Biochemistry and Biophysics Reports 5 (2016) 105–110

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

Human carboxylesterase 2: Studies on the role of glycosylation for enzymatic activity

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ARTICLE INFO

Article history:
Received 11 September 2015
Received in revised form
16 November 2015
Accepted 18 November 2015
Available online 19 November 2015

Keywords:
Carboxylesterase
Deglycosylation
Glycosylation
hCES
Site directed mutagenesis

ABSTRACT

Human carboxylesterase 2 (hCES2) is a glycoprotein involved in the metabolism of drugs and several environmental xenobiotics, whose crystallization has been proved to be a challenging task. This limitation could partly be due to glycosylation heterogeneity and has delayed the disclosure of the 3D structure of hCES2 which would be of upmost relevance for the development of new substrates and inhibitors. The present work evaluated the involvement of glycans in hCES2 activity and thermo stability in an attempt to find alternative active forms of the enzyme that might be adequate for structure elucidation.

Partial or non-glycosylated forms of a secreted form of hCES2 have been obtained by three approaches: (i) enzymatic deglycosylation with peptide N-glycosidase F; (ii) incubation with the inhibitor tunicamycin; ii) site directed mutagenesis of each or both N-glycosylation sites.

Deglycosylated protein did not show a detectable decrease in enzyme activity. On the other hand, tunicamycin led to decreased levels of secreted hCES2 but the enzyme was still active. In agreement, mutation of each and both N-glycosylation sites led to decreased levels of secreted active hCES2. However, the thermostability of the glycosylation mutants was decreased.

The results indicated that glycans are involved, to some extent in protein folding in vivo, however, removal of glycans does not abrogate the activity of secreted hCES2.

1. Introduction

Carboxylesterases (CES) are a subset of esterases that belong to the α/β hydrolase family [35]. These enzymes catalyze the hydrolysis of esters, thioesters and amides, freeing the respective carboxylic acid and alcohol [14]. Carboxylesterases are responsible for detoxifying exogenous substrates, like pesticides and other environmental toxicants and are also involved in metabolizing drugs and/or activating pro-drugs [24]. In fact, after oxidative enzymes such as cytochrome (CYP) P450 enzymes and UDP-glucuronosyltransferases (UGTs), esterases are the third major class of enzymes involved in the metabolic clearance of currently administered therapeutic drugs [13,34]. Specific inhibitors for CESs have been reported, such as bis-*p*-nitrophenyl phosphate (BNPP) and benzyl [29,39]. Due to their potential pharmacological application, the search for individual CESs specific inhibitors is, in fact, an active research field [20,28,32,40,41,5,7,8].

hCES have different tissue distribution, localization within the cell and substrate preferences [14] with some overlapping. Moreover they have different genetic variants resulting from alternative splicing, single nucleotide polymorphisms (SNPs) and multiple copy variants that have been shown to influence drug metabolism and clinical outcomes [16,23]. Human CES enzymes encompass a signal peptide of approximately 17 to 22 aminoacid residues responsible for their targeting to the endoplasmic reticulum (ER) where they are retained due to a carboxy-terminal ER retention signal – the H-X-E-L (Histidine – X – Glutamic Acid – Leucine) consensus sequence that interacts with the KDEL (Lysine – Aspartic Acid – Glutamic Acid – Leucine) receptor present in the luminal side of the ER [26]. This is in accordance with the absence of CES from human plasma, contrary to what happens in most rodents [1,25].

Three main human carboxylesterases have been most studied

Abbreviations: CES, carboxylesterases; hCES, human carboxylesterases

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http://dx.doi.org/10.1016/j.bbrep.2015.11.018

among the five carboxylesterases listed in the Human Genome Organization database: hCES1, hCES2 and hCES3 [23]. Human CES1 has traditionally been the most studied hCES, however, increased attention has been devoted to hCES2 due to its role in the activation of prodrugs, its potential application in prodrug-activating gene therapies [30,38] and also its involvement in pre-systemic metabolism of orally administered drugs. Human CES2 is found in different tissues like the liver and kidney being in fact the major intestinal carboxylesterase [6] and it has been reported to be downregulated in some cancers [15] while overexpressed in others [35].

