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Progesterone regulates the expression and activity of two mouse isoforms of the glycoprotein folding sensor UDP-Glc: Glycoprotein glucosyltransferase (UGGT)

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ABSTRACT

UDP-Glucose:glycoprotein glucosyltransferase (UGGT) is a central component of the endoplasmic reticulum (ER) glycoprotein-folding quality control system, which prevents the exit of partially folded species. UGGT activity can be regulated by the accumulation of misfolded proteins in the ER, a stimulus that triggers a complex signaling pathway known as *unfolded protein response* (UPR) which is closely associated with inflammation and disease. In this work, we investigated the effect of progesterone (P4) on the expression and activity of UGGT in a mouse hybridoma. We detected the expression of two UGGT isoforms, UGGT1 and UGGT2, and demonstrated that both isoforms are active in these cells. Interestingly, the expression of each isoform is regulated by high physiological P4 concentrations. This work provides the first evidence of a hormonal regulation of UGGT isoform expression and activity, which might influence the glycoprotein quality control mechanism. These findings could contribute to the study of pathologies triggered by the accumulation of misfolded proteins.

1. Introduction

The endoplasmic reticulum (ER) is responsible for the coordination of protein biosynthetic and secretory activities in the cell; it assists the maturation of proteins destined for the extracellular environment, the cellular plasma membrane and the exo/endocytic compartments. The fidelity of the maturation process is assured by a stringent mechanism known as the *ER quality control system* that inhibits the secretion of folding intermediates, unassembled subunits of oligomeric complexes, and misfolded polypeptides. This system has been highly studied for N-glycosylated proteins, where the enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT) is a central player [1,2]. UGGT operates as a gatekeeper as it senses glycoprotein conformation and prevents the transport of non-native polypeptides out of the ER [3].

N-glycosylation occurs by the addition of preassembled core glycans (N-acetylglucosamine2-mannose9-glucose3) to nascent polypeptides emerging in the ER lumen [4]. Sequential trimming of the two outermost glucose residues, by glucosidase I and gluosidase II respectively, allows the protein bound N-glycans to associate with the ER lectins calnexin and calreticulin. This interaction promotes proper protein folding by preventing protein aggregation and by facilitating the activity of ERp57, a protein disulfide isomerase associated with the lectins that catalyzes the formation of intra- and intermolecular disulfide bonds. Release from the lectin anchor is followed by glucosidase II cleavage of the innermost glucose residue. Native polypeptides transit the ER, but nonnative polypeptides are tagged for reassociation with the lectins by reglucosylation on N-glycans catalyzed by UGGT. UGGT recognizes hydrophobic amino acid patches exposed in molten globule-like conformers [5,6] as well as hydrophobic surfaces exposed in not fully assembled oligomeric complexes [7]. Lectin binding cycles continues until glycoproteins either acquire their native structures or are recognized as irreparably misfolded species and are retro-translocated to the cytosol for degradation by proteasomes [8,9].

Many physiological stimuli and fluctuations in intracellular homeostasis disrupt protein folding in the ER, generating the accumulation of misfolded or unfolded proteins in the organelle. This condition is known as ER stress and induces a coordinated adaptive program called the *unfolded protein response* (UPR) [10]. The UPR alleviates stress by up-regulating protein folding and degradation pathways in the ER and inhibiting protein synthesis. In particular, UGGT expression is regulated during UPR [11,12]. It was demonstrated that the UPR intracellular signaling pathway responds to metabolic,

Abbreviations: DJN, Deoxynojirimycin; P4, Progesterone; UGGT, UDP-Glucose: glycoprotein glucosyltransferase

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oxidative stress, and inflammatory response pathways [13]. The cross-talk between the UPR and inflammation was observed mostly in specialized cell types that require the trafficking of large amounts of proteins through the ER, such as B lymphocytes [14,15]. Moreover, the UPR has recently been associated with a variety of diseases including metabolic diseases, inflammatory diseases, cancer and neurodegenerative diseases such as Alzheimer, Parkinson and multiple sclerosis [16,17]. Therefore, signaling components of the UPR are emerging as potential targets for novel treatments of human disorders.

On the other hand, while progesterone (P4) has historically been considered within the context of reproductive functions, it is now clear that this hormone has effects on the immune system, cardiovascular system, kidney function, adipose tissue, behavior and respiratory system [18–21]. Furthermore, P4 has been reported to exert neuroprotective effects in numerous experimental models. In a model of traumatic brain injury, P4 regulates the maturation of the NGF neurotrophin [22]. A completed phase II, randomized, double-blind, placebo-controlled clinical trial assessing the efficacy of P4 treatment for acute traumatic brain injury yielded promising results. Fahnestock and colleagues have described that pro-NGF is increased in Alzheimer´s disease brains [23]. Physiologically concentrations of P4 have been shown to significantly attenuate amyloid β-peptide-induced toxicity [24]. However, there are only a few reports regarding P4 regulation of the UPR [25] and, to our knowledge, there are no reports exploring specifically the effects of P4 on the components of the ER glycoprotein quality control system.

