Glu-Ala

The sequencing yield was only 5-10%, which may mean that the protein is predominantly N-blocked, as are native testis ACE and wild-type hTACE (4,10,11). The sequence indicates that mature hTACEΔ36N was generated after cleavage(s) of the signal peptide as follows:

....V P S Q E ↑ A ?↑ S L V T D E A E A....

(Signal peptide underlined; Fig. 1.) Interestingly, this places either a Glu in the -1 or a Gln in the -3 positions, both of which are considered unfavorable for signal sequence processing (14).

Table 1. Amino acid composition of mutant and wild-type recombinant testis ACE

Amino acid	Residues per mol hTACE		hTACEΔ36N	
Asx	63.7	(64)	62.6	(63)
Glx	83.3	(83)	74.8	(76)
Ser	43.0	(43)	36.2	(36)
Gly	34.8	(35)	40.7	(34)
His	23.3	(23)	19.2	(20)
Arg	29.4	(29)	31.2	(29)
Thr	41.7	(42)	32.1	(32)
Ala	51.9	(51)	48.7	(47)
Pro	37.1	(36)	35.6	(35)
Tyr	25.9	(26)	24.8	(26)
Val	29.1	(32)	29.3	(30)
Met	16.9	(17)	16.3	(17)
Ile	23.3	(25)	23.4	(25)
Leu	65.4	(66)	64.5	(66)
Phe	24.8	(26)	25.3	(26)
Lys	31.7	(30)	30.4	(30)

Results are the average of four analyses, performed as described (13); values based on the predicted sequences are in parentheses. Underlined are residues expected to be decreased after deletion of the 36-residue N-terminal sequence (Fig. 1).

Catalytic properties. hTACEΔ36N is indistinguishable from wild-type hTACE in terms of the hydrolysis of furanacryloyl-L-Phe-Gly-Gly, chloride activation, and inhibition by lisinopril (not shown) under conditions described previously (11).

Electrophoretic mobility and enzymatic deglycosylation. hTACEΔ36N has an $M_r \approx 85$ kDa (Fig. 2A), whereas wild-type hTACE typically runs as a broad smear at $M_r \approx 100$ kDa, likely due to variable glycosylation by the CHO cells (10,11). Treatment of hTACEΔ36N with

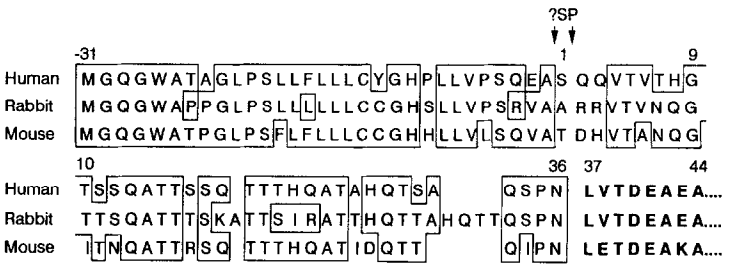

Figure 1. N-terminal sequences of testis ACE preprotein from human (4,6), rabbit (5), and mouse (7). The signal peptide cleavage site (?SP) is known only for the rabbit protein (A/R) (5); the arrows indicate possible cleavage sites in the human and mouse proteins. The sequence from -31 to +36 [numbering refers to the human sequence (4)] is unique to testis ACE; the boxed residues are conserved. Residues 1 to 36 constitute the N terminus of mature testis ACE and have a high content of Ser/Thr (47, 44, and 35%) and Gln (19, 12, and 17%) (human, rabbit, and mouse, respectively). The sequence (bold) from residue 37 through the C terminus is identical to the C-terminal domain (i.e. second half) of somatic ACE. In plasmid pLEN-ACEΔ36N the cDNA encoding residues 2 to 36 was deleted.