Contrary to hCES1, which has a substrate preference for esters with small alcohol group, hCES2 hydrolyzes preferentially substrates with a large alcohol group and a small acyl group as is the case of irinotecan [11], some angiotensin receptor blockers as well as aspirin, having a consequent effect in their bioavailability [22,27].

While hCES1 is so far the only human CES form that has a fully known structure [2], the tertiary structure of hCES2 has been elusive to many scientists for more than a decade. hCES2 has two potential N-glycosylation sites, at Asn103 and Asn267 [26]. The purified native human enzyme has been shown to be sensitive to endoglycosidase H (Endo H), which hydrolyzes Asn-linked high mannose oligosaccharides [21]. On the other hand, recombinant hCES2 from HEK-293T cells has been efficiently deglycosylated with PNGase F but not Endo H, indicating that it was further modified at the Golgi apparatus [12]. However, taking into account that the efficiency of glycosylation can be affected by conformational limitations and by the acidicity of "X" residue (any amino acid except proline) in the Asn-X-Ser/Thr sequons where N-glycosylation may occur [31], the number of occupied N-glycosylation sites has not in fact been confirmed. Accordingly, for rat carboxylesterases, the intracellular form is sensitive to Endo H whereas the secreted form is only sensitive to PNGase F [17].

The effect of glycosylation on hCES2 activity is not known and the data available on other carboxylesterases is not always consensual. For example, Hydrolase C, a rat carboxylesterase, has been shown to be inactive when expressed in *E. coli*, a prokaryotic organism, but active when expressed in the baculovirus/insect cell (*Spodoptera frugiperda* 21 – Sf21) system [36,37]. In addition, the expression of a recombinant rabbit liver carboxylesterase in *E. coli* yielded an inactive protein which was attributed to the lack of N-glycosylation, whereas an active protein was obtained from yeast cell expression system (*Pichia pastoris*), capable of N-glycosylation [18]. However, despite previous claims that, for full activity of recombinant hCES1, expressed in the baculovirus-insect cell system, proper glycosylation (especially N-linked glycosylation) was necessary [10], difficulties in obtaining an active form of hCES-1 from *E. coli* have recently been attributed to issues related to the protein being expressed in inclusion bodies and with low purity [3]. The authors have refolded the enzyme via buffer exchange and obtained an active form, thus questioning previous claims on the need of proper glycosylation for production of a recombinant active form. Additionally, [17] found that native deglycosylation of rat carboxylesterases (hydrolases A and B), purified from rat liver microsomes, has no effect on enzyme activity. The authors hypothesized that these data may point towards a higher relevance of glycosylation for protein stability and solubilization and not as much for enzymatic activity of mature proteins.

The 3D structure of hCES2 has not been deciphered yet, mostly because its crystallization has been a challenging task. Glycoproteins are many times difficult to crystallize due to the heterogeneity of N-glycan structures and conformations at the surface of proteins and therefore we considered the production of non-glycosylated forms of hCES2in order to obtain alternatives for crystallization and diffraction studies. However, since these new forms would only be useful for structural studies if they remained active, the main goal of the present work was to evaluate the impact of glycosylation in hCES2 activity. For that purpose, several strategies [4] were tested to prevent or remove glycosylation, and test the impact on enzyme activity and stability, namely: recombinant hCES2, secreted from HEK-293T cells [12], was enzymatically deglycosylated (1); N-glycosylation was inhibited during production (2) or the N-glycosylation sites were mutated (3).

2. Results and discussion

2.1. Effect of enzymatic degycosylation on enzyme activity

In order to test if N-glycosylation was relevant for enzyme activity, the supernatant of HEK293T cells transfected with pCI-neo hCES2-10xHis [12] was subjected to enzymatic deglycosylation with PNGase F under denaturing and native conditions. In both conditions, a downward shift in molecular weight of under 10 kDa was observed by Western Blot (Fig. 1A). In native conditions the results indicated incomplete digestion that could not be improved with a longer incubation period, or by increasing the amount of enzyme. This can be explained by limited accessibility of the enzyme to one of the N-glycosylation sites. Deglycosylation of purified hCES2-10xHis [12] showed a decreased level of detection using the glycoprotein detection method ProQ Emerald 300 with the deglycosylated bands only being faintly detected probably due to non-specific binding or to O-glycosylation (Fig. 1B).

