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Genetic Engineering of IgG-Glucuronidase Fusion Proteins

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Abstract

β -Glucuronidase (GUSB) is a lysosomal enzyme that could be developed as a brain therapy for Type VII Mucopolysaccharidosis. However, GUSB does not cross the blood-brain barrier (BBB). To enable BBB transport of the enzyme, human GUSB was re-engineered as a fusion protein with the chimeric monoclonal antibody (MAb) to the human insulin receptor (HIR). The HIRMAb crosses the BBB on the endogenous insulin receptor, and acts as a molecular Trojan horse to ferry into brain the GUSB. The 611 amino acid GUSB was fused to either the carboxyl or amino terminus of the heavy chain of the HIRMAb. This study illustrates the differential retention of functionality of IgG-enzyme fusion proteins depending on how the fusion protein is engineered.

Keywords

blood-brain barrier; drug targeting; monoclonal antibody; lysosomal enzyme

Introduction

β -Glucuronidase (GUSB), is a lysosomal enzyme that could be developed as a therapeutic molecule for either antibody-directed enzyme prodrug therapy (ADEPT) (deGraaf et al, 2002), or enzyme replacement therapy of Mucopolysaccharidosis Type VII (MPS-VII) (LeBowitz et al, 2004). In ADEPT, the enzyme is co-administered with a prodrug comprised of a β -glucuronide moiety, which is activated with the GUSB release of the sugar part of the prodrug. MPS-VII is a genetic disease leading to an inactive GUSB enzyme and the accumulation of lysosomal storage products. In the case of ADEPT or MPS-VII, it is necessary to treat the brain. However, GUSB enzyme therapy is not effective in the brain, as the large molecule GUSB enzyme does not cross the brain capillary endothelial wall, which forms the blood-brain barrier (BBB) in vivo.

The delivery of large molecule lysosomal enzymes, such as iduronidase (IDUA), which is the enzyme absent in MPS Type I, is possible following the re-engineering of the enzyme as an IgG-enzyme fusion protein (Boado et al, 2008). The IgG fusion partner is a peptidomimetic monoclonal antibody (MAb), which acts as a molecular Trojan horse to ferry the enzyme across the BBB (Boado et al, 2007a). A molecular Trojan horse is an endogenous peptide, or peptidomimetic MAb, that undergoes receptor-mediated transport across the BBB via endogenous peptide receptors (Pardridge, 2008). The BBB insulin receptor or transferrin receptor transport circulating insulin or transferrin, respectively, and also transports certain receptor-specific MAb's.

The delivery of IDUA across the BBB of the primate brain was made possible by fusion of the IDUA to the carboxyl terminus of the heavy chain of a chimeric MAb against the human insulin receptor (HIR), and this fusion protein was designated HIRMAb-IDUA (Boado et al, 2008). The fusion protein was shown to retain the bi-functional properties of the 2 fusion partners, i.e., high affinity binding to the HIR comparable to the HIRMAb, and IDUA enzyme activity comparable to recombinant IDUA. In the present study, human GUSB is fused to the heavy chain of the chimeric HIRMAb. The engineering of intron-free expression plasmids encoding the heavy chain (HC) and light chain (LC) of the chimeric HIRMAb has been described previously (Boado et al, 2007b). Initially, the GUSB was fused to the carboxyl terminus of the antibody heavy chain, and this fusion protein is designated HIRMAb-GUSB (Figure 1, left panel). Subsequently, the GUSB was fused to the amino terminus of the heavy chain of the HIRMAb, which is designated GUSB-HIRMAb (Figure 1, right panel). The 2 IgG-GUSB fusion proteins were tested for GUSB enzyme activity and for IgG binding to the HIR.