Therefore, the aim of this work was to study the effect of P4 on the expression and activity of UGGT in a mouse hybridoma. We employed a physiological range of P4 doses in this study: from 10^{-10} M to 10^{-5} M [21,26–34]. The lowest level of P4 (10^{-10} M) is found in sera from women during the follicular phase of the menstrual cycle as well as in men, whereas intermediate concentrations are achieved in sera from women along the lutheal phase of the menstrual cycle (10^{-9} M to 10^{-8} M) and during pregnancy (10^{-7} M). The highest P4 concentrations (10^{-6} to 10^{-5} M) are found locally at the sites of progesterone synthesis such as gonads, adrenal glands, placenta and brain. Importantly, relevant effects, inside and outside the reproductive system, were attributed to the different concentrations tested [18–21,24,26–34]. We detected the expression of two UGGT isoforms in the hybridoma: UGGT1 and UGGT2. Moreover, our results indicate that both isoforms are active and can be regulated by high doses of P4. These findings could contribute to the study of pathologies triggered by the accumulation of misfolded proteins.

2. Materials and methods

2.1. Hybridoma cell cultures

The hybridoma 112D5 secreting IgG1 anti-DNP mAb was obtained by Morelli et al. [35]. Cells (5×10^5 cells/ml) were cultured at 37 °C and 5% CO_2 during 48 h in RPMI 1640 (Gibco-Invitrogen, Carlsbad, USA) supplemented with 0.2 % $NaHCO_3$, 10% heat-inactivated fetal bovine serum (Gibco-Invitrogen, Carlsbad, USA), 20 μg/ml penicillin, 20 μg/ml streptomycin, 2 mM L-glutamine and 1 mM pyruvate in the absence or presence of P4 (10^{-5}, 10^{-6}, 10^{-8}, 10^{-9} and 10^{-10} M; progesterone-water soluble, Sigma-Aldrich, St. Louis, USA) and in the presence of 5 mM deoxynojirimycin (DJN; glucosidase I and II inhibitor; Sigma-Aldrich). Cells were recovered by centrifugation at 400*g* for 10 minutes. Each experiment was repeated 6 times. Cell viability was determined by Trypan Blue exclusion. Anti-DNP activity was assessed by ELISA as reported previously [36].

2.2. Cell proliferation assay

Cell proliferation was estimated by measuring DNA synthesis. Cells were cultured in the conditions described above in a 96-well plate and were pulsed 18 h before harvesting with 1 μCi/well [^{3}H]-Thymidine 20 Ci/mmol (New England Nuclear Corp.; MA, USA). The cells were subsequently harvested onto glass fiber filters, which were washed, dried and counted in a Packard 1600 TR scintillation counter. Each experiment was repeated 3 times.

2.3. Microsomes preparation

Cells were suspended in 5 mM Tris–HCl pH 7.4 supplemented with 0.1 M sucrose, β-mercaptoethanol and a protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 μg/ml leupeptin, all Sigma-Aldrich, St. Louis, USA). The cell suspension was sonicated at 60 Hz (2×15 s). The cell lysate was then centrifuged at 11,000*g* for 10 min, the supernatant was recovered and further centrifuged at 200,000*g* for 1 hour. The pellet was suspended in 10 mM Tris–HCl pH 8.0. Finally, protein concentration was determined by Bradford method using BSA as a standard.

2.4. UGGT enzymatic activity assay

UGGT activity was measured in the hybridoma microsomal fractions as the incorporation of [^{14}C]-Glc on denatured tyroglobulin as described by Trombetta et al. [37]. Briefly, reaction mixtures contained, in a 100 μl final volume: Tris–HCl 10 mM pH 8.0, $CaCl_2$ 10 mM, Tritón X-100 0.6 %, UDP-[^{14}C]-Glc 6 μM 285 Ci/M (New England Nuclear Corp.; MA, USA), deoxynojirimycin 150 μM (Sigma-Aldrich, St. Louis, USA), 300 μg of microsomal fraction and 500 μg of urea-denatured thyroglobulin. Reaction mixtures were incubated for 15 minutes at 37 °C and the reaction was stopped with 1 ml of 10% trichloroacetic acid (TCA). Radioactivity was measured in protein pellets after several washes with TCA with a Packard 1600 TR scintillation counter. Each determination was done by triplicate from six independent cell cultures. Enzyme activity was obtained as cpm/μg of microsomal proteins, the value from non-treated cells was considered as a unit (arbitrary unit = 1) and the rest of the values were compared to it.

2.5. Identification of mouse UGGT sequences and prediction of the sub-cellular localization

Mouse UGGT protein sequences were identified by homology search employing the biosequences analysis software HMMER (HMMER 3.0; http://hmmer.org/), which is based in the use of hidden Markov models (HMMs) of protein's domains. Briefly, the *Homo sapiens* HUGT1 (NP_064505.1) and HUGT2 (NP_064506.3) protein sequences were searched iteratively against the UniProtKB/Swiss-Prot (release 2012_08) and UniProtKB/TrEMBL (release 2012_7) databases employing the "jackhmmer" program. Then, the criteria for *Mus musculus* homologues sequence selection was based on the *E* value, which threshold was set at 0.0001, and the presence of two characteristic features of UGGT: the conserved motif DQDXXN at the C-terminal catalytic domain [3] and the UDP-g GGTase protein domain (PF06427), as established in the Pfam database, a collection of protein families represented by multiple sequence alignments and hidden Markov models [38].

