Biochemical and Biophysical Research Communications xxx (2016) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The effect of structural motifs on the ectodomain shedding of human angiotensin-converting enzyme

Nailah Conrad [a, 1], Sylva L.U. Schwager [a], Adriana K. Carmona [b], Edward D. Sturrock [a, *]

[a] Department of Integrative Biomedical Sciences, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory 7925, South Africa
[b] Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de Sao Paulo, Sao Paulo, Brazil

ARTICLE INFO

Article history:
Received 6 October 2016
Accepted 16 October 2016
Available online xxx

Keywords:
Ectodomain shedding
Glycosylation
Protein-protein interactions
Cellular processing
Stalk
Cleavage

ABSTRACT

Somatic angiotensin converting enzyme (sACE) is comprised of two homologous domains (N and C domains), whereas the smaller germinal isoform (tACE) is identical to the C domain. Both isozymes share an identical stalk, transmembrane and cytoplasmic domain, and undergo ectodomain shedding by an as yet unknown protease. Here we present evidence for the role of regions distal and proximal to the cleavage site in human ACE shedding. First, because of intrinsic differences between the N and C domains, discrete secondary structures (α-helix 7 and 8) on the surface of tACE were replaced with their N domain counterparts. Surprisingly, neither α-helix 7 nor α-helix 8 proved to be an absolute requirement for shedding. In the proximal ectodomain of tACE residues H^{610}-L^{614} were mutated to alanines and this resulted in a decrease in ACE shedding. An N-terminal extension of this mutation caused a reduction in cellular ACE activity. More importantly, it affected the processing of the protein to the membrane, resulting in expression of an underglycosylated form of ACE. When E^{608}-H^{614} was mutated to the homologous region of the N domain, processing was normal and shedding only moderately decreased suggesting that this region is more crucial for the processing of ACE than it is for regulating shedding. Finally, to determine whether glycosylation of the asparagine proximal to the Pro1199-Leu polymorphism in sACE affected shedding, the equivalent P^{623}L mutation in tACE was investigated. The P^{623}L tACE mutant showed an increase in shedding and MALDI MS analysis of a tryptic digest indicated that N^{620}WT was glycosylated. The absence of an N-linked glycan at N^{620}, resulted in an even greater increase in shedding. Thus, the conformational flexibility that the leucine confers to the stalk, is increased by the lack of glycosylation reducing access of the sheddase to the cleavage site.

1. Introduction

A number of membrane proteins are enzymatically cleaved or 'shed' from the cell surface, resulting in the modulation of biological events and providing novel therapeutic approaches to diverse diseases by targeting shedding. Angiotensin-converting enzyme (ACE) plays a pivotal role in blood pressure regulation and fertility. The identification of novel hereditary ACE mutations that result in increased ACE shedding has advanced our understanding of the role of ACE shedding in health and disease [1,2]. Extensive biochemical and molecular analysis has helped to elucidate: how the ACE sheddase recognises its target protein, where in the target protein shedding occurs, how the process is regulated, and the identity of the ACE sheddase(s) [3,4]. These findings point to the potential therapeutic role of targeting shedding in regulating tissue ACE levels in cardiovascular and other diseases where ACE is implicated in the aetiology.

Somatic ACE (sACE) is comprised of two domains (N and C) and a single C domain variant is cleaved at a much faster rate than sACE [5]. In addition, testis ACE is cleaved 2–3 fold more rapidly than sACE albeit at the same site in the stalk [6]. This points towards the N domain of sACE negatively regulating shedding. However, a mutant where the N domain was fused to the stalk and anchored in

Abbreviations: ACE, angiotensin-converting enzyme; sACE, somatic ACE; tACE, testis ACE; TNF-α protease inhibitor, TAPI; phorbol 12,13-dibutyrate, PDBu; phenylmethylsulphonyl fluoride, PMSF.

* Corresponding author. Department of Integrative Biomedical Sciences, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory 7925, Cape Town, South Africa. Tel.: +27 21 406 6312; fax: +27 21 406 6479.
E-mail address: Edward.Sturrock@uct.ac.za (E.D. Sturrock).
[1] Present address: Department of Human Biology, Faculty of Health Sciences, University of Cape Town, South Africa.

http://dx.doi.org/10.1016/j.bbrc.2016.10.155
0006-291X/

the membrane was very poorly shed and suggested that the mere presence of a single domain was not sufficient for cleavage off the membrane [7,8]. This indicated that the C domain may have a recognition motif that interacts with the sheddase and regulates ectodomain cleavage. Domain swop-over mutants reinforced the notion of a sheddase recognition motif within the ectodomain of the C domain as the double C domain mutant was cleaved within the linker region as well as at the stalk, which does not occur in sACE [9]. This, taken together with the fact that sACE is shed less efficiently than testis ACE (tACE), suggests that the recognition motif may be occluded in the C domain of sACE.

This was proposed by Chattopadhyay et al. who hypothesised that a 5-residue motif in the ectodomain directly proximal to the cleavage site alters shedding of rabbit tACE [10]. The proximal ectodomain is followed by the stalk region which starts at residue W^{616} according to Chubb et al., [11]. It is 17 residues N-terminal of the cleavage site, 13 residues N-terminal to P^{623} and 10 residues N-terminal to the seventh potential glycosylation site of the C domain. Therefore, the region directly proximal to the cleavage site has many important hallmarks.

