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RESEARCH

Generation of Polyclonal Antibodies Against Recombinant Human Glucocerebrosidase Produced in *Escherichia coli*

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Abstract Deficiency of the lysosomal glucocerebrosidase (GCR) enzyme results in Gaucher's disease, the most common inherited storage disorder. Treatment consists of enzyme replacement therapy by the administration of recombinant GCR produced in Chinese hamster ovary cells. The production of anti-GCR antibodies has already been described with placenta-derived human GCR that requires successive chromatographic procedures. Here, we report a practical and efficient method to obtain anti-GCR polyclonal antibodies against recombinant GCR produced in *Escherichia coli* and further purified by a single step through nickel affinity chromatography. The purified GCR was used to immunize BALB/c mice and the induction of anti-GCR antibodies was evaluated by enzyme-linked immunosorbent assay. The specificity of the antiserum was also evaluated by western blot analysis against recombinant GCR produced by COS-7 cells or against endogenous GCR of human cell lines. GCR was strongly recognized by the produced antibodies, either as cell-associated or as secreted forms. The detected molecular masses of 59–66 kDa are in accordance to the expected size for glycosylated GCR. The GCR produced in *E. coli* would facilitate the production of polyclonal (shown here) and monoclonal antibodies and their use in the characterization of new biosimilar recombinant GCRs coming in the near future.

Keywords Glucocerebrosidase · Polyclonal antibody · Gaucher's disease · Recombinant protein expression · Lysosomal storage disorder

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Introduction

Glucocerebrosidase (GCR; EC 3.2.1.45) is a membrane-associated lysosomal enzyme [1, 2] that catalyses the hydrolysis of glucocerebroside to glucose and ceramide [3–5]. More than 300 mutations were identified in the GCR gene [6, 7], which result in insufficient GCR activity, leading to Gaucher's disease, the most prevalent human lysosomal storage disorder [6, 8–10]. This disease is characterized by an accumulation of glycolipids in macrophages, mainly in liver, spleen and bone marrow, causing functional abnormalities in these organs. The disease can be classified in three forms according to absence (Type I) or presence and severity of lesions in central nervous system (Types II and III) [9, 11–13]. The most common and safe treatment of Gaucher's disease consists of enzyme replacement therapy (ERT) in which an active enzyme is given to the patients

with Types I and III diseases by intravenous infusions [5, 8, 14]. ERT has proven to be effective over a period of 15 years, stopping and reversing many symptoms of the disorder [7, 15]. The therapy was first introduced in 1991 by the use of a placenta-derived product that was further replaced in 1994 by a recombinant form, produced in Chinese hamster ovary (CHO) cells [7]. Currently, new enzymatic preparations are being investigated and include gene-activated GCR using human fibroblasts [16, 17] and plant-derived recombinant human GCR [18, 19]. Anti-GCR antibodies are important and useful tools to characterize GCR produced in a number of expression systems, including insect cells [9, 20], plant cells [18] and mammalian cells [21–23]. Anti-GCR antibodies can also be used to discriminate between non-neuropathic and neuropathic cases of this disorder, as demonstrated by Ginns and collaborators [24, 25], and to characterize the molecular forms of the enzyme in normal individuals and in patients with Types I, II and III forms of the disease [3, 11, 26–29]. So far, anti-GCR antibodies (both monoclonal and polyclonal) have been produced against purified GCR from human placenta [24, 30, 31]. However, this is a limited source that requires many complex steps for GCR purification [32]. Here, we describe the production of murine anti-human GCR polyclonal antibodies against a recombinant GCR expressed in *Escherichia coli* and purified by a nickel-charged chromatography column. The potential use of the antibodies to characterize GCR was shown through immunoblotting analysis of cellular extracts and culture supernatants from COS-7 cells transiently transfected with mammalian expression plasmids containing the human GCR cDNA under the control of the adenovirus major late promoter (Ad MLP). In addition, the antibodies were capable to recognize endogenous GCR in ECV 304 and HeLa human cell lines. The results demonstrated the feasibility to produce polyclonal antibodies against recombinant GCR obtained in *E. coli*. The specificity and quality of the produced antiserum were described, as well as the functionality of the mammalian expression plasmids, confirming that they may be used for stable transfection of CHO cells in the future.