Materials and methods

Engineering of human GUSB expression vector

Human GUSB cDNA corresponding to amino acids Met₁-Thr₆₅₁ of the human GUSB protein (NP_000172), including the 22 amino acid signal peptide, and the 18 amino acid carboxyl terminal propeptide, was cloned by reverse transcription (RT) polymerase chain reaction (PCR) using the oligodeoxynucleotides (ODNs) and methods described previously (Zhang et al, 2008). PCR products were resolved in 1% agarose gel electrophoresis, and the expected major single band of ~2.0 kb corresponding to the human GUSB cDNA was isolated. The cloned human GUSB was inserted into the pcDNA eukaryotic expression plasmid as described previously (Zhang et al, 2008), and this GUSB expression plasmid was designated pCD-GUSB. The entire expression cassette of the plasmid was confirmed by bi-directional DNA sequencing.

Engineering of HIRMAb heavy chain-GUSB fusion protein expression vectors

The genetically engineered HIRMAb used in this study is a chimeric antibody, and the engineering of intron-less expression plasmids encoding the HC and LC of the chimeric HIRMAb were reported previously (Boado et al, 2007b). The pCD-HC-GUSB plasmid expresses the fusion protein wherein the carboxyl terminus of the heavy chain (HC) of the HIRMAb is fused to the amino terminus of human GUSB, minus the 22 amino acid GUSB signal peptide, and minus the 18 amino acid carboxyl terminal GUSB propeptide. This heavy chain fusion protein is designated HC-GUSB, and the pCD-HC-GUSB expression plasmid was cloned by PCR using the pCD-GUSB as template. The forward PCR primer introduces “CA” nucleotides to maintain the open reading frame and to introduce a Ser-Ser linker between the carboxyl terminus of the CH3 region of the HIRMAb HC and the amino terminus of the GUSB minus the 22 amino acid signal peptide of the enzyme. The HC-GUSB fusion protein includes an amino terminal 19 amino acid IgG signal peptide (Boado et al, 2007b). The GUSB reverse PCR primer introduces a stop codon, “TGA,” immediately after the terminal Thr of the mature human GUSB protein (Boado et al, 2007b). The fusion of the GUSB monomer to the carboxyl terminus of each HC is shown in Figure 1 (left panel), and this fusion protein is designated HIRMAb-GUSB.

The fusion protein wherein the GUSB is fused to the amino terminus of the HC of the HIRMAb is designated GUSB-HIRMAb (Figure 1, right panel), and this heavy chain fusion protein was expressed from the pCD-GUSB-HC plasmid DNA. The pCD-GUSB-HC plasmid expresses the fusion protein wherein the amino terminus of the heavy chain (HC) of the HIRMAb, minus its 19 amino acid signal peptide, is fused to the carboxyl terminus of human GUSB, including the 22 amino acid GUSB signal peptide, but minus the 18 amino acid carboxyl terminal GUSB

propeptide, The pCD-GUSB vector was used as template for PCR. The GUSB 18 amino acid carboxyl terminal propeptide in pCD-GUSB was deleted by site-directed mutagenesis (SDM). The latter created an AfeI site on the 3'-flanking region of the Thr⁶³³ residue of GUSB, and it was designated pCD-GUSB-AfeI. The carboxyl terminal propeptide was then deleted with AfeI and HindIII (located on the 3'-non coding region of GUSB). The HIRMAb HC open reading frame, minus the 19 amino acid IgG signal peptide and including the HIRMAb HC stop codon, was generated by PCR using the HIRMAb HC cDNA as template (Boado et al. 2007b). The PCR generated HIRMAb HC cDNA was inserted at the AfeI-HindIII sites of pCD-GUSB-AfeI to form the pCD-GUSB-HC. A Ser-Ser linker between the carboxyl terminus of GUSB and amino terminus of the HIRMAb HC was introduced within the AfeI site by the PCR primer used for the cloning of the HIRMAb HC cDNA. The fusion of the GUSB monomer to the amino terminus of each HC is depicted in Figure 1 (right panel), and this fusion protein is designated GUSB-HIRMAb.