The plasma ACE Pro1199Leu polymorphism observed by Kramer et al. [12] was associated with a 5-fold increase in shedding. Eyries et al. [2] conducted further investigations into this polymorphism by generating a sACE $P^{1199}L$ variant as well as a C domain variant ($P^{623}L$ - tACE numbering). In both CHO-K1 and COS cell lines the $P^{1199}L$ mutant was shed 1.5-fold more efficiently than wild-type. Thus, the introduction of the proline has a significant effect on shedding. Based on a 2D structural analysis, it was suggested that proline induces conformational constraints by forming twists in the structure. Leucine, on the other hand, is more flexible allowing for better access by the sheddase. This plasma ACE polymorphism showed that the stalk region was more important for cleavage secretion than the cleavage site *per se*. The mutation of P^{623} could also allow glycosylation of the normally unglycosylated N^{620} [13]. An introduction of a glycan in this region could re-direct the sheddase to a more favourable position and thus increase shedding, as shown when an O-glycosylated region was introduced into the stalk region [4].

In this study the effects of distal ectodomain chimeric mutants of helix 7 and helix 8, and A^{261} QH on ACE shedding and activity were investigated. Furthermore, the importance of the ectodomain proximal to the cleavage site was studied by using a series of stalk alanine mutants and a $P^{623}L$ tACE mutant.

2. Materials and methods

2.1. Construction of tACE mutants

The construction of the ACE mutant constructs pcDNA tACE H7, pcDNA tACE H8 and pcDNA tACE AQH was described before in Balyasnikova et al. [14]. tACE Ala610-614 was synthesised using the tACE ALA For and Rev primers (5'-GAGAACGAGCT***AGC***TG***C***GG***C***-G***GC***GGCGGGCTGGCC-3', 5'-GGCCAGCCCGCCGCCGCCGCAGCTAG-CTCGTTCTC-3'). Ndom 608-614 was produced with the tACE ST Ndom F and tACE ST Ndom R primers (5'-CTCCGCACGGA-GAAC***C***A***A***C***A***G***A***ATGGGGAAGTACTGGGCTGGCCGC-3', 5'-GCGGCCA-GCCCAGTACTTCCCCATTCTGTTGGTTCTCCGTGCGGAG-3') and tACE Pro623Leu were synthesised with the P2LF and P2LR primers (5'-GCAGTACAACTGGACGC***T***GAAT***T***CCGCTCGCTCC-3', 5'-GGAGCG-AGCGGAATTCAGCGTCCAGTTGTACTGC-3'). The template DNA for these mutants were wild-type tACE. tACE Ala 608-614 was produced with the tACE ala2F and tACE ala2R primers (5'-CTCCGCACGGAGAA***T***G***CTGC***AGCTGCGGCGGCGGCG-3', 5'-CGC-CGCCGCCGCAGCTGCAGCATTCTCCGTGCGGAG-3'). The DNA template used for this mutant was the pGEM tACE Ala610-614 mutant so that an additional 2 alanine residues were added upstream of residues 608 and 609. tACE NP/DL was produced with the primers P2LDF and P2LDR (5-CCGCAGTAC***G***ACTGGACGCTGAAT***AG***CGCT CGCTCAG-3', 5'-CTGAGCGAGCGCTATTCAGCGTCCAGTCGTACTGC-GG-3') using pGEM tACE Pro623Leu as the DNA template to produce a double mutant containing leucine at position 623 (L^{623}) and convert the asparagine 620 (N^{620}) to aspartate to knock out the glycosylation sequon.

2.2. Expression of ACE constructs in CHO-K1 cells

All constructs were transfected into CHO-K1 cells via the calcium phosphate method using the Pro®Fection Mammalian Transfection System (Promega, USA). Shedding assays were conducted by growing CHO-K1 cells expressing tACE proteins to confluence in a 6-well plate. The complete growth medium was removed, cells were washed with phosphate-buffered saline (PBS) and replaced with 500 μl minimal medium Optimem (Gibco), supplemented either with 1 μM of phorbol 12, 13-dibutyrate (PDBu) or 50 μM TAPI (Peptides International). Cell lysates were harvested by adding 500 μl of triton lysis buffer [1% Triton X-100, 50 mM Hepes pH 7.5, 0.5 M NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF)] to each well. The assay was conducted over 4 h. The percentage ACE shed was calculated as the ratio of total ACE activity in the medium to total ACE activity in the medium and cell lysate.

2.3. Western blot analysis

Medium and cell lysate samples underwent western blot analysis as previously described [15,16].

2.4. ACE activity assays

ACE activity assays were performed as described in Schwager et al. [17]. Protein purification tACE Pro623Leu protein was purified using Lisinopril-sepharose column. The purified protein was subjected to PNGaseF digest and samples were prepared for MALDI MS according to [18].

3. Results

3.1. Effect of distal ectodomain motifs on ACE shedding

It has been previously shown that regions in the ectodomain likely act as a sheddase recognition motif [19,20]. Fig. 1A shows the surface representation of tACE crystal structure with the distal ectodomain mutants tACE H7, tACE H8 and tACE AQH. Ectodomain shedding of tACE H7, tACE H8 and tACE A261QH was analysed by ACE activity assays (Fig. 1B) in both media and cell lysates. All three constructs were shed under basal conditions with similar efficiency to wild-type tACE. Moreover, they showed an increase in shedding on addition of phorbol ester (1 μM) and a decrease in shedding in the presence of the hydroxamate inhibitor TAPI (50 μM).

3.2. The proximal ectodomain region of tACE does not affect shedding

To analyse the effect of the proximal ectodomain and stalk region on shedding, we substituted residues with alanines (Fig. 2A). When the 5-residue motif (H^{610}-L^{614}) in human tACE was replaced with alanines (tACEAla 610-614) there was 6-fold a reduction in soluble tACE activity in the medium. N-terminal extension of the mutation by 2 residues (tACEAla 608-614) led to a further decrease

Please cite this article in press as: N. Conrad, et al., The effect of structural motifs on the ectodomain shedding of human angiotensin-converting enzyme, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.10.155