Enzyme activity was assessed on supernatants subjected to native deglycosylation, (PNGase+) and compared with the control (PNGase-) as described in the Materials and Methods section. No evidence of decay in total hydrolytic activity was observed as specific activities were respectively 93.9 ± 3.7 pM s^{-1} μg^{-1} (PNGase+) and 88.9 ± 6.4 pM s^{-1} μg^{-1} for PNGase- and PNGase+ samples, respectively. It can therefore be concluded that the presence of N-glycans in the mature form of the protein – fully processed through the cell machinery – is not essential for protein activity.

These results are in accordance with the observations of [17] which refered that rat liver hydrolase A and B remained active after enzymatic deglycosylation.

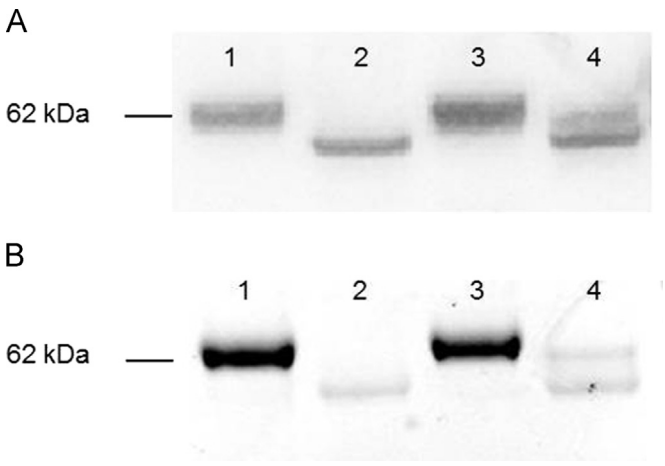

Fig. 1. Enzymatic deglycosylation of hCES2-10xHis with PNGase F. (A) Western Blot demonstrating size reduction upon denaturing and native deglycosylation of supernatants (25 μg of total protein per well). (B) Denaturing and native deglycosylation of purified hCES2-10xHis and staining with ProQ Emerald 300 (5 μg of total protein per well). Lanes: 1 – hCES2-10xHis under denaturing conditions, (PNGase F -); 2 – hCES2-10xHis under denaturing conditions (PNGase F +); 3 – hCES2-10xHis under native conditions (PNGase F -); 4 –hCES2-10xHis under native conditions deglycosylated (PNGase F +).

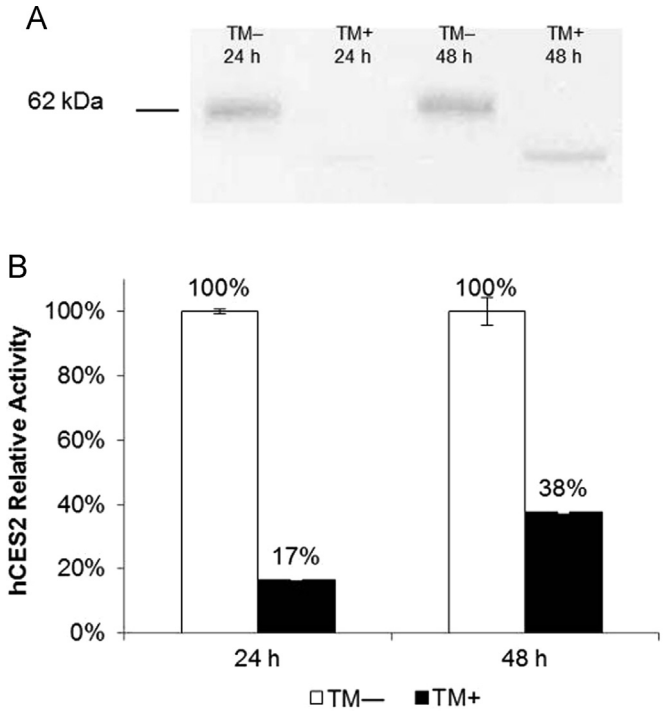

Fig. 2. Supernatant from cell cultures treated with tunicamycin (TM+) or DMSO (TM−) were harvested at 24 and 48 h. (A) Western Blot of the supernatants, 25 μg of total protein per well; (B) Relative activity of hCES2-10xHis in the presence or absence of tunicamycin. Results are the average of three determinations, error bars represent the standard deviation ($n=3$).