Next, the potential sub-cellular localization of the selected proteins was predicted by the TargetP software [39]. This software analyses the N-terminal region of the proteins in the search of signal peptides and determines the probability of the protein to localize in cellular compartments or secretion.

2.6. Gene expression analysis

UGGT2 mRNA expression was determined by RT-PCR. Total RNA from hybridoma cells was extracted with TRI Reagent (Molecular Research Center Inc; Ohio, USA) following the protocol provided by the manufacturer. Then, cDNA was synthesized from total RNA using random primers (Invitrogen, Carlsbad, USA) and MMLV reverse transcriptase (Promega; Madison, USA). We included a control without

reverse transcriptase. The PCR reaction was then performed with specific primers (Integrated DNA Technologies; Iowa, USA): forward ACTTTCC CACATTGCTTTGGGCTCACTTTCCCACATTGCTTTGGGCTC and reverse AGA AGGTTATTGGTGGTGCCCTCA. PCR products were separated on a 2.1% agarose gel and visualized with ethidium bromide.

2.7. Preparation of antibodies

The sequence bearing the 619–643 residues (DLKEMNTEELKGAV LEKMVGTFVDL) of UGGT2 (NP_001074721.2) that had no similarity with UGGT1 (NP_942602.2; Supplementary Fig. 1) or any other protein, as observed in the mouse protein data banks, was selected in order to synthesize multiantigenic peptides (MAPs; Genbiotech). Polyclonal antibodies specific for UGGT2 were raised in rabbits using four branched MAPs. Rabbits were immunized subcutaneously with 1 mg/ml of MAPs plus Freund's adjuvant on days 0, 7, 16 and 25. Afterwards, the animals were anesthetized and sacrificed and the immune sera were employed for UGGT2 detection.

2.8. Protein expression analysis

UGGT1 and UGGT2 protein expression was analyzed by Western Blot. Equal amounts of microsomal proteins (1 μg) were loaded onto SDS-PAGE gels (7.5% polyacrylamide) and then transferred to nitrocellulose (Hybond-ECL, GE Healthcare, Pittsburgh, USA). The membranes were blocked with 5% nonfat dried milk in 50 mM Tris–HCl, 150 mM NaCl pH 7.5, 1% Tween-20 (T-TBS) at 4 °C overnight. Then, blots were incubated with a polyclonal rabbit anti-rat UGGT (obtained and provided by Dr. A. Parodi, Fundación Instituto Leloir, Buenos Aires, Argentina) or a polyclonal rabbit anti-mouse UGGT2 (obtained by us, as described in 2.7) at room temperature for 1 hour. After four washes of 5 minutes each with T-TBS, the blots were incubated at room temperature for 1 hour with polyclonal goat anti-rabbit IgG conjugated to horseradish peroxidase (ZyMAX, Invitrogen; Carlsbad, USA). Finally, blots were developed with enhanced chemiluminescence reagent (Amersham ECL Advance Western Blotting Detection Kit; GE Healthcare, Pittsburgh, USA) and exposed to X-ray film (AGFA CB) from 15 seconds to 6 minutes. Protein expression was determined by densitometry of the blot bands using the *ImageJ 1.42q* software (National Institutes of Health, USA). Densitometry value from non-treated cells was considered as a unit (arbitrary unit = 1) and the rest of the data were related to it.

Since protein expression was analyzed in cell microsomes, loading controls could not be included. Therefore, as described before [36], six western blots from independent cell cultures were carried out in each case, the samples were loaded in different gel positions in each SDS-PAGE in order to avoid misinterpretation of the results as a consequence of any alteration during the protein transference.

2.9. Gene silencing experiments

Two dsRNAs for each target gene (UGGT1 and UGGT2) were custom synthesized by Invitrogen (Carlsbad, USA). The sequences of the dsRNAs are the followings: UGGT1 (Ugcgl1: NM_198899.2), oligo 1: UAGGU CCAGAUACUCUCGCTT and oligo 2: GCGAGAGUAUCUGGACCUATT). UGGT2 (Ugcgl2: NM_001081252.2): oligo 1: CAGGUGAUGCUCGU CUGUUUAUAAA and oligo 2: UUUAUAAACAGACGAGCAUCACCUG). Scrambled sequences of each dsRNAs were also designed (UGGT1: GCGUGACUAGGUCAAGCUATT and UAGCUUGACCUAGUCACGCTT. UGGT2: CAGAGGUUCGCCUGUUUAUUUGAAA and UUUCAAAUAAAC AGGCGAACCUCUG). 1×10^5 hybridoma 112D5 cells were transfected with 1 to 150 nM dsRNA employing Lipofectamine RNAiMAX (Invitrogen, Carlsbad, USA) in OptiMEM (Invitrogen, Carlsbad, USA), according to the protocols depicted by the manufacturer. Cultures were performed during 48 and 72 h in 24-well plates at 37 °C in RPMI (GIBCO-Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum. The highest specific inhibition was achieved with 150 nM dsRNA for UGGT1 oligos and 5 nM for UGGT2 oligos after culturing for 72 h. Besides, P4 10^{-5} M or 10^{-6} M was added 24 h after the transfection in some cases.