Materials and Methods

GCR cDNA Amplification and Cloning Procedures

The human GCR cDNA was obtained by purification of the total RNA from ECV 304 cells (ATCC CRL 1998), followed by the cDNA synthesis through RT-PCR (results not shown). The 1,519-bp cDNA encoding human GCR lacking the signal peptide was amplified by PCR using the forward primer (5′ GGA TCC GCC CGC CCC TGC ATC CCT AAA A 3′) containing a *Bam*HI restriction site, and the reverse primer (5′ GAA TTC GTG CCT CCT TGA GTA TCT 3′) containing an *Eco*RI restriction site. PCR was performed using Pfx DNA polymerase (Invitrogen, Carisbad, CA, USA), 0.2 mM of each deoxynucleoside triphosphate and 20 pmol of each primer. PCR amplification conditions were as follows: 94°C, 5 min; 30 cycles of 94°C, 1 min; 47°C, 45 s; 68°C, 3 min; and a single step of 68°C, 7 min for final extension. PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, USA). DH5α *E. coli* competent cells were transformed with the ligation product for propagation and amplification of the recombinant DNA. Positive clones were confirmed by DNA sequencing using an automated DNA sequencer (ABI 3100) based on the dideoxytermination method [33]. The resulted plasmid pGEM-T-GCR was digested and the insert was subcloned into the *Bam*HI and *Eco*RI sites of the pAE, an *E. coli* expression vector based on T7 promoter, which allows the expression of recombinant proteins in fusion with a six histidine tag ($his_{\times 6}$ tag) at the N-terminus [34]. For GCR expression in mammalian cells, the human GCR cDNA was amplified and subcloned in the pED expression vector, kindly provided by Dr. R. J. Kaufman (Howard Hughes Medical Institute, University of Michigan), which provides high expression levels of heterologous proteins based on a dicistronic expression system [35]. Three plasmid constructs were obtained for expression in mammalian cells (1) A GCR cDNA cloned with its own signal peptide; (2) A GCR cDNA fused to an Ig kappa-chain signal peptide (Igκ); (3) A GCR cDNA fused to Igκ and to a $his_{\times 6}$ tag at the GCR N-terminus to facilitate protein purification by nickel charged chromatography.

Expression in *E. coli*

Escherichia coli BL21 SI competent cells (Gibco/BRL) were transformed with the pAE-GCR plasmid and grown in plates overnight at 30°C in LB medium without NaCl and containing 100 μg/mL ampicillin (LBON-amp). A single colony was inoculated in 20 mL of medium and grown overnight at 30°C. After 16 h, the culture was diluted 100-fold in 1 L of LBON-amp medium. When OD_{600} reached 0.6, NaCl was added to the medium at final concentration of 300 mM for induction of the recombinant GCR expression. After 3 h, cells were collected by centrifugation, resuspended in 100 mL lysis buffer (300 mM NaCl, 100 mM Tris–HCl) pH 8.8 and lysed in French Pressure (Thermo Electron Corporation, San Jose, CA, USA). Cellular lysates were centrifuged at 26,000×*g* for 15 min. The insoluble inclusion bodies were washed with 20 mL binding buffer (500 mM NaCl, 50 mM Tris–HCl) pH 8.8 containing 1 M urea. After centrifugation, the inclusion bodies were dissolved in 20 mL binding buffer containing 5 mM β-mercaptoethanol and 8 M urea.

Purification of Recombinant Human GCR

Glucocerebrosidase was purified by affinity chromatography using a column (1.0 cm diameter) containing 5 mL nickel-charged chelating Sepharose (GE Healthcare, Buckinghamshire, UK). The resin was equilibrated with binding buffer containing 5 mM β-mercaptoethanol and 8 M urea. After adsorption of GCR protein extracted from the inclusion bodies, the resin was washed with binding buffer containing 8 M urea and 5, 20, 40 and 60 mM imidazole (10 volumes each). GCR was eluted with 5 volumes of the same solution containing 500 mM imidazole and eluted fractions were analyzed by 10% SDS-PAGE. Quantification of purified GCR was based on a standard curve generated with bovine serum albumin (BSA) and further densitometry analysis in a 10% SDS-PAGE.

