Biochemical Pharmacology 87 (2014) 352–359

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

N-Glycosylation during translation is essential for human arylacetamide deacetylase enzyme activity

Kyotaka Muta, Tatsuki Fukami *, Miki Nakajima, Tsuyoshi Yokoi

Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma-machi, Kanazawa, Japan

ARTICLE INFO

Article history:
Received 10 August 2013
Accepted 3 October 2013
Available online 11 October 2013

Keywords:
Arylacetamide deacetylase
N-Glycosylation
Enzyme activity
Protein folding
Phenacetin

ABSTRACT

Human arylacetamide deacetylase (AADAC) can hydrolyze clinical drugs such as flutamide, phenacetin, and rifamycins. AADAC is a glycoprotein, but the role of glycosylation remains unclear. In the present study, we investigated the effect of glycosylation on AADAC enzyme activity. Immunoblot analysis of mutant AADACs that contained an asparagine (N, Asn) to glutamine (Q, Gln) substitution at either residue 78 or 282 (N78Q or N282Q) showed a different migration compared with the wild-type protein. A mutant AADAC that contained N to Q substitutions at both residue 78 and 282 (N78Q/N282Q) showed a similar migration to AADAC in human liver microsomes (HLM) treated with endoglycosidase H (Endo H), which produces deglycosylated proteins. This result indicated that AADAC was glycosylated at both N78 and N282. Mutant types of AADAC with the N282Q and the N78Q/N282Q substitutions showed dramatically lower phenacetin hydrolase activity than did the wild-type protein. The treatment of wild-type AADAC-expressing HuH-7 cells with tunicamycin, which produces unglycosylated protein, decreased AADAC enzyme activity. However, the treatment of the HLM with Endo H caused no decrease of AADAC activity. Thus, the oligosaccharide chain, per se, was not important for AADAC activity in the mature form. The mutant types of AADAC containing the N282Q and the N78Q/N282Q substitutions were not detected by immunoblotting analysis after non-reducing SDS-PAGE, suggesting that the glycosylation of AADAC at N282 was important for proper protein folding. Overall, this study found that the translational, but not post-translational, *N*-glycosylation of AADAC plays a crucial role in regulating AADAC enzyme activity.

1. Introduction

Post-translational modifications, such as phosphorylation, acetylation, methylation, and ubiqutination [1], are covalent processing events that change the properties of a protein. Glycosylation is also a common and highly diverse co- and post-translational modification reaction, and more than 50% of all proteins in humans are glycosylated [2,3]. Protein glycosylation can be categorized into two types: *O*-linked and *N*-linked. *N*-linked glycans are added to asparagine (N, Asn) residues at the consensus amino acid sequence, Asn-X-(Ser/Thr), where X is any amino acid except for proline. *N*-Glycosylation plays crucial roles in various cellular processes including protein folding, protein stabilization, substrate recognition, protein secretion, and intracellular trafficking [4].

The role of glycosylation of some drug-metabolizing enzymes has been elucidated. Kroetz et al. [5] reported that carboxylesterase 1 (CES1), which is a major hydrolase in the human liver, is glycosylated, and the treatment of CES1-exspressing *Spodoptera frugiperda* Sf9 cells with tunicamycin, an inhibitor of oligosaccharyl-transferase (OST), showed a decrease in *p*-nitrophenylacetate hydrolase activity. In addition, our previous study showed that the tunicamycin treatment of UDP-glucuronosyltransferase (UGT) 1A9-expressing HEK293 cell eliminated the 4-methylumbeliferon (4-MU) glucuronidation activity, and the substitution of the N71 and N344 residues with glutamine (Q, Gln) also attenuated the 4-MU glucuronidation activity [6]. These studies indicate that unglycosylation of CES1 and UGT1A9 causes a decrease in enzyme activity. However, in the case of paraoxonase 1, the substitution of N residues with Q residues showed little effect on the enzyme activity [7]. Thus, the role of protein glycosylation seems to be different depending on the enzyme.

Abbreviations: AADAC, arylacetamide deacetylase; CES, carboxylesterase; DMSO, dimethyl sulfoxide; Endo H, endoglycosidase H; ER, endoplasmic reticulum; α-Glc, α-glucosidase; GPT, GlcN-Ac-1-phosphate transferase; HLM, human liver microsomes; PNGase F, peptide: *N*-glycosidase F; SNP, single nucleotide polymorphism.

* Corresponding author at: Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan. Tel.: +81 76 234 4438; fax: +81 76 234 4407.
E-mail address: tatsuki@p.kanazawa-u.ac.jp (T. Fukami).

0006-2952/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2013.10.001

Human arylacetamide deacetylase (AADAC), which is a serine hydrolase expressed in the human liver [8], is localized to the endoplasmic reticulum (ER) lumen [9]. AADAC was first identified as the enzyme that catalyzes the deacetylation of 2-acetylaminofluorene [10], and we recently found that AADAC is involved in the hydrolysis of clinical drugs, such as flutamide, phenacetin, and rifamycins [11–13]. These hydrolase activities may have a clinical impact on aspects related to the onset of toxicity and detoxification. For instance, the hydrolyzed metabolite of flutamide, 5-amino-2-nitrobenzotrifluoride (FLU-1), is involved in liver injury as a result of subsequent *N*-hydroxylation [14,15]. The hydrolyzed metabolite of phenacetin (*p*-phenetidine) is considered to be a cause of renal failure and hematotoxicity [16–18]. The hydrolyzed metabolite of rifampicin, 25-desacetylrifampicin, can neither induce cytochrome P450 3A4 nor produce the cytotoxic effect of the parent compound [13].