2.10. Statistical analysis

UGGT activity data, obtained as cpm/μg protein, was transformed with square root function prior to statistical analysis and then normalized to be easily compared with the protein expression results. One-way analysis of variance (ANOVA) and "Newman–Keuls Multiple Comparison Test" were performed to compare mean differences using GraphPad Prism 5-Graphics Software. *P* values <0.05 were considered significant.

3. Results

Hybridoma cells were cultured with different P4 concentrations (10^{-5}, 10^{-6}, 10^{-8}, 10^{-9} and 10^{-10} M) during 48 h. According to previously published results [36], cell viability (94 ± 2%) and cell proliferation (215295 ± 17447 cpm) were not altered with any of the hormone doses. Moreover, anti-DNP titer was constant along the studied conditions.

3.1. UGGT activity is regulated by P4 in a dose dependent way

We first analyzed whether UGGT activity could be regulated by P4. To this aim, the enzyme activity was determined in microsomes from hybridoma cells cultured during 48 h in the presence of increasing P4 concentrations. Results showed that only the highest P4 doses employed could regulate UGGT activity: 10^{-6} M decreased the activity by 56% and 10^{-5} M increased it by 25% (Fig. 1).

3.2. Hybridoma 112D5 expresses at least two UGGT isoforms whose expression is modulated by P4

In order to determine whether the modulation of UGGT activity by P4 was due to a regulation at the protein level, we analyzed UGGT protein expression by western blot employing a rat anti-UGGT antibody (obtained from Dr. Parodi, Fundación Instituto Leloir, Buenos Aires, Argentina) that cross reacts with mouse UGGT. In contrast to that observed in the activity assays, P4 10^{-5} M significantly decreased UGGT protein expression, while P4 10^{-6} M had no effect on it (Fig. 2a). These unexpected findings prompted us to hypothesize that the cells expressed at least two UGGT isoforms; while both of them might have been detected in the activity assay, only one might have been recognized by the primary antibody in the western blot. The presence of two UGGT isoforms was previously reported in humans and were named HUGT1 and HUGT2, but only the former displayed reglucosylating activity in vitro [11].

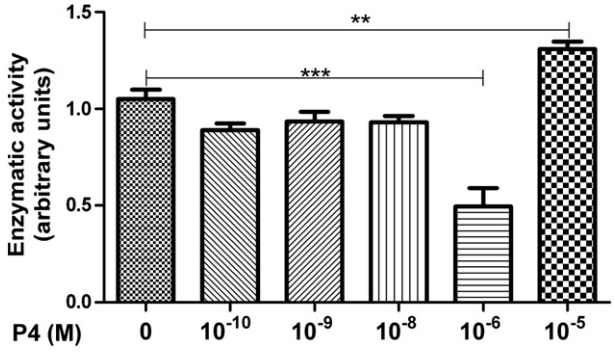

Fig. 1. Progesterone effects on UGGT activity. 112D5 hybridoma cells were incubated for 48 h with increasing doses of progesterone (P4, from 10^{-10} to 10^{-5} M). Then, UGGT enzyme activity was determined in microsomal fractions. The bars represent the mean value plus the standard deviation of six independent cell cultures. $^{**}p < 0.01$; $^{***}p < 0.001$.