Immunization of Mice

Five- to 7-week-old female BALB/c mice were immunized intraperitoneally with 10 μg of purified human GCR in $Al(OH)_3$. Immunizations were performed over a period of 4 weeks, with booster doses at every week. Mice were bled by the retrorbital plexus 1 week after each immunization and the blood was incubated for 30 min at 37°C. The clot was removed by centrifugation and the pooled sera collected from supernatants were stored at −20°C until use.

Enzyme-Linked Immunosorbent Assay

A Microtiter plate (Maxisorp-NUNC) was incubated at 4°C for 16 h with 10 μg/mL of purified GCR in 0.05 M carbonate–bicarbonate buffer pH 9.6. After three washes with 0.05% tween 20/phosphate-buffered saline pH 7.4 (PBS-T), the plate was blocked with 10% (m/v) non-fat dried milk in PBS-T for 1 h at 37°C. The blocking buffer was removed with three washes of PBS-T, and dilutions of anti-GCR serum were added to the plate in 1% (m/v) bovine serum albumin (BSA)-PBS-T. The plate was incubated at 37°C for 1 h. After washing, proper dilutions of a peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO, USA) were added to the plate that was incubated for an additional hour at 37°C. The plate was washed and developed by the addition of 8 mg o-phenylenediamine (OPD) in 20 mL of a 0.2 M citrate–phosphate buffer pH 5.0, in the presence of 10 μL H_2O_2. The reaction was stopped by adding 4 M H_2SO_4, and the titer was defined as the last dilution in which the absorbance at 492 nm reached 0.1. The identification of IgG subclasses and IgM isotype in the serum was performed using goat anti-mouse IgG subtypes and goat anti-mouse IgM, respectively (Southern Biotech., Birmingham, AL, USA). In these cases, an additional incubation step was performed with a peroxidase-conjugated anti-goat IgG.

Transient Transfection of COS-7 Cells

COS-7 cells (ATCC CRL 1651) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carisbad, CA, USA) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL anfotericin B (Invitrogen, Carisbad, CA, USA) and 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil). Transient transfection was carried out with pED expression vector containing the GCR cDNA in the three cloning strategies by Lipofectamine 2000 (Invitrogen, Carisbad, CA, USA), according to the manufacturer's instructions. The cells were washed three times with PBS 48 h after transfection and the culture medium was replaced by DMEM lacking FBS. After 24 h of incubation, both the culture medium and the cells were collected, centrifuged at $277 \times g$ for 10 min, and stored at −20°C until use.

Culture of Human Cell Lines

HeLa (ATCC CCL 2) and ECV 304 (ATCC CRL 1998) cells were maintained as described for COS-7 cells. After three washes with PBS, the cells were collected, centrifuged at $277 \times g$ for 10 min, and stored at −20°C until use.

Western Blotting Analysis

COS-7, HeLa and ECV 304 cell pellets were lysed in twofold SDS-PAGE sample buffer, containing 8 M urea, 1.5 mM EDTA and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The suspensions were fractionated on a 10% SDS-PAGE and electro-transferred to nitrocellulose or PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was blocked with 10% (m/v) non-fat dried milk in PBS-T for 16 h. Membrane was washed three times for 10 min with PBS-T, and further incubated with a 1:5,000 dilution of the murine anti-human GCR polyclonal antibodies, in 5% non-fat dried milk-PBS-T, for 1 h. The membrane was washed three times with PBS-T and incubated with a proper dilution of anti-mouse IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO, USA) in 5% non-fat dried milk-PBS-T, washed, and revealed with ECL or ECL Plus detection reagent (GE Healthcare, Buckinghamshire, UK), following manufacturer's instructions.

PNGase F Digestion

PNGase F digestion was performed in samples of COS-7 cells culture supernatants previously precipitated with 10%

(v/v) trichloroacetic acid (TCA) and washed with cold acetone. The samples were heated for 10 min at 100°C in glycoprotein denaturing buffer, according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). After addition of G7 reaction buffer, 10% NP-40 and PNGase F, the reactions were incubated at 37°C for 16 h. Samples were subjected to 10% SDS-PAGE followed by western blotting analysis, as described above.