Transient expression of GUSB, HIRMAb-GUSB, and GUSB-HIRMAb fusion proteins in COS cells

COS cells were plated in 6-well cluster dishes, and were transfected with (a) pCD-GUSB, (b) dual transfected with pCD-LC and pCD-HC-GUSB, or (c) dual transfected with pCD-LC and pCD-GUSB-HC, where pCD-LC is the expression plasmid encoding the light chain (LC) of the chimeric HIRMAb, as described previously (Boado et al, 2008). Transfection was performed using Lipofectamine 2000, with a ratio of 1:2.5, ug DNA:uL Lipofectamine 2000, and conditioned serum free medium was collected at 3 and 7 days. GUSB enzyme activity was measured in the medium. For production of larger amounts of fusion protein, COS cells were transfected in 10xT500 flasks. The 3 day and 7 day medium was pooled, and the 2 L of serum free conditioned medium was concentrated to 400 mL with tangential flow filtration followed by purification with protein A affinity chromatography, as described previously (Boado et al, 2008).

SDS-PAGE and Western blotting

The purity of protein A purified fusion protein produced by COS cells was evaluated with 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 5% β -mercaptoethanol. Immunoreactivity was tested with a primary goat antiserum against human IgG heavy and light chains (Vector Labs, Burlingame, CA), as described previously (Boado et al, 2008).

HIR receptor assay

The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with an ELISA. CHO cells permanently transfected with the HIR ECD were grown in serum free media (SFM), and the HIR ECD was purified with a wheat germ agglutinin affinity column, as previously described (Boado et al, 2007a). The HIR ECD was plated on 96-well dishes and the binding of the HIRMAb, the HIRMAb-GUSB fusion protein, or the GUSB-HIRMAb fusion protein to the HIR ECD was detected with a biotinylated goat anti-human IgG (H+L) secondary antibody, followed by avidin and biotinylated peroxidase (Vector Labs, Burlingame, CA). The concentration of either HIRMAb or fusion protein that gave 50% maximal binding, ED₅₀, was determined with a non-linear regression analysis (subroutine PAR of the BMDP2007 Statistical Software, Statistical Solutions, Dublin).

GUSB enzyme assay

The GUSB enzyme activity was determined with a fluorometric assay using 4-methylumbelliferyl β -L-glucuronide (MUGlcU), which was purchased from Molecular Probes-Invitrogen (Carlsbad, CA). This substrate is hydrolyzed to 4-methylumbelliferone (4-

MU) by GUSB, and the 4-MU is detected fluorometrically with a Farrand filter fluorometer using an emission wavelength of 450 nm and an excitation wavelength of 365 nm. A standard curve was constructed with known amounts of 4-MU (Sigma-Aldrich, St. Louis, MO). The assay was performed at 37°C with 60 min incubations at pH=4.8, and was terminated by the addition of glycine-carbonate buffer (pH=10.5).

Results

DNA sequencing of the expression cassette of the pCD-HC-GUSB expression plasmid encompassed 4,321 nucleotides (nt), including a 714 nt cytomegalovirus (CMV) promoter, a 9 nt Kozak site (GCCGCCACC), a 3,228 nt HC-GUSB fusion protein open reading frame, and a 370 nt bovine growth hormone (BGH) transcription termination sequence. The plasmid encoded for a 1,075 amino acid protein, comprised of a 19 amino acid IgG signal peptide, the 113 amino acid variable region of the HIRMAb heavy chain, the 330 amino acid human IgG1 constant region, a 2 amino acid linker (Ser-Ser), and the 611 amino acid human GUSB minus the enzyme signal peptide and carboxyl terminal propeptide. The GUSB sequence of the HIRMAb-GUSB fusion protein was 100% identical to Leu²³-Thr⁶³³ of human GUSB (NP_000172). The predicted molecular weight of the heavy chain fusion protein, minus glycosylation, is 119,306 Da, with a predicted isoelectric point (pI) of 7.83. DNA sequencing of the pCD-GUSB-HC expression cassette showed the plasmid expressed 1,078 amino acid protein, comprised of a 22 amino acid GUSB signal peptide, the 611 amino acid GUSB, a 2 amino acid linker (Ser-Ser), the 113 amino acid variable region of the HIRMAb heavy chain, and the 330 amino acid human IgG1 constant region. The GUSB sequence of the GUSB-HIRMAb fusion protein was 100% identical to Met¹-Thr⁶³³ of human GUSB (NP_000172). Both IgG-GUSB fusion proteins were formed from the same light chain of the chimeric HIRMAb, which was comprised of a 20 amino acid IgG signal peptide, a 108 amino acid variable region of the HIRMAb light chain, and a 106 amino acid constant region of human IgG kappa (Boado et al, 2007b).