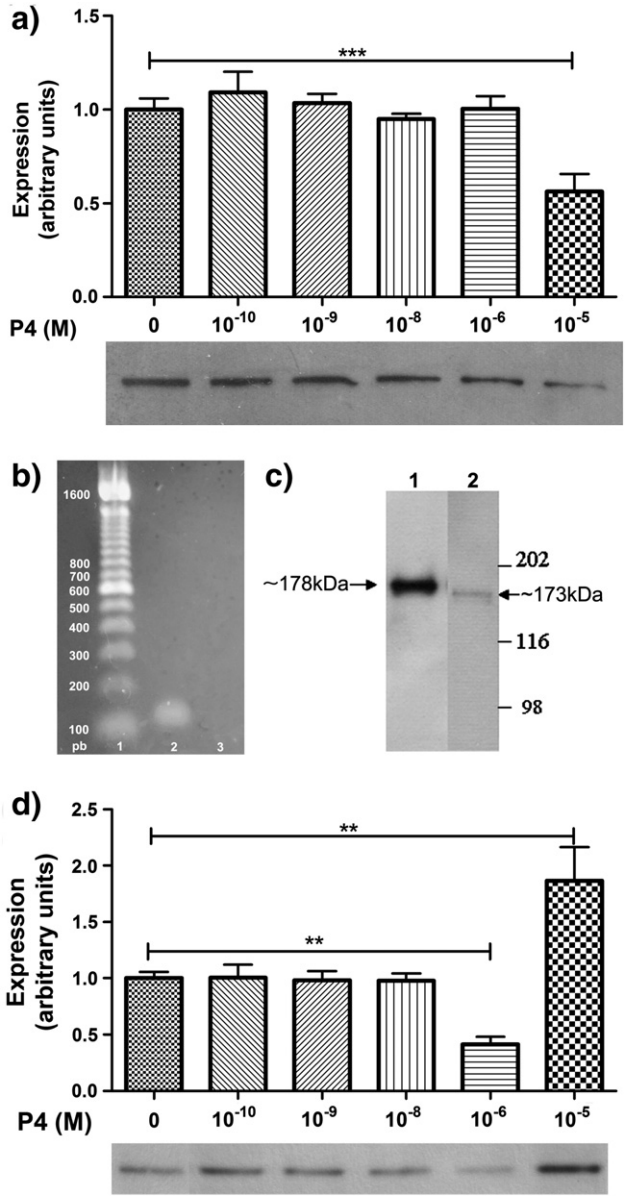

Fig. 2. Progesterone effects on UGGT expression. (a) UGGT expression determined by western blot using a rabbit anti-rat UGGT. Hybridoma cells were incubated for 48 h with increasing doses of progesterone (P4, from 10^{-10} to 10^{-5} M). The bars of the graph represent the mean densitometry value plus the standard deviation of six independent cell cultures. A representative western blot is shown below the graph (b) PCR amplification of cDNA synthesized from total RNA from the 112D5 hybridoma cells: lane 1, molecular weight standard; lane 2, UGGT2 amplicon of 131 bp; lane 3, negative control (cDNA synthesis reaction in the absence of reverse transcriptase). Products were resolved in a 2.1% agarose gel. (c) Western blot of microsomes from the 112D5 hybridoma employing rabbit anti-rat UGGT (lane 1) and rabbit anti-mouse UGGT2 (lane 2). (d) UGGT2 expression in hybridoma cells treated for 48 h with P4 as assessed by western blot using a polyclonal anti-mouse UGGT2. The bars of the graph represent the mean densitometry value plus the standard deviation of six independent cell cultures. $^{**}p < 0.01$; $^{***}p < 0.001$.

Consequently, we next searched for mouse homologues of HUGT1 and HUGT2 in *UniProtKB* databases, employing the HMMER software. Then, *Mus musculus* proteins with *E* values < 0.0001, with the presence of the Pfam protein domain PF06427 and the presence of the conserved motif DQDXXN at the C-terminal catalytic domain were selected as true homologues. This strategy allowed the identification of two full sequences, one homologue to HUGT1 and one homologue to HUGT2. The sequence annotated as mouse UGGT1 (NP_942602.2) shared 91% of protein identity and 96% of protein similitude to HUGT1, whereas the sequence annotated as mouse UGGT2 (NP_001074721.2) shared 79% of protein identity and 91% of protein similitude to HUGT2. Additionally, both sequences bear a signal peptide, as determined by the TargetP software [39], and an ER retrieval sequence at the C-terminus: HEEL for UGGT1 and HDEL for UGGT2 (Supplementary Fig. 1), which is consistent with the protein's cellular function at the ER and the observations reported for rat and human UGGT homologues [11,40]. Interestingly, there are only few reports regarding mouse UGGT1 protein expression and, until now, there are no evidences reporting mouse UGGT2 protein expression.

Therefore, we first designed specific primers for the detection of UGGT2. Results showed that hybridoma cells synthesize UGGT2 mRNA (Fig. 2b, lane 2). This result is supported by the numerous ESTs (*expressed sequence tags*) characterized for this transcript in a variety of mouse tissues (http://www.ncbi.nlm.nih.gov/UniGene; Mm.213406). Then, we raised a specific UGGT2 antibody to identify the protein expression. The antibody was generated by immunizing rabbits with multi-antigenic peptides bearing the 619–643 residues of UGGT2. Importantly, this sequence is not present in UGGT1 (Supplementary Fig. 1). The specificity of this antiserum was analyzed by western blot employing microsomes from hybridoma 112D5. We identified a band of approximately 173 kDa employing the UGGT2 antibody, in agreement with the predicted molecular weight of UGGT2. The western blot analysis employing the rat UGGT antibody detected mouse UGGT1 specifically, while the UGGT2 antibody prepared by us reacted uniquely with UGGT2 (Fig. 2c). UGGT2 band was specific, as stripping and re-probing with secondary antibody alone did not produce any other bands. In agreement with these results, Arnold et al. observed that the same rat anti-UGGT antibody employed by us recognized HUGT1 and not HUGT2 [11,41]. Therefore, albeit the relative abundance of each isoform cannot be determined by western blot, these results demonstrated that the mouse hybridoma expresses at least two UGGT isoforms.

Therefore, we next sought to determine whether the discrepancies observed between UGGT activity and expression in P4-treated cells were due to the presence and activity of UGGT2. Hence, we repeated the western blots of P4-treated cells using the UGGT2 polyclonal antibody. We observed a decrease in UGGT2 expression when cells were treated with P4 10^{-6} M and an increase in UGGT2 expression when P4 concentration was 10^{-5} M (Fig. 2d). These results are consistent with the variations observed in UGGT activity, suggesting that both isoforms are active in mouse when using the standard activity assay.

3.3. UGGT1 and UGGT2 show in vitro biological activity and are independently regulated by progesterone

In order to shed light to the contribution of each isoform to the general UGGT activity and to the regulation exerted by P4, we performed isoform-specific knockdowns by RNA interference, employing double stranded RNA oligos (dsRNA).