Results

GCR Expression in *E. coli* and Purification

The human GCR cDNA without the nucleotide sequence coding for its signal peptide was cloned into the pAE vector [34] at *Bam*HI and *Eco*RI sites in frame with a $his_{\times 6}$ tag at the N-terminus to facilitate GCR purification through a nickel charged resin. The expected protein band of 56 kDa for unglycosylated GCR [36] was detected in NaCl-induced *E. coli* BL21 SI cells by 10% SDS-PAGE analysis (Fig. 1a). The GCR expression was observed in soluble and insoluble fractions (Fig. 1b, lanes 1 and 2, respectively). GCR purification was performed in a single chromatographic step, as described in Material and Methods, from inclusion bodies dissolved in binding buffer containing 8 M urea. After washing steps with increasing concentrations of imidazole, a significant amount of the GCR protein was successfully eluted with 500 mM imidazole, as shown in Fig. 1b (lanes 3–8). The final purification yield for GCR was estimated to be approximately 0.8 mg/L culture (data not shown).

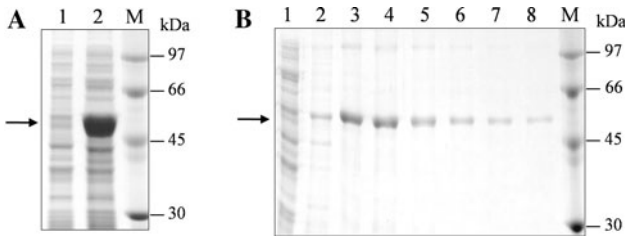

Fig. 1 Expression, purification and characterization of recombinant GCR by 10% SDS-PAGE. **a** GCR expression in *E. coli* BL21 SI cells. *Lanes 1, 2* cellular extracts before and after induction with 300 mM NaCl, respectively. **b** GCR purified from inclusion bodies by nickel affinity chromatography. *Lane 1* soluble cellular fraction, *lane 2* insoluble fraction containing the inclusion bodies dissolved in 8 M urea-binding buffer, *lanes 3–8* fractions eluted with 500 mM imidazole. *M* molecular mass standard. GCR bands are indicated by the *arrows*. The gels were stained with Coomassie Blue

Production of GCR Antiserum

The purified recombinant GCR was inoculated in BALB/c mice with $Al(OH)_3$ as adjuvant, leading to the induction of anti-GCR IgG and IgM in the pooled sera, as evaluated by ELISA. The highest IgG titer was about 1:80,000 observed 1 week after the fourth immunization (Fig. 2a). IgG1 was the main subclass present in the serum, followed by IgG2a, IgM and IgG3 (Fig. 2b).

Immunodetection of GCR Expressed in COS-7 Cells

The mammalian expression pED vector containing the human GCR cDNA with its own signal peptide (pED-GCR), with its signal peptide replaced by the Igκ signal peptide (pED-IgκGCR), or fused to a $his_{\times 6}$ tag between the Igκ signal peptide and the GCR mature sequence (pED-Ig$\kappa_{6\times\text{his}}$GCR) were transfected in COS-7 cells for transient expression of GCR, in order to evaluate the specificity of the produced antibodies. The presence of glycosylated GCR bands with the molecular mass of approximately 64 kDa, expected for the cell-associated GCR [21], was detected in lysates of COS-7 cells transfected by the three plasmids (pED-Ig$\kappa_{6\times\text{his}}$GCR, pED-GCR and pED-Ig$\kappa$GCR) (Fig. 3, lanes 1, 2 and 4). An additional band of 56 kDa was detected in extract of COS-7 cells transfected with pED-GCR (Fig. 3, lane 2). A 56-kDa band that corresponds to non-glycosylated GCR [36] was also observed in lane 5 that contains the recombinant GCR purified from *E. coli*, used to produce the specific anti-GCR antibody. No band was visualized in