Transfection of COS cells in a 6-well format with the pCD-GSUB resulted in high GUSB enzyme activity in the conditioned medium at 7 days (Table I, Experiment A). However, there was no specific increase in GUSB enzyme activity following dual transfection of COS cells with the pCD-HC-GUSB and pCD-LC expression plasmids (Table I, Experiment B). However, the low GUSB activity in the medium could be attributed to the low secretion of the HIRMAb-GUSB fusion protein, as the medium IgG was only 23 ± 2 ng/mL, as determined by a human IgG-specific ELISA described previously (Boado et al, 2007b). Therefore, COS cell transfection was scaled up to 10xT500 plates, and the HIRMAb-GUSB fusion protein was purified by protein A affinity chromatography. IgG Western blotting demonstrated the expected increase in size of the fusion protein heavy chain (Figure 2). The HIR receptor assay showed there was no decrease in affinity for the HIR following fusion of the 611 amino acid GUSB to the carboxyl terminus of the HIRMAb heavy chain (Figure 3). However, the GUSB enzyme activity of the HIRMAb-GUSB fusion protein was low at 6.1 ± 0.1 nmol/hr/ug protein.

Dual transfection of COS cells in a 6-well format with the pCD-LC and pCD-GUSB-HC expression plasmids resulted in higher GUSB enzyme activity in the conditioned medium at 7 days, as compared to dual transfection with the pCD-LC and pCD-HC-GUSB plasmids (Table I, Experiment C). However, the GUSB-HIRMAb fusion protein was also secreted poorly by the COS cells, as the medium human IgG concentration in the 7 day conditioned medium was only 13 ± 2 ng/mL, as determined by ELISA. COS cell transfection was scaled up to 10xT500 plates, and the GUSB-HIRMAb fusion protein was purified by protein A affinity chromatography. SDS-PAGE demonstrated the expected increase in size of the fusion protein heavy chain (Figure 4). The GUSB enzyme activity of the purified GUSB-HIRMAb fusion protein was high at 226 ± 8 nmol/hr/ug protein, which is 37-fold higher than the specific GUSB

enzyme activity of the HIRMAb-GUSB fusion protein. However, the HIR receptor assay showed there was a marked decrease in affinity for the HIR following fusion of the GUSB to the amino terminus of the HIRMAb heavy chain, which resulted in a 19-fold reduction in receptor binding affinity (Figure 5).

Discussion

The results of this study are consistent with the following conclusions. First, fusion genes have been genetically engineered, which encode a fusion protein wherein the amino terminus of human GUSB is fused to the carboxyl terminus of the CH3 region of the HC of the chimeric HIRMAb (Figure 1, left panel, HIRMAb-GUSB fusion protein), or the carboxyl terminus of human GUSB is fused to the amino terminus of the heavy chain of the chimeric HIRMAb (Figure 1, right panel, GUSB-HIRMAb fusion protein). Second, following expression in COS cells, the HC of the fusion protein is about 80 kDa larger than the HC of the chimeric HIRMAb (Figure 4), and the fusion protein HC reacts with an anti-human IgG antibody (Figure 2). Third, the HIRMAb-GUSB fusion protein retains high affinity binding to the HIR (Figure 3), but demonstrates a >95% reduction in GUSB enzyme specific activity (Methods). Fourth, the GUSB-HIRMAb fusion protein has high GUSB enzyme specific activity, 226 ± 8 nmol/hr/ug protein (Methods), but has a 95% reduction in binding affinity to the HIR (Figure 5).