2.2. Effect of inhibition of N-glycosylation with tunicamycin

As previously referred, different authors have presented contradictory results relative to the effect of glycosylation in carboxylesterase activity [19,3,4,9], and therefore we hypothesized that, although the removal of glycans post synthesis might not affect activity, the presence of glycans during protein synthesis could be necessary for the correct folding of the enzyme. In order to test this hypothesis, hCES2-10xHis expression was achieved in the presence of tunicamycin, an inhibitor of the formation of the N-glycan precursor [10,31].

As expected, this process resulted in the production of a lighter form of the protein, as confirmed by Western Blot, which is consistent with the absence of glycosylated residues (Fig. 2A). An accentuated decrease in hCES2-10xHis expression is also apparent from relative band intensity (2:100 after 24 h and 8:100 after 48 h) in the presence (TM+) and absence (TM−)of tunicamycin, despite similar cell viability (the use of a housekeeping control, for normalization purposes, was not possible since analyses were performed in supernatants). Such decay is not likely to be due to reduced antibody affinity towards the non glycosylated form since this has not been observed when deglycosylation was achieved through enzyme hydrolysis (Fig. 1A).

The lower levels of protein observed in the presence of tunicamycin might be due to a folding limitation for the non-glycosylated protein with subsequent degradation due to the quality control mechanism of glycoproteins involving calnexin in the endoplasmic reticulum [33] since we observed that tunicamycin did not affect cell viability or total protein concentration. The lower levels detected in the supernatant may also be due to intracellular accumulation of the non-glycosylated protein. Further experiments are required to clarify this issue.

Accordingly, supernatants from TM+ cultures had significantly lower hydrolytic activity towards 4-MUBA than those of the control cultures. However, taking into account that fainter bands were observed for the TM+ cultures, the observed reduction in 4-MUBA hydrolysis is more likely to be due to the lower amount of hCES2-10His expressed in the presence of tunicamycin rather than from an effective decrease in enzyme catalytic ability. Moreover, the reduction in activity is not as accentuated as the decay in band intensity. Therefore it is possible to claim that hCES2 does not fully lose its catalytic activity even when glycosylation is prevented at early stages of protein production (Fig. 2B). These results are in accordance with results from other authors that demonstrated to be possible to produce an active recombinant non-glycosylated hCES1 [3].

2.3. Mutation of N-glycosylation sites of hCES2

In order to fully confirm the number of glycosylation sites, as well as the individual relevance of each site for activity, glycosylation was prevented through a different approach that consisted in using site directed mutagenesis to replace the asparagine residues in the Asn-X-Ser/Thr sequons, by glutamine thus precluding binding of the sugar moieties to those putative sites.

For that purpose, each or the two potential N-glycosylation sites were mutated resulting in three mutated forms of the protein which lacked either the first (p.Asn175Gln, Glyco1), the second (p.Asn340Gln, Glyco2) or both (p.Asn175Gln/Asn340Gln, Glyco1+2) glycosylation sites (Fig. 3).

Through Western Blot it was possible to confirm a decrease in the molecular weight of the mutant forms present in the cell culture supernatants in comparison with the positive control (fully glycosylated hCES2-10xHis); as expected the difference in weight was larger in the case of the double mutant (Fig. 4A) and consistent with the decrease observed upon culturing in the presence of tunicamycin. These results unequivocally confirmed that the two previously described putative glycosylation sites of hCES2 [26] are in fact glycosylated.

Relative expression of hCES2-10xHis also decreased in the case of the mutants according to relative band intensities (Fig. 4A): 100%, 73%, 66% and 22% respectively for PC, Glyco2, Glyco1 and Glyco1+2. These results are in agreement with those obtained for protein expression in presence of tunicamycin. In this case it could also be due to folding impairment in the absence of the glycans that lead to protein degradation in context of the quality control mechanism associated with calnexin in the endoplasmic reticulum, or it could be due to intracellular accumulation of the protein.

