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Surface plasmon resonance analysis of complex formation of therapeutic recombinant lysosomal enzymes with domain 9 of human cation-independent mannose 6-phosphate receptor

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ABSTRACT

The efficacy of enzyme replacement therapy (ERT) for lysosomal storage diseases (LSDs) possibly depends on the cellular uptake of recombinant lysosomal enzymes (LEs), and it is known that cation-independent mannose 6-phosphate receptor (CI-M6PR) on the cell membrane is predominantly involved in the endocytosis of many LEs. To examine the biomolecular interaction between therapeutic LEs and CI-M6PR, we biophysically analyzed the complex formation of four LEs available with domain 9 of human CI-M6PR, a binding site of the receptor, by means of surface plasmon resonance (SPR) biosensor assays. The results revealed that the affinity of the LEs for domain 9 of the receptor increased in the following order: laronidase, agalsidase beta, idursulfase, and alglucosidase alfa; and the high affinity of laronidase for domain 9 of CI-M6PR was due to fast complex formation rather than slow dissociation of the complex. The affinity of the enzymes for domain 9 of CI-M6PR almost coincided with their cellular uptake. The SPR biosensor assay is sensitive and provides important information for the development of effective therapeutic LEs for LSDs.

1. Introduction

Lysosomal storage diseases (LSDs) are a group of inherited metabolic disorders characterized by accumulation of biological substrates in lysosomes, caused by a deficiency of a specific lysosomal enzyme (LE) or a defect of a lysosome-related molecule [1,2]. The impairment of lysosomal function due to storage of undegraded or partially degraded substrates leads to a range of clinical manifestations of various organs and tissues. Efforts to develop effective therapies such as hematopoietic stem cell transplantation, enzyme replacement therapy (ERT), small molecule therapy, and gene therapy for LSDs have been made for the last 2–3 decades, which have provided results, and ERT especially has been successfully used for many LSDs [1,2].

ERT is based on the intravenous administration of a recombinant LE. The administered enzyme is incorporated from the blood into cells through endocytosis, and then exhibits catalytic activity, replacing that of the missing or dysfunctional one [3,4]. Although various cell-surface receptors may be involved in the uptake of LEs, the cation-independent mannose 6-phosphate receptor (CI-M6PR) is thought to play a major role in the uptake of many LEs [3–6]. It is known that CI-M6PR is a type I integral membrane glycoprotein with a molecular weight of approximately 300 kDa, and that it recognizes mannose 6-phosphate (M6P) residues at the non-reducing ends of the sugar chains of LEs [7].

Previously, we established a method for measuring M6P residues on glycoproteins, and determined the numbers of M6P residues on some recombinant LEs [8]. Then, we examined the binding of the exposed M6P residues of recombinant LEs with domain 9 of CI-M6PR, one of the binding sites of the receptor, by means of Western blotting. The results suggested that the levels of exposed M6P residues of the recombinant LEs were associated with their binding with domain 9 of CI-M6PR [8]. However, not only the level of M6P residues but also other factors such as the structures of the sugar chains of LEs possibly influence the binding of LE molecules with the receptor [4,9,10], and elucidation of the mechanisms of the complex formation between recombinant LEs with CI-M6PR will be required to develop therapeutic enzymes that can be effectively incorporated into the target cells.

Abbreviations: ERT, enzyme replacement therapy; LSD, lysosomal storage disease; LE, lysosomal enzyme; CI-M6PR, cation-independent mannose 6-phosphate receptor; SPR, surface plasmon resonance; M6P, mannose 6-phosphate; PBS, phosphate-buffered saline;; CD-M6PR, cation-dependent mannose 6-phosphate receptor.

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In this study, we examined the kinetics of the complex formation between LEs and domain 9 of human CI-M6PR by means of surface plasmon resonance (SPR) biosensor assays, and compared the results with those of cellular uptake examinations.

2. Materials and methods

2.1. Materials

Agalsidase beta (Fabrazyme®: therapeutic recombinant α-galactosidase A for Fabry disease), laronidase (Aldurazyme®: therapeutic recombinant α-L-iduronidase for mucopolysaccharidosis type I), idursulfase (Elaprase®: therapeutic recombinant iduronate sulfatase for mucopolysaccharidosis type II (this enzyme produced by Shire H.G.T. (Lexington, MA; Current company name: Takeda Pharmaceutical Co., Tokyo, Japan) is marketed by Sanofi K.K. (Tokyo, Japan) in Japan), and alglucosidase alfa (Myozyme®: therapeutic recombinant acid α-glucosidase for Pompe disease) were purchased from Sanofi K.K. His-tagged-domain 9 of human CI-M6PR was produced by the Glycoscience & Glycotechnology Research Group, Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan) [8,11]. Other chemicals of analytical grade were obtained from usual commercial sources.

2.2. Protein determination

Protein was measured with a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL) using bovine serum albumin as a standard, according to the manufacturer's instructions.

2.3. Determination of binding-kinetics parameters of complex formation between LEs and domain 9 of human CI-M6PR by means of SPR biosensor assays

The interaction between recombinant LEs (agalsidase beta, laronidase, idursulfase, and alglucosidase alfa) and