The HIRMAb-GUSB and GUSB-HIRMAb fusion proteins are both secreted poorly, as dual transfection of COS cells with the heavy chain and light chain expression plasmids results in human IgG levels of 10-13 ng/mL in the 7 day conditioned medium (Results). In contrast, a HIRMAb-IDUA fusion protein has been engineered, and the HIRMAb-IDUA fusion protein is secreted well to the medium following dual transfection of COS cells, which resulted in medium IgG levels >1,000 ng/mL (Boado et al, 2008). The low secretion of the IgG-GUSB fusion proteins was not due to the low secretory capacity of the GUSB enzyme, as this protein was actively secreted to the medium, resulting in medium enzyme activity of 6892 ± 631 nmol/hr/mL following transfection with pCD-GUSB (Table 1, Experiment A). Given a specific activity of 2,000 nmol/hr/ug protein for recombinant GUSB (Sands et al, 1994), the medium concentration of the GUSB enzyme was >3,000 ng/ml, or >300-fold higher than the IgG-GUSB fusion proteins. The finding of the present study on the low secretion by COS cells of IgG-GUSB fusion proteins parallels prior observations showing a 10-fold reduction in secretion by transfected COS cells of a GUSB fusion protein, wherein the amino terminus of the GUSB was fused to the carboxyl terminus of a single chain Fv (ScFv) antibody (Haisma et al, 1998). GUSB enzyme activity of the ScFv-GUSB fusion protein was not measured (Haisma et al, 1998). In another construct, the amino terminus of mature GUSB was fused to the carboxyl terminus of an engineered antibody comprised of the variable region of the heavy chain (VH) and the CH1 domain of the human IgG3 constant-region, and the GUSB enzyme activity was 720 nmol/hr/ug protein, which indicated the GUSB enzyme activity was retained in this construct (Bosslet et al, 1994). However, this IgG3-GUSB fusion protein differs from the fusion protein engineered in the present study in 2 respects. First, the hinge region of human IgG3 is longer and more flexible than the hinge region of the human IgG1 hinge region used in the present study. Second, the GUSB was fused to the entire CH1-hinge-CH2-CH3 sequence of the constant-region of human IgG1 in the present study. This may have constrained the GUSB in a conformation that resulted in a loss of enzyme activity. The loss of GUSB enzyme activity with the engineering of the HIRMAb-GUSB construct is unlikely to be due to the placement of the GUSB enzyme in the dimeric configuration shown in Figure 1 (left panel). GUSB normally forms a dimer in its native conformation (Gehrmann et al, 1994). The loss of GUSB enzyme activity of the HIRMAb-GUSB fusion protein may be due the the initial folding of the IgG heavy chain, which is translated within host cell before the translation and folding of the GUSB part of the IgG-enzyme fusion protein. Conversely, the GUSB part of the fusion protein

is translated and folded before the IgG part of the fusion protein with the GUSB-HIRMAb fusion protein (Figure 1, right panel).

In the case of the GUSB-HIRMAb fusion protein (Figure 1, right panel), the carboxyl terminus of the mature GUSB is fused to the amino terminus of the heavy chain of the HIRMAb. This construct is similar to the GUSB-IGF2 fusion protein reported previously (LeBowitz et al, 2004), wherein the mature GUSB, minus the 18 amino acid carboxyl terminal propeptide, is fused, via a 3 amino acid linker, to the 60 amino acid human insulin-like growth factor (IGF)-2. The IGF-2 was used as a molecular Trojan horse to ferry the GUSB into brain, owing to the expression of IGF-1 and IGF-2 receptors on the BBB (Duffy et al, 1988). However, the IGFs are >99% bound by IGF-binding proteins (IGFBP) in the plasma, and such IGFBP binding effects may impair IGF transport across the BBB in vivo (Pan et al, 2000). Nevertheless, the GUSB enzyme activity was preserved following expression of the GUSB-IGF2 fusion protein (LeBowitz et al, 2004). This observation parallels that of the present study, which shows high GUSB enzyme activity for the GUSB-HIRMAb fusion protein (Results), when the GUSB is fused to the amino terminus of the IgG heavy chain (Figure 1, right panel). However, the binding affinity of the HIRMAb for the HIR is a function of the complementarity determining regions (CDRs) of the heavy chain of the HIRMAb, and these CDRs are located near the amino terminus of the heavy and light chains of the antibody (Boado et al, 2007a). Fusion of the 80 kDa GUSB to the amino terminus of the heavy chain of the HIRMAb results in a 19-fold reduction in GUSB-HIRMAb fusion protein binding to the HIR (Figure 5). This is an unacceptable loss of HIR binding affinity and predicts the GUSB-HIRMAb fusion protein would be transported across the BBB in vivo poorly.