Cell viability in the presence of lipofectamine (the transfection reagent) alone was about 70% and this value was not altered in the presence of lipofectamine plus the UGGT1 or UGGT2 dsRNA; thus indicating that the viability reduction observed for silenced cells with respect to non-treated cells was due solely to lipofectamine toxicity and not to the enzyme specific inhibition. Even though UGGT knockout is lethal in mice, our result is consistent with data observed in other cell cultures in which viability was indistinguishable from that of the wild type cells [42]. Silenced cells showed similar anti-DNP titer to wild type ones in every tested condition; suggesting that UGGT isoforms are dispensable for IgG1 maturation and further secretion. This result is supported by similar data obtained by us when cells were cultured in the presence of 5 mM deoxynojirimycin (DJN), an inhibitor of glucosidase I and II which prevents the entry of a glycoprotein to the lectin cycle in the ER.

In the absence of P4, the UGGT1 dsRNA decreased approximately 82% UGGT1 expression (Fig. 3a), without affecting that from UGGT2

(Fig. 3b), whereas UGGT enzymatic activity decreased about 60% (Fig. 3c). This result shows that UGGT1 is active in the murine hybridoma and represents the main contributor in the activity assay. On the other hand, UGGT2 dsRNA reduced a 60% UGGT2 expression (Fig. 3b), did not alter UGGT1 expression (Fig. 3a) and diminished a 20% the UGGT activity (Fig. 3c). In this case, this result suggests that UGGT2 is also active in our working conditions, even though to a lesser extent than UGGT1. All these results are specific since the incubation with scramble dsRNA of each isoform did not produce any variations in the variables measured (Fig. 3a–c).

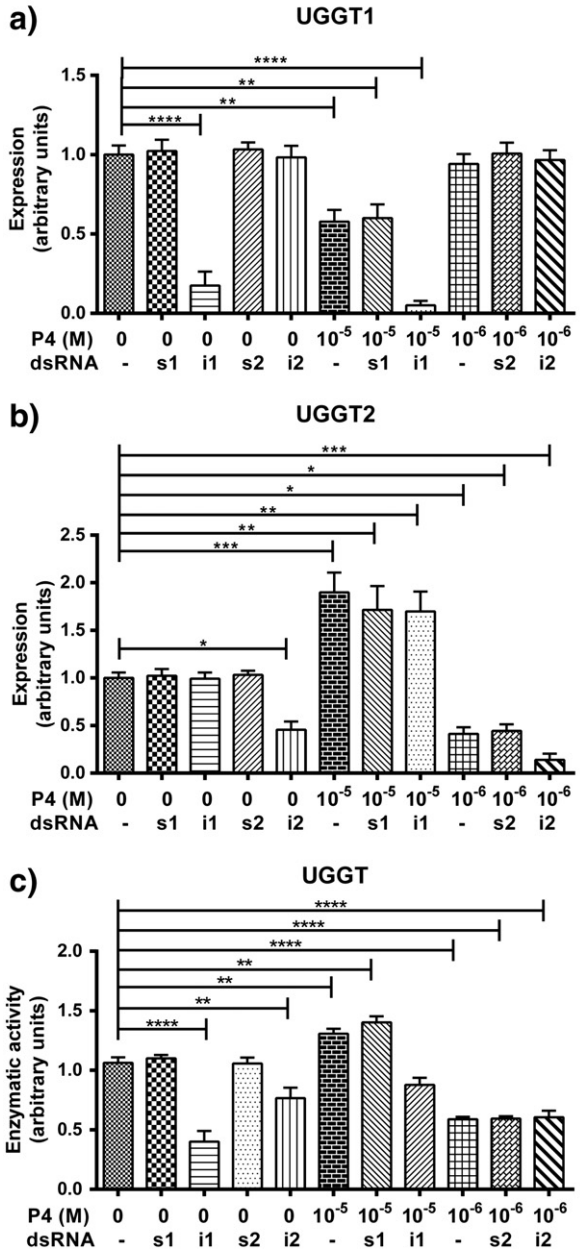

Fig. 3. UGGT1 and UGGT2 gene silencing experiments. 112D5 hybridoma cells were transfected employing lipofectamine with double-stranded RNA oligonucleotides (dsRNA) specific for UGGT1 (i1) or UGGT2 (i2) for 72 h. In addition, tests were performed by transfecting cells with scramble dsRNA (includes the nucleotides present in each dsRNA but in a different sequence (s1 = scramble for UGGT1 and s2 = scramble for UGGT2). Progesterone (P4 10^{-5} M or 10^{-6} M) was added to the culture medium 24 h after transfection in the indicated cases. Then, (a) UGGT1, (b) UGGT2 expression and (c) UGGT activity were determined in the cell microsomes. The bars of each graph represent the mean plus the standard deviation of three independent cell cultures. Pictures of a representative western blot are shown in each case. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

When UGGT1 dsRNA treated cells were incubated with P4 10^{-5} M, UGGT1 expression was almost abolished (Fig. 3a), UGGT2 expression was significantly increased to a similar level to that observed in wild-type cells treated with P4 10^{-5} M only (Fig. 3b) and UGGT activity was similar to non-treated cells (Fig. 3c). These results suggest that the increased UGGT2 expression observed in the presence of P4 10^{-5} M does not respond to a compensatory mechanism triggered by the reduction in UGGT1 expression or vice versa. Instead, they would indicate that P4 10^{-5} M exerts a specific regulatory mechanism over each isoform.