The supernatants containing the mutated forms of hCES2-10xHis were tested for enzymatic activity (normalized to total

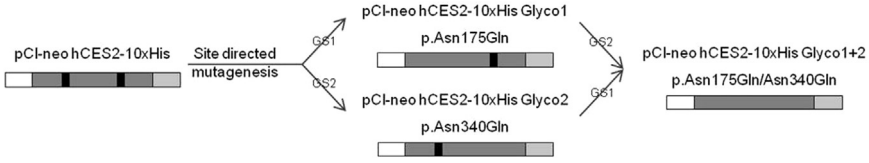

Fig. 3. Schematic representation of pCI-neo hCES2-10xHis mutant plasmids lacking one (p.Asn175Gln, p.Asn340Gln) or two (p.Asn175Gln/Asn340Gln) glycosylation sites. The coding sequence of each plasmid contained a signal peptide (white rectangle) and the 10xHis tag (light gray rectangle). Glycosylation sites are represented by the black rectangles.

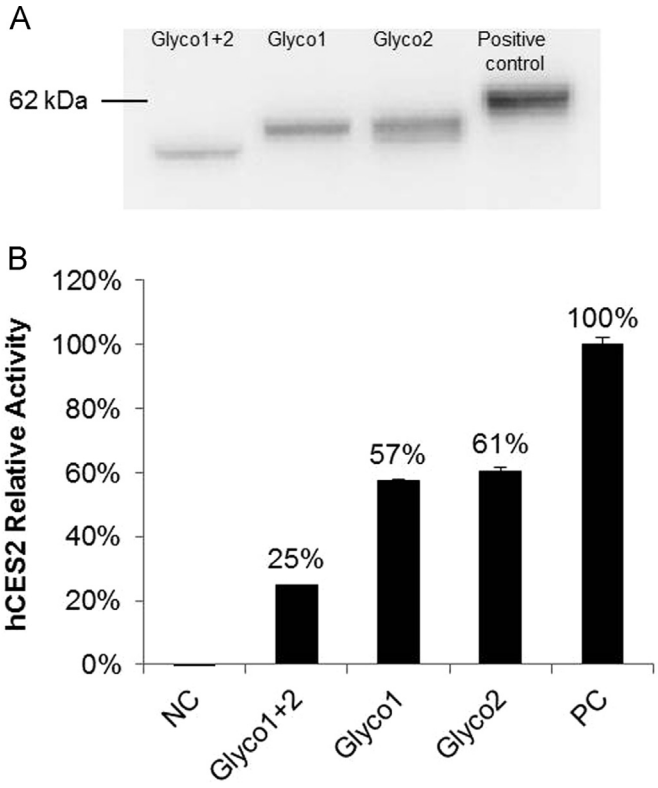

Fig. 4. Glycosylation site mutants and positive control (fully glycosylated hCES2-10xHis) present in cell culture supernatants were analyzed. (A) Western blot analysis, 25 μg of total protein per well. (B) Relative activity of the mutants comparing to the positive control. Results are the average of three determinations; error bars represent the standard deviation of the triplicates. PC – positive control, hCES2-10xHis; Glyco 1 – hCES2-10xHis lacking the first glycosylation site; Glyco 2 – hCES2-10xHis lacking the second glycosylation site; Glyco 1+2 – hCES2-10xHis lacking both glycosylation sites; NC – negative control, supernatant of non transfected cells.

protein present). Wild type hCES2-10xHis demonstrated the highest specific activity toward 4-MUBA, while Glyco 1 and Glyco 2 retained approximately 60% of the control activity and Glyco 1+2 dropped to 25% of activity (Fig. 4B).

These results thus indicate that the loss in the relative activity of the supernatants is more likely due to lower expression of the mutants than to loss of catalytic ability of the protein upon loss of glycosylation. This conclusion contradicts the observation of other authors who have not been able to obtain active carboxylesterases (rat and rabbit) upon expression in prokaryotic systems [18,36,37]) but are in agreement with recent studies demonstrating that it is possible to obtain active hCES-1 from *E. coli* upon post purification refolding, despite the lack of glycosylation machinery in prokaryotes [3].