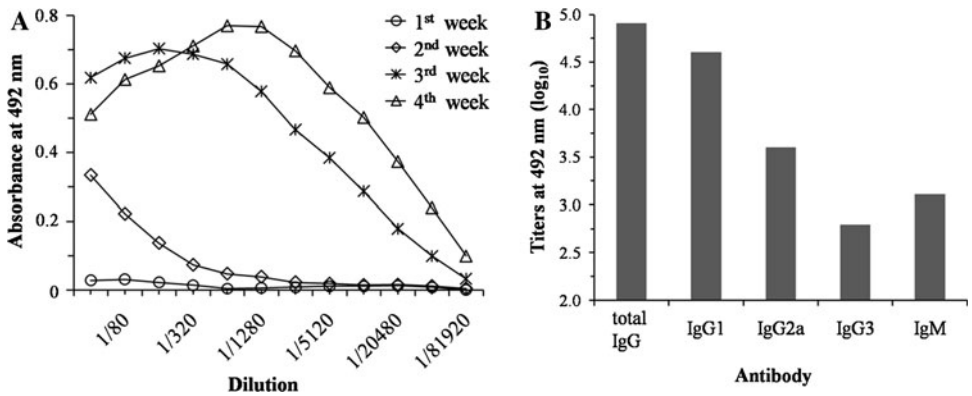

Fig. 2 Production of anti-GCR antibodies in BALB/c mice intraperitoneally immunized during 4 weeks with booster doses at every week with purified recombinant GCR from *E. coli*. The ELISA assays were performed by coating the 96-well plates with 10 μg/mL of the purified GCR protein. **a** Induction of total IgG in the pooled sera. **b** Log_{10} of total IgG, IgG1, IgG2a, IgG3 and IgM titers

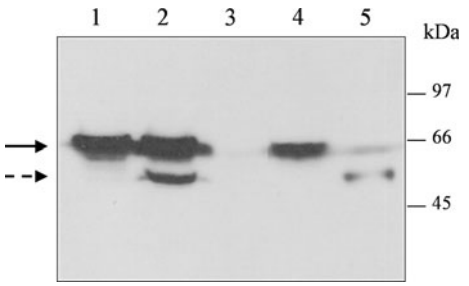

Fig. 3 Western blotting analysis of COS-7 cell extracts using murine anti-GCR polyclonal antibodies raised against the recombinant GCR purified from *E. coli*. *Lanes 1, 2 and 4* COS-7 cell extracts transfected with plasmids pED-Ig$\kappa_{6\times his}$GCR, pED-GCR and pED-IgκGCR, respectively, *lane 3* non-transfected COS-7 cell extract (negative control), *lane 5* recombinant human GCR purified from *E. coli* (positive control). The glycosylated GCR of approximately 64 kDa is indicated by a *solid arrow*. The 56-kDa protein, corresponding to non-glycosylated GCR, is indicated by a *dashed arrow*

the negative control (non-transfected COS-7 cells) (Fig. 3, lane 3).

Deglycosylation Assay of Recombinant GCR

To evaluate the secretion of GCR into the culture supernatant, media collected from cultures of COS-7 cells transfected with pED-IgκGCR plasmid were analyzed and also submitted to digestion with the N-glycosidase PNGase F. The western blotting shows that the non-digested GCR migrated with a molecular mass of approximately 66 kDa (Fig. 4, lane 3), which is in agreement to the expected mass for the glycosylated enzyme secreted from mammalian cells [1, 21]. The digested sample presented a shift to a lower molecular mass protein, similar to the recombinant protein produced from *E. coli* (56 kDa) (Fig. 4, lanes 4 and 5), as expected for non-glycosylated GCR. No bands were detected in the culture medium of non-transfected COS-7 cells (negative control) (Fig. 4, lanes 1 and 2).

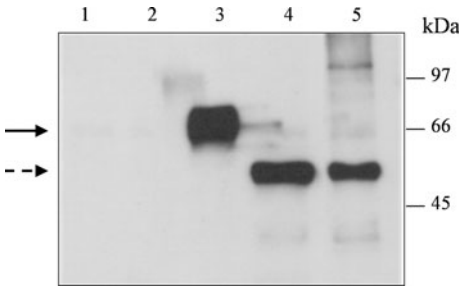

Fig. 4 PNGase F digestion of secreted recombinant GCR from COS-7 cells, detected by western blotting analysis using the murine polyclonal antibodies against recombinant GCR produced in *E. coli*. The media were precipitated with 10% TCA previously to PNGase F digestion. *Lanes 1, 2* culture medium of non-transfected COS-7 cells before and after digestion, respectively, *lanes 3, 4* culture medium of transfected COS-7 cells with plasmid pED-IgκGCR before and after digestion, respectively, *lane 5* recombinant human GCR purified from *E. coli*. The glycosylated GCR of approximately 66 kDa and the non-glycosylated GCR of 56 kDa are indicated by *solid* and *dashed arrows*, respectively