At last, the incubation of UGGT2 dsRNA treated cells with P4 10^{-6} M further reduced UGGT2 expression from 60% to approximately 90% (Fig. 3b), UGGT1 expression remained unaltered (Fig. 3a) and UGGT activity was further reduced from 20% to approximately 50% (Fig. 3c). Having blocked almost all UGGT2, the only source of enzymatic activity would derive from UGGT1, supporting the observation that UGGT1 is active in the murine hybridoma.

Altogether, these results indicate that both UGGT1 and UGGT2 proteins expressed by the hybridoma 112D5 contribute to the UGGT activity assay. In addition, P4 can independently regulate the expression of each isoform.

4. Discussion

Given the therapeutic benefits reported for progesterone in human diseases associated with the accumulation of misfolded proteins, in the present work we investigated whether this hormone could regulate the expression and activity of UGGT, an UPR-related enzyme. Interestingly, we detected the existence of two active isoforms of UGGT in the mouse and provided evidences indicating that P4 regulates the expression and biological activity of both of them.

This study demonstrates that UGGT activity in hybridoma cells responds to the highest P4 concentrations of the physiological doses tested. We found that P4 10^{-6} M decreased UGGT activity while P4 10^{-5} M increased it. However, P4 10^{-6} M did not modify UGGT protein expression while P4 10^{-5} M reduced it significantly. These contradictory results led us define two possible scenarios. On the one hand, cross-linking experiments revealed that UGGT can associate to a series of chaperones in the ER [43], suggesting that the enzyme activity might be regulated through associated proteins. This situation might be consistent with the observed results when cells were cultured with P4 10^{-6} M; however, it was difficult to associate with the effects of P4 10^{-5} M. The second possibility arose from the finding of two cDNAs encoding human UGGT homologues, named HUGT1 and HUGT2 [11]. Despite the high degree of sequence identity between HUGT1 and HUGT2, the authors reported that recombinant HUGT2 was not functional. Considering this finding and our experimental evidences, we proposed that the 112D5 murine hybridoma expresses at least two UGGT isoforms, both of them active as proved by using the standard UGGT assay, while only one of them was detected by western-blot. Therefore, we searched for the mouse homologues of HUGTs employing *hidden Markov models* by means of the HMMER software. Interestingly, we found only two proteins, being one of them highly similar to HUGT1 and the other highly similar to HUGT2; these proteins were named UGGT1 and UGGT2 respectively. Importantly, until now, there was only clear experimental evidence for the existence of UGGT1 protein [44]. In this work, we first determined UGGT2 mRNA in the hybridoma and then we detected the protein expression employing a specific antibody generated by us. In conclusion, we demonstrated that the 112D5 hybridoma expresses two UGGT isoforms, where UGGT1 was recognized by the antibody employed previously while UGGT2 was detected by the antibody obtained in this work.

The biological activity of UGGT1 has been widely demonstrated in different organisms [41,45], including in the mouse [46]. In this work,

we presented evidences that are also in favor of UGGT1 activity, being the strongest and clearest one that observed in the iRNA experiments (Fig. 3): in cells treated with UGGT1 dsRNA, UGGT1 expression diminished approximately 82% whereas the UGGT total activity decreased 60%. Considering that in this condition UGGT2 expression was not altered, this result indicates that UGGT1 is active in the murine hybridoma. Furthermore, it suggests that UGGT1 specific activity is the main contributor to the total UGGT activity. Interestingly, several evidences support the hypothesis that UGGT2 is also biologically active in the mouse hybridoma cells. Despite not having exceeded a 60% inhibition in UGGT2 expression in the UGGT2 silencing experiments, a significant decrease in UGGT activity (20%) was observed. This observation argues in favor of an active UGGT2 isoform. However, in spite of the fact that both UGGT isoforms were able to transfer UDP-Glc *in vitro*, our results indicate that UGGT2 depicted a lower contribution to the *in vitro* activity assay than UGGT1. This fact could either indicate that the assay conditions are optimized for UGGT1 and/or that UGGT2 has a lower specific enzymatic activity. Moreover, it is interesting to note that the specific inhibition of the expression of one isoform did not induce the expression of the other. Therefore, it is likely that UGGT1 and UGGT2 fulfill different roles in the cellular environment, probably by focusing their activity on a different set of substrates. Recently, Buzzi et al. [45] demonstrated that uggt2 is an essential gene in *Caenorhabditis elegans* and presented several evidences supporting the hypothesis that the two homologues have distinct biological functions.