2.4. Thermal stability of N-glycosylation mutants

As previously referred, the tridimensional structure of hCES2 has not yet been determined. According to our experience this task has not been achieved due to difficulties in obtaining crystals of adequate size and stability for allowing diffraction studies (results not shown) which may be related to enzyme stability. Considering that conformation of the protein, its stability and crystallizing aptitude may be influenced by the glycosylation profile, thermal stability of the glycosylated protein and of the mutants was evaluated by quantifying the activity of the supernatants subjected to a temperature gradient (Fig. 5).

Stability of the mutants subjected to higher temperatures, in comparison with the respective control (activity observed at

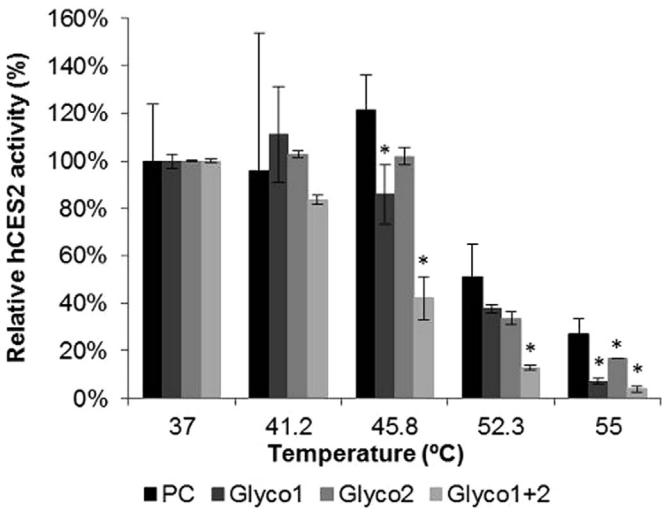

Fig. 5. Relative activity of glycosylation site mutants and of fully glycosylated hCES2-10xHis (PC) after being subjected to a temperature gradient (normalized to the activity observed at 37 °C in each case). Results are average of three determinations; error bars represent the standard deviation of the triplicates. PC – positive control, hCES2-10xHis; Glyco 1 – hCES2-10xHis lacking the first glycosylation site; Glyco 2 – hCES2-10xHis lacking the second glycosylation site; Glyco 1+2 – hCES2-10xHis lacking both glycosylation sites. * Indicates a statistically significant difference ($P < 0.05$) in activity observed in the mutants in relation to the activity of the PC at the same temperature.

37 °C), was not significantly affected at 41.2 °C. However, higher temperatures promoted a relative decrease in activity that was more accentuated in the case of the double mutant.

3. Conclusions

In conclusion, N-glycosylation of hCES2 is relevant for thermostability of the enzyme and also for its in vivo folding or secretion, but is not crucial for enzyme activity. Since the partial or non-glycosylated mutants are still secreted to some extent in an active form they constitute promising candidates for future structural studies.

4. Materials and methods

4.1. Cell culture and transfection

Suspension adapted Human embryonic kidney cells (HEK-293T; ATCC CRL-11268) were cultured using Frestyle 293 medium (Gibco, Life Technologies; USA). The cells were sub-cultured twice a week using an inoculum of 0.3×10^6 cells/mL and incubated in a humidified atmosphere of 8% CO_2 at 37 °C, with orbital agitation at 130 rpm.

To determine cell viability and concentration the Trypan Blue exclusion method was used; the cellular suspension samples were diluted in 0.1% Trypan Blue (Gibco, Life Technologies; USA) solution prepared in PBS (Gibco, Life Technologies; USA) and the cells were counted twice on a Fuchs-Rosenthal haemacytometer (Marienfeld; Lauda-Konigshofen, Germany).

The pCIneo hCES2-10xHis mammalian expression vector, was generated as previously described [12]. Briefly, human recombinant *CES2* gene synthetically synthesized with a C-terminal 10xHis tag (GeneArt; Regensburg, Germany) was cloned in pCI-neo plasmid (Promega; Madison, USA.) using *SalI* and *NotI* restriction endonucleases (New England Biolabs; Ipswich, USA). For the formation of a full transcript containing the C-terminal histidine tag,

the stop codon of *CES2* gene was deleted.