In summary, the present studies describe the bi-functional properties of 2 different constructs of IgG-GUSB fusion proteins (Figure 1). In the case of the HIRMAb-GUSB fusion protein, there is retention of HIR binding activity, but a loss of GUSB enzyme activity. In the case of the GUSB-HIRMAb fusion protein, there is retention of the GUSB enzyme activity, but a loss of HIR binding activity. In future studies, the enzyme activity of the Trojan horse-GUSB fusion protein may be restored by use of an alternative BBB molecular Trojan horse. Or, the enzyme activity of the HIRMAb-GUSB fusion protein may be restored by an increase in the length of the linker between the carboxyl terminus of the IgG heavy chain and the amino terminus of the lysosomal enzyme. Nevertheless, these properties may be specific to IgG-GUSB fusion protein, since prior studies demonstrate retention of both lysosomal enzyme activity, and high HIR binding activity of a HIRMAb-IDUA fusion protein (Boado et al, 2008).

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HIRMAb-GUSB

(enzyme fused to carboxyl terminus of MAb)

GUSB-HIRMAb

(enzyme fused to amino terminus of MAb)

Figure 1.

Structures of HIRMAb-GUSB fusion proteins. In the structure on the left, the human GUSB protein without its 22 amino acid signal peptide, or its 18 amino acid carboxyl terminal propeptide, is fused to the carboxyl terminus of the heavy chain of the chimeric HIRMAb. In the structure on the right, the human GUSB protein with its 22 amino acid signal peptide, but without its 18 amino acid carboxyl terminal propeptide, is fused to the amino terminus of the heavy chain of the chimeric HIRMAb.

Human IgG Western Blot

Figure 2.

Western blot with an anti-human IgG primary antibody, where the immunoreactivity of the HIRMAb-GUSB fusion protein is compared to the chimeric HIRMAb. Both the HIRMAb-GUSB fusion protein and the HIRMAb have identical light chains on the anti-IgG Western. The size of the HIRMAb-GUSB fusion heavy chain, 130 kDa, is about 80 kDa larger than the size of the heavy chain of the HIRMAb, owing to the fusion of the 80 kDa GUSB to the 50 kDa HIRMAb heavy chain.

Figure 3. Binding of either the chimeric HIRMAb or the HIRMAb-GUSB fusion protein to the HIR extracellular domain (ECD) is saturable. The ED₅₀ of HIRMAb-GUSB binding to the HIR ECD is comparable to the ED₅₀ of the binding of the chimeric HIRMAb.

Figure 4. Reducing SDS-PAGE of the HIRMAb and the GUSB-HIRMAb fusion protein shows both proteins have identical light chains, and that the size of the GUSB-HIRMAb fusion heavy chain, 130 kDa, is about 80 kDa larger than the size of the heavy chain of the HIRMAb.

Figure 5. Binding of either the chimeric HIRMAb or the GUSB-HIRMAb fusion protein to the HIR extracellular domain (ECD) is saturable. The ED₅₀ of HIRMAb-GUSB binding to the HIR ECD, 4.8 ± 0.4 nM, is over 19-fold elevated compared to the ED₅₀ of the binding of the chimeric HIRMAb, 0.25 ± 0.03 nM,.

Table 1

GUSB enzyme activity in COS cells following transfection

Experiment	Treatment	Medium GUSB activity (nmol/hour/mL)
A	Lipofectamine 2000 only	65 ± 1
	pCD-GUSB	6892 ± 631
B	Lipofectamine 2000 only	76 ± 3
	pCD-HC-GUSB, pCD-LC	72 ± 3
C	Lipofectamine 2000 only	162 ± 7
	pCD-HC-GUSB, pCD-LC	155 ± 2
	pCD-GUSB-HC, pCD-LC	1119 ± 54

Mean ± SE (n=3 dishes per point).