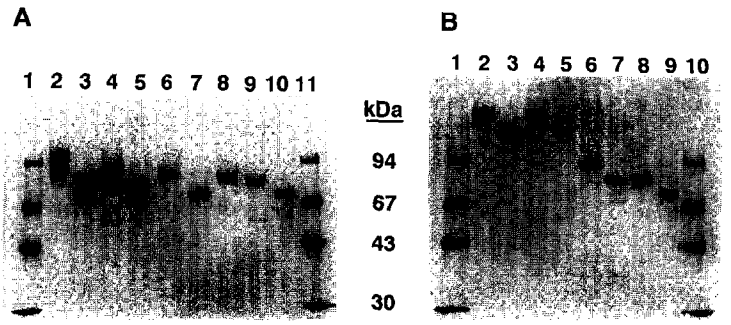

Figure 2. SDS-polyacrylamide gel elctrophoresis of untreated and enzymatically deglycosylated ACE proteins. (A) Recombinant wild-type hTACE (lanes 2-5) and mutant hTACEΔ36N (lanes 6-10) were digested (5 μg per lane) with one or more glycosidases (Genzyme) (10): Lanes 2 and 6, untreated; lanes 3 and 7, N-Glycanase (0.5 U); lanes 4 and 9, neuraminidase (15 mU) and O-Glycanase (2 mU); lanes 5 and 10, N-Glycanase, neuraminidase, and O-Glycanase; lane 8, neuraminidase only. Lanes 1 and 11, mol. wt. markers. (B) Native rabbit ACE from lung (lanes 2-5) and testis (lanes 6-9), treated as described for lanes 2-5 in (A).

N-Glycanase reduced the M_r to ~70 kDa (Fig. 2A, lane 7); additional sequential digestion with neuraminidase and O-Glycanase resulted in little additional change (Fig. 2A, lane 10). The theoretical polypeptide mol. wt. is 71 kDa. Treatment with neuraminidase alone or with neuraminidase followed by O-Glycanase decreased the M_r to 80 and 78 kDa, respectively (Fig. 2A, lanes 8 and 9). Therefore, hTACEΔ36N consists of ~18% carbohydrate by weight, of which 90% is N-linked and 10% O-linked (Table 2); ~30% of the total sugars is sialic acid.

In contrast, treatment of the wild-type hTACE with N-glycanase resolved the protein into two bands at M_r = 71 and 81 kDa (Fig. 2A, lane 3). Further digestion with neuraminidase and O-Glycanase resulted in a decrease in the M_r of the upper band from 81 to 74 kDa (Fig. 2A, lane 5); many-fold overdigestion with these glycosidases reduces hTACE to a single species at $M_r \approx 70$ kDa (10). Treatment of hTACE only with neuraminidase and O-Glycanase produced a broad band of ~85 kDa, similar in size to native hTACEΔ36N (Fig. 2A, lane 4). Thus, hTACE consists of ~30% carbohydrate by weight, 60% N-linked and 40% O-linked (Table 2).

For comparison, native rabbit testis ACE and somatic ACE from rabbit lung and human kidney were treated in the same manner (Fig. 2B; human kidney ACE not shown). Although the native rabbit testis ACE runs as a sharper band than hTACE, it similarly consists of ~25% carbohydrate by weight of which 55% is N-linked and 45% is O-linked. Significantly, somatic ACE consists of only ~18% carbohydrate, of which little or none is O-linked (Table 2).

Quantitative carbohydrate analysis. Compared to hTACE, hTACEΔ36N contains markedly lower molar amounts of sugars, particularly N-acetylgalactosamine (GalNAc), N-acetylglucosamine, galactose (Gal), and N-acetylneuraminic acid (the principal sialic acid) (Table 2). Most striking is the complete absence of GalNAc, which is significant because the structure Gal→GalNAc→Ser/Thr is a common core region of mucin-like O-linked oligosaccharide chains