Although AADAC has two potential glycosylation sites (N78 and N282) [19], the role of glycosylation remains unclear. In this study, we investigated the role of *N*-glycosylation on the enzyme activity and the protein folding of human AADAC.

2. Materials and methods

2.1. Materials

Flutamide, FLU-1, phenacetin, rifampicin, castanospermine, 1-deoxynojirimycin, and thapsigargin were purchased from Wako Pure Chemicals (Osaka, Japan). *p*-Phenetidine, tunicamycin, and *O*-glycosidase were purchased from Sigma–Aldrich (St. Louis, MO). Endoglycosidase H (Endo H) and peptide: *N*-glycosidase F (PNGase F) were obtained from New England Biolabs (Beverly, MA). 25-Desacetylrifampicin was obtained from Toronto Research Chemicals (Toronto, Canada). Human liver microsomes (HLM, pooled, $n = 50$) were obtained from BD Gentest (Woburn, MA). Monoclonal mouse anti-human AADAC antibody was obtained from Abnova (Taipei, Taiwan). Rabbit anti-human GAPDH antibody and mouse anti-KDEL antibody were obtained from Imagenex (San Diego, CA) and Stressgen (Ann Arbor, MI), respectively. IRDye680-labeled goat anti-rabbit secondary antibody and Odyssey Blocking Buffer were obtained from Li-COR Biosciences (Lincoln, NE). Perfect NT Gel M was from Dream Realization & Communication (Tokyo, Japan). *Taq* DNA polymerase was obtained from Greiner Japan (Tokyo, Japan). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). All other chemicals and solvents were of the highest or analytical grade commercially available.

2.2. Treatment of HLM with Endo H, PNGase F, or O-glycosidase

HLM were adjusted to a concentration of 6 mg/mL using a denaturing buffer with a final concentration of 0.5% sodium dodecyl sulfate (SDS) and 40 mM dithiothreitol (DTT), followed by denaturation at 95 °C for 10 min. The denatured protein (30 μg) was incubated with 250 U of Endo H, 2.5 mU of *O*-glycosidase, or 300 U of PNGase F in 50 mM sodium citrate buffer (pH 5.5) in a final volume of 10 μL at 37 °C for 1 h, and was then subjected to SDS-PAGE and immunoblot analysis. For the measurement of enzyme activity or SDS-PAGE under non-reducing conditions, HLM or cell homogenates expressing AADAC (prepared as described later), were treated with Endo H under non-denaturing conditions as follows: HLM or cell homogenates (30 μg) were incubated with or without 400 U of Endo H in 50 mM citrate buffer (pH 5.5) in a final volume of 10 μL at 37 °C for 1 h.

2.3. SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed using HLM (30 μg) and HuH-7 cells (50 μg) as reported previously [11]. Briefly, protein sources were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-P (Millipore Corporation, Billerica, MA). The antibodies used in this study were a monoclonal mouse anti-human AADAC antibody (1:200), rabbit anti-human GAPDH antibody (1:1000), or mouse anti-KDEL antibody (1:200). The band intensity was quantified using ImageQuant TL Image Analysis software (GE Healthcare, Buckinghamshire, UK). The relative expression level of each AADAC was determined by the band intensity and the expression of wild-type AADAC was defined as 1 U.

2.4. Construction of the AADAC-mutant expression plasmids and transient transfection into HuH-7 cells

The AADAC-mutant expression plasmids were constructed using the pTargeT/AADAC cDNA vector, which was constructed in our previous study [11], as a template. The N78 residues and N282 residues in AADAC were substituted with Q using a QuickChange Site-Directed Mutagenesis kit (Stratagene: La Jolla, CA). The primer sets to construct the expression plasmids of the single mutants N78Q or N282Q are shown in Table 1. The expression plasmid of the double mutant, N78Q/N282Q, was constructed by introducing N78Q into the expression plasmid of the N282Q single mutant using the following primer pair: AADAC N78Q-S and AADAC N78Q-AS (Table 1). The sequences of each AADAC cDNA were confirmed using a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing kit (GE Healthcare) with a Long-Read Tower DNA sequencer (GE Healthcare). In this study, the reference nucleotide sequence of AADAC was NM_001086.2. Human hepatocellular carcinoma (HuH-7 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). HuH-7 cells were seeded (6×10^5 cells) in 6-well plates and were transfected with the respective AADAC expression plasmid (8 μg) using Lipofectamine 2000. After 48 h of incubation, the HuH-7 cells were harvested and suspended in a small amount of TGE buffer (10 mM Tris–HCl, 20% glycerol, 1 mM EDTA, pH 7.4) and disrupted using the freeze–thaw method three times. Each protein expression level was determined by the band intensity from the immunoblot analysis, as described above.

Table 1
Primers used for site-directed mutagenesis of human AADAC.

Primer	Sequence
AADAC N78Q-S	5′-CCCACCAACCTCAGATGAACAGGTCACTGTGACTGAG-3′
AADAC N78Q-AS	5′-CTCAGTCACAGTGACCTGTTCATCTGAGGTTGGTGGG-3′
AADAC N282Q-S	5′-CAAGTCATCTCTTCAAATTTGTTCAGTGGAGTTCCCTGCTCCC -3′
AADAC N282Q-AS	5′-GGGAGCAGGGAACTCCACTGAACAAATTTGAAGAGATGACTTG-3′

The underlined portions represent the mutated nucleotides.