Immunodetection of Endogenous GCR in Human Cell Lines

In order to evaluate the potential of the produced polyclonal antibody to recognize GCR in human cells, extract of HeLa and ECV 304 cells were submitted to immunoblotting assay. Extracts of both human cell lineages showed an expected band around 59 kDa, corresponding to the mature-glycosylated GCR (Fig. 5, lanes 5 and 6). The glycosylated recombinant GCR ($\sim$64 kDa) from COS-7 cells transfected with pED-IgκGCR plasmid was used as positive control (Fig. 5, lane 1), as well as the non-glycosylated recombinant GCR purified from *E. coli* (56 kDa) (Fig. 5, lane 3). The band around 58 kDa detected in non-transfected COS-7 cells (Fig. 5, lane 2) possibly corresponds to endogenous GCR, since ECL Plus reagent and PVDF membrane were used here to increase the sensitivity of the assay which allowed the detection of endogenous human GCR.

Discussion

Anti-GCR antibodies are important tools for the characterization of GCR, the lysosomal enzyme that causes the Gaucher's disease when its enzymatic activity is impaired. Specific monoclonal and polyclonal antibodies have already been produced using mice or rabbit immunized with placenta-derived GCR [24, 30, 31], after complex procedures of enzyme purification [32]. Although polyclonal antibodies do not provide a continuous supply like monoclonal antibodies, there is no restriction to produce

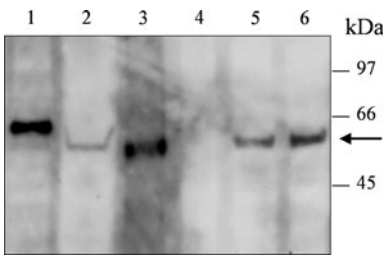

Fig. 5 Western blotting analysis of endogenous GCR in HeLa and ECV 304 human cells, using the produced murine anti-GCR polyclonal antiserum. *Lanes 1, 2* cellular extract of COS-7 cells transfected with the pED-IgκGCR plasmid and non-transfected COS-7 cells, respectively (control samples), *lane 3* recombinant human GCR of 56 kDa purified from *E. coli* (positive control), *lane 4* molecular mass standard, *lanes 5, 6* non-transfected HeLa and ECV 304 cell lysates, respectively. The endogenous-glycosylated GCRs from HeLa and ECV 304 cells are indicated by the *arrow*

the antigen for animal immunization purposes. Moreover, polyclonal antibodies have the advantage of being obtained in a relative short time, with little financial investment compared with monoclonal antibodies [37, 38], and can be successfully used in immunological techniques. The ability of polyclonal reagents to recognize a multiplicity of epitopes is interesting in some immunological assays, where the detection of a molecule could be compromised by the use of a single epitope [37]. Furthermore, the use of recombinant *E. coli*-derived GCR would also facilitate the production of monoclonal antibodies using immunized mice with this antigen. In addition to its applicability to discriminate the different molecular masses of GCR, both in healthy individuals and Gaucher's patients [11, 26–29], the anti-GCR antibodies can be used to analyze new recombinant enzyme formulations, proposed for therapeutic use. Since GCR is a glycosylated protein, its expression has been investigated in complex eukaryotic systems, such as plant and mammalian cells [17, 18, 22]. However, bacteria systems may represent an interesting alternative to obtain the enzyme for antibody production purposes. *E. coli* constitutes the most well-established expression platform that promotes high protein yield with low cost. Although in bacteria systems the GCR is inactive [36], specific GCR antiserum can be produced using a non-glycosylated GCR. Thus, in this work we described the GCR expression in *E. coli* and showed the production of anti-GCR polyclonal antibodies in BALB/c mice immunized with the purified protein. The GCR cDNA was not codon-optimized but the protein was successfully produced in *E. coli* BL21 SI. In addition, the use of pAE vector [34] to clone the GCR cDNA in frame with a his_{6x} tag at N-terminus allowed GCR purification by a single chromatographic step through nickel charged resin. The purified GCR was used to immunize mice, leading to high titers of antibodies, as evaluated by ELISA and shown in Fig. 2. Since this approach was very efficient, immunization of larger animal species can also be adapted to scale up the production of anti-GCR antiserum in larger quantities.