Table 2. Carbohydrate content of recombinant and native ACE proteins

	ACE proteins				
Sugar	hTACEΔ36N	hTACE	RT-ACE	RL-ACE	HK-ACE
	Enzymatic deglycosylation (% carbohydrate by weight)				
Total	18	30	25	18	18
N-linked	16	18	13	18	18
O-linked	2	12	12	0	0
	Quantitative analysis (residues per mol)				
NAc-galactosamine	0	8.3	8.9	0	0
NAc-glucosamine	35.4	45.8	27.0	47.9	60.2
galactose	12.9	30.1	16.2	42.7	28.9
mannose	11.3	13.8	23.6	38.3	37.3
fucose	7.0	7.9	1.6	8.6	14.7
xylose	1.7	1.1	2.9	1.4	1.4
ribose	0.9	0.3	0.3	0	0.1
NAc-neuraminic acid	7.8	22.9	4.6	4.6	5.9
NGl-neuraminic acid	1.1	1.1	0.1	1.1	0
Total	78	131	85	145	149

Enzymatic deglycosylations were done as described in Fig. 2. Percentages are with reference to the undigested protein; these values are approximate only because electrophoresis of glycoproteins is inherently inaccurate. Quantitative analyses were performed by high-performance liquid chromatography of protein hydrolysates derivatized with 1-phenyl-3-methyl-5-pyrazolone for reducing sugars (15) or 1,2-diamino-4,5-methylenedioxybenzene for neuraminic acids (16). Abbreviations: RT, rabbit testis; RL, rabbit lung; HK, human kidney; NAc, N-acetyl; NGl, N-glycolyl.

(17). Notable too is that mannose, which is only a minor component of O-linked chains (18), is present in equal amounts in hTACEΔ36N versus hTACE. Significantly, native testis ACE, like wild-type hTACE, contains GalNAc, whereas this sugar is absent from native somatic ACE.

Taken together, these data indicate that the Ser- and Ser-rich N-terminal region of testis ACE contributes 80-90% of O-linked sugars in this protein. Moreover, it is clear that heavy O-glycosylation is a special chracteristic of the testis isozyme that is attributable to the unique N-terminal sequence for which there is no homolog in the somatic protein. However, the biological function of this sequence remains unresolved. One possibility is that this sequence may be important in the targeting of the enzyme. Unlike somatic ACE, which resides as an ectoenzyme on the surfaces of epithelial and endothelial cells (1), testis ACE appears to be intracellular in the cytoplasmic droplets of spermatids (19) where it is likely bound to vesicular elements by the C-terminal membrane-spanning sequence that it shares with somatic ACE (4,10). However, this cannot be tested in CHO cells, because in these cells wild-type hTACE is transported to the cell surface and is not sequestered intracellularly (10).

A second possibility is that the N-terminal sequence contributes to the stability of testis ACE, since it is known that O-glycosylated regions are relatively resistant to proteases (20). However, such O-glycosylated regions are frequently adjacent to the membrane anchors of

integral membrane proteins, constituting a protected "stalk" (21). Since testis ACE is C-terminally anchored whereas the O-glycosylated sequence is at the N terminus, such a role is unlikely. This is borne out by the spontaneous proteolytic release into the culture medium of full-length membrane-bound hTACE expressed in CHO cells (10). Moreover, once secreted into the culture media, hTACEΔ36N does not differ from hTACE in its stability.

Lastly, this sequence may function in the clearance of the testis isozyme from the epididymal lumenal fluid. Testis ACE-containing cytoplasmic droplets shed by maturing spermatozoa disintegrate in the epididymal lumen (22), but only the somatic isozyme can be recovered from seminal plasma (23), indicating that the testis isozyme is either rapidly degraded or actively removed by the epididymal epithelium. In the rat the contents of disintegrated cytoplasmic droplets are actively endocytosed and degraded by the clear cells of the epididymal epithelium, possibly by a receptor-mediated process (22). Thus, the unique N-terminal sequence of testis ACE may constitute a specific ligand for receptor-mediated endocytosis by epididymal epithelial cells.

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