Transient transfection of HEK-293T was performed using polyethylenimine (PEI, Polysciences; Eppelheim, Germany). A transfection solution of 1 mL containing fresh medium and plasmid DNA:PEI at a ratio of 1:3, in which DNA concentration was 5 μg/mL, was vortexed, incubated for 10 minutes (min) at room temperature and added dropwise to the cell culture. Cell growth and viability was followed throughout a time period of 72 h (hours). The cell culture was then centrifuged for 10 min at 300 × *g*, and the supernatant was harvested.

Quantification of total protein in cell culture supernatant was performed using the Bradford Assay in triplicate according to the manufacturer's instructions in a 96 well plate with Coomassie Brilliant Blue G-250 (Bio-Rad, USA).

4.2. SDS-PAGE and Western Blot

Ice cold ethanol was added to the samples (100 μg of total protein) in a ratio of 1:4 and incubated for 2 h at −20 °C, for protein precipitation. Precipitated protein was resuspended in Sample Buffer and Reducing Agent (20 μL) and heated to 70 ºC for 20 min. 10 μL of each sample (50 μg of total protein) were loaded onto the wells of a NuPAGE BisTris 4–12% (w/v) acrylamide gel, using the X-Cell Surelock mini cell system and MES (2-(*N*-morpholino)ethanesulfonic acid) Running Buffer. Electrophoresis ran for 40 min at 200 V. All of the reagents and materials used were from Life Technologies, USA.

The gel was transferred onto a PVDF (polyvinylidene difluoride) or nitrocellulose membrane, using the Transblot Turbo Semi Dry Transfer System (Bio-Rad, USA) for 25 min at 25 V. The membrane was blocked in 5% (w/v) milk (skim milk for microbiology, Merck, USA) in 0.05% (v/v) Tween 20 (Merck, USA)/Tris Buffered Saline (TBS, Sigma-Aldrich, USA) overnight. The membrane was incubated for 1 h with the goat anti-hCES2 primary antibody (R&D Systems, USA) diluted 1:200, followed by 1 h in secondary antibody, HRP-mouse anti-goat, diluted 1:5000. The antibodies were diluted in 1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich, USA) in 0.05% (v/v) Tween 20/TBS solution. Washings were done with Tween/TBS. Detection was by chemilluminescence using ECL Amersham (GE Life Sciences, UK), and images were collected using ChemiDoc (Bio-Rad, USA).

4.3. Enzymatic deglycosylation through digestion with PNGase F

HEK-293T transfected cell supernatants containing hCES2 10xHis were concentrated using Amicon Ultra-15, PLTK Ultracel-PL Membrane, 30 kDa (Merck Milipore, USA) and quantified for total protein concentration.

Glycoproteins from concentrated supernatants were deglycosylated using peptide N-glycosidase F (PNGase F; ProZyme, USA). The reactions were performed under two different sets of conditions: the native reaction (1) consisted in adding 50 mU of enzyme (PNGaseF) to 100 μg of sample in the reaction buffer, while the denaturing reaction (2) required heating at 100 °C for protein denaturing, prior to adding 10 mU of PNGaseF. Both reactions were incubated overnight at 37 °C. Deglycosylation was monitored by SDS-PAGE and Western Blot.

Purified hCES2-10xHis [12]) was also subjected to deglycosylation with PNGase F and deglycosylation was monitored by SDS-PAGE, followed by staining ProQ Emerald 300 (Life Technologies, USA) that stains glycosylated proteins.

4.4. Enzymatic inhibition of N-glycosylation

In order to produce a non-glycosylated form of hCES2-10xHis, HEK293T cells transfected with pCI-neo hCES2-10 × His were incubated for 4 h at 37 °C, 8% CO2 and 130 rpm orbital agitation, after which the cell culture was split and centrifuged (200 × *g*, 10 min). The supernatant was then replaced with fresh medium containing either 2 μg/mL of tunicamycin solubilized in dimethylsulfoxide (DMSO) or, as control, the same volume of DMSO. The cell cultures were again incubated for 48 h under the same conditions, and cell viability was assessed at several time points. Total protein concentration and esterase activity were determined as previously described.