The experiments performed in the presence of P4 alone and plus dsRNA demonstrated that P4 specifically regulates the expression of UGGT1 and UGGT2, affecting UGGT total activity. In addition, they suggest that P4 could also modulate UGGT1 and UGGT2 specific activity. On the one hand, P4 10^{-5} M decreased UGGT1 expression (50%), increased UGGT2 expression (80%) and, in spite of the fact that in the absence of P4 UGGT1 behaved as the main contributor of the activity assay, in this case UGGT activity showed a mild increase (25%). Moreover, when UGGT1 knock-down cells were treated with P4 10^{-5} M, the increase in UGGT2 expression persists (80%) while an almost complete decrease in UGGT1 expression was observed without detecting changes in the enzyme activity. These observations suggest that P4 10^{-5} M, besides its effects on UGGT1 and UGGT2 expression, might also up-regulate UGGT2 specific activity. On the other hand, cells treated solely with UGGT2 dsRNA or P4 10^{-6} M showed an equivalent UGGT1 expression and a similar down-regulation of UGGT2 expression (50–60%). However, the enzyme activity in the latter condition was much lower. These results not only indicate that P4 10^{-6} M down-regulates the expression of UGGT2 but also suggest that it might down-regulate UGGT1 specific activity. This idea is supported by the results obtained when UGGT2 knock-down cells were cultured in the presence of P4 10^{-6} M, where although UGGT1 expression was similar to the two previous conditions and UGGT2 expression was almost abolished, the enzyme activity was reduced to the level observed for wild-type cells in the presence of P4 10^{-6} M (50%).

As stated before, P4 could possibly regulate UGGT isoform activity through associated proteins. It is important to note that the ER contains a complex network of molecular complexes that includes chaperones and foldases [47] and, as previously mentioned, UGGT can associate to one of them [43]. In addition, UGGT was also found in a protein complex with the 15-kDa Selenoprotein (Sep15), which participates in the formation of disulfide bonds and its expression is regulated by dietary selenium and redox homeostasis [44]. Although it was not specified in these works, the isoform present in both complexes was UGGT1, as we could determine after analyzing the sequences reported in both of them. These observations support the hypothesis that the specific activity of UGGT1 could be regulated through the modulation of associated proteins. Although until now, there is no evidence of the presence of UGGT2 in protein complexes, we cannot discard that it might require an associated protein for catalytic activity.

The fact that P4 10^{-6} M and 10^{-5} M produced opposite effects on UGGT activity in hybridoma cells is intriguing. P4 is a steroid hormone that typically binds to its intracellular receptors (nPR), which mediate many of the physiological effects of P4 by regulating gene expression. The expression of nPR in the hybridoma was already described [48] and confirmed by us (data not shown). However, P4 also exerts non-transcriptional effects that have been shown to rely on membrane P4 receptors (mPR). What is more, evidence suggesting a cross-talk between nPR and mPR have recently emerged [49]. Until now, the mPR family includes mPR α, β and γ and the recently described mPR δ and ε [49–52]. In addition, the progesterone receptor membrane component 1 (PGRMC1) is another prospective candidate mediating the non-genomic actions of progesterone. Until now, we detected the expression of mPRγ in the hybridoma's plasma membrane. Interestingly, employing a P4-BSA-FITC conjugate (which is unable to diffuse through the cell membrane) we observed, by flow cytometry, hormone binding to the hybridoma plasma membrane at P4 10^{-5} M and not at P4 10^{-6} M. Moreover, our studies suggest that both nuclear and membrane receptors are differentially involved in the regulation of UGGT1 and UGGT2 expression and activity (unpublished data). We are currently studying the complex intracellular mechanism regulated by P4 concentrations that might be operating in the hybridoma.

The difference in activity between UGGT2 and HUGT2 could be related to some variations along the primary sequence that could affect their conformation and/or enzymatic activity. The catalytic site boundaries have been defined by Arnold et al. [11]. Interestingly, UGGT2, unlike HUGT2, retains the lysine 1294 in the catalytic site, an amino acid present in all UGGT homologues except HUGT2. Nevertheless, the differences in activity might also arise from the recombinant nature of HUGT2.

On the other hand, IgG1 titer was not altered neither in UGGT silenced cells (UGGT1 or UGGT2) nor in those cells cultured in the presence of DJN. These results indicate that the inability to enter into the lectin cycle and consequently in the ER glycoprotein quality control mechanism (including UGGT1 and UGGT2) did not affect, in particular, hybridoma IgG1 maturation and secretion. This observation is consistent with several evidences showing that IgG1 proper assembly and secretion is regulated by the molecular chaperone BiP; the mechanism implicated was described in detail by Feige et al. [53].

Finally, it was previously reported that UGGT activity can be modulated by the accumulation of misfolded proteins in the ER triggered by several stressors such as tunicamycin, an *N*-glycosylation blocking compound or the ionophore A-23187 [11,12]. In this work we show the first evidence of the regulation of UGGT activity by a physiological stimulus. Interestingly, we also observed a correlation between P4 concentration in serum from healthy women along the two phases of the menstrual cycle and UGGT activity from B lymphocytes isolated from the same blood samples (unpublished data).

5. Conclusions

This work reports the expression and activity of two UGGT mouse isoforms. Moreover, it provides the first evidence of a hormonal regulation of both UGGT1 and UGGT2 protein expression and activity, bringing a new approach to study the regulation of the quality control mechanism of glycoproteins by P4 in health and disease. These results might be especially important in the study of pathologies triggered by the accumulation of misfolded proteins as well as for the study of sex differences in the manifestations of some diseases.

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