Moreover, in order to evaluate the efficacy of the antibody to detect wild-type glycosylated GCR, we have cloned the GCR cDNA into the mammalian pED expression vector [35]. Three different constructs were used for transient transfection of COS-7 cells. In the pED-GCR construct, the native GCR signal peptide was maintained, while in the pED-IgκGCR plasmid, the GCR mature sequence was fused to secretory Igκ signal peptide aiming to obtain high levels of the GCR into the culture medium. In addition, the pED-Ig$\kappa_{6\times his}$GCR plasmid expresses a GCR with a $his_{\times 6}$ tag at the N-terminus to facilitate the protein purification. In the western blotting analysis, using the produced antibodies, protein bands of 56 kDa and 64–66 kDa were detected as the non-glycosylated and glycosylated forms of GCR, respectively (Figs. 3, 4). According to the literature, the human GCR is a monomer of 497 amino acids that contains five putative *N*-glycosylation sites, four of which are normally occupied [9]. Its molecular size ranges from 59 to around 69 kDa [27], according to the complexity of the carbohydrate chains. As reviewed by Fabrega et al. [22], three bands have been reported to represent glycosylated GCR. Initially, the cleavage of the GCR leader peptide yields a non-glycosylated 56-kDa polypeptide that becomes the core of glycosylation reactions. A precursor form of approximately 63 kDa is synthesized with high-mannose-type oligosaccharide chains, and subsequent processing of the carbohydrates moieties results in the intermediary sialylated complex-type structures of 66–69 kDa. These enzyme forms are transported to the lysosomes and modified by lysosomal exoglycosidases, resulting in the 59-kDa GCR mature form [22, 29, 39].

GCR bands with approximately 64 kDa were recognized by the antibody in cell lysates transfected with the three plasmids: pED-GCR, pED-IgκGCR and pED-Ig$\kappa_{6\times his}$GCR. Additionally, a 56-kDa protein band, corresponding to a non-glycosylated GCR, was detected only in pED-GCR (Fig. 3, lane 2). In this case, the presence of the native GCR leader peptide possibly resulted in the highest levels of the GCR expression, causing accumulation of proteins to be directed to glycosylation process. On the other hand, the secreted GCR observed in the culture supernatant of cells transfected with the pED-IgκGCR plasmid displayed 66 kDa and the non-glycosylated form was not observed (Fig. 4, lane 3). The 56-kDa protein band was detected only after the deglycosylation reaction with PNGase F, a glycosidase that removes all N-linked carbohydrate (Fig. 4, lane 4). GCR secretion is a glycosylation-dependent process [21]. In addition, GCR is a membrane-associated lysosomal enzyme that, under natural conditions, is secreted from the cells at very low levels or even not secreted. However, the use of strong expression systems can lead to significant amount of GCR into the culture medium, as reported by Leonova and Grabowski [21]. These authors detected a molecular mass of approximately 69 kDa of secreted GCR from CHO and human fibroblast cells, while a 64 kDa was observed for intracellular GCR. Thus, the molecular forms of GCR detected by transient expression analysis here are in accordance to those described in the literature.

In addition to transient transfection analysis of COS-7 cells, the immunoblotting assay of human cell lineages demonstrated the potential of the polyclonal anti-GCR antibodies to recognize human samples. The molecular mass of 59 kDa detected in HeLa and ECV 304 cell lysates (Fig. 5, lanes 5 and 6) are in accordance to the expected GCR mature form [22, 29, 39].

In conclusion, we showed that the GCR expressed in *E. coli*, although inactive, was capable to induce the production of specific anti-GCR polyclonal antibodies, useful tools to characterize GCR in clinical samples or to monitor recombinant GCR expression. Furthermore, the success of the transient expression of GCR in mammalian cells indicates that the expression plasmids characterized here can be used for stable transfection in CHO cells for GCR production.

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