4.5. Mutation of N-glycosylations sites

For confirmation of the putative N-glycosylation sites, the asparagine residue from the Asn-X-Ser/Thr sequons in pCIneo hCES2-10xHis mammalian expression vector were substituted by a glutamine using the GeneArt Mutagenesis Kit (Life Technologies, USA, ref. A13282) according to the manufacturer's instructions. These mutations preclude N-glycosylation.

Two pairs of primers were designed, one pair for each glycosylation site, resulting in two single mutants: N175Q and N340Q. A double mutant plasmid, N175Q/N340Q, was also obtained through two consecutive mutagenesis reactions, one for each set of primers (Table 1). Mutations were confirmed by DNA sequencing.

HEK-293T cells were transfected with these plasmids as previously described. The supernatants were quantified for total protein and tested for ester hydrolysis activity.

4.6. Enzyme activity assays

For the evaluation of enzymatic activity of the expressed enzymes the hydrolysis of 4-methylumbelliferyl acetate (4-MUBA) to 4-methylumbelliferone (4-MUB) was measured by spectrofluorimetry (FL800 Bio-Tek Instruments, Winooski, VT, USA). Every assay, including controls, was performed in triplicate, and results are presented as the average ± standard deviation. Correction for non-enzymatic hydrolysis was performed.

The substrate stock solution, 15 mM 4-MUBA (Sigma-Aldrich, USA), was prepared in DMSO (Sigma-Aldrich, USA). This solution was diluted to 7.5 mM in a 1:1 mixture of Hank's Balanced Salt Solution (HBSS, Life Technologies, USA)/DMSO. For determination of total esterase activity, 5 mM of substrate was added to the sample diluted in HBSS to a final reaction volume of 250 μL. Reactions were followed at 37 °C, at λ_{ex}=350 and λ_{em}=450 nm for 15 min, in 50 s intervals and enzymatic activity was determined from the linear portion of the curve. A stock solution of 4-MUB (Sigma-Aldrich, USA) 15 mM was also prepared in 5 mL DMSO and then diluted in HBSS, to prepare the calibration curve in the range 0.002–0.075 mM .

For the determination of specific hCES2 activities, inhibition assays were performed, in the presence of 0.025 mM loperamide (Sigma-Aldrich, USA), selective inhibitor of hCES2 [9], while for determination of specific CES activities assays were performed in the presence of 0.5 mM of Bis(4-nitrophenyl) phosphate (BNPP, Sigma-Aldrich, USA), which is a CES inhibitor [19]. The inhibitors were added prior to substrate addition. Final DMSO concentration

Table 1
Primers designed for the site directed mutagenesis reactions. T_m: melting temperature; GC – Guanine Cytosine Content. GS1 pair of primers was used to mutate the first, while the GS2 was used to mutate the second glycosylation site.

Name	Sequence (5′–3′)
GS1 Fw	TAGCCAGTTCCAGATGACCTTCCCTTCCGACTCCATGTCT
GS1 Rv	GGAAGGTCATCTGGAACTGGCTAAGAAACTCTGACTCCACT
GS2 Fw	GGTGGTGGCCCAGCTGTCTGCCTGTGACCAAGTTG
GS2 Rv	ACACCGAGACAGCTGGGCCACCACCGTGGAGATGACA

in the reaction mixture never exceeded 1% (v/v) in order not to affect hCES catalytic activity [33].

4.7. Thermal stability

Supernatant samples were subjected to a non-linear temperature gradient using a BioRad IQ Cycler IQ5 (Bio-Rad, USA) for 3 min. After incubation the samples were centrifuged at 13,000 rpm, 4 °C for 15 min. The pellets, which contained denatured protein, were discarded, and the supernatants were tested for enzymatic activity as described above.

Acknowledgment

This work was funded by Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/44025/2008, PTDC/EBB-BIO/111530/2009 and PEst-OE/EQB/LA0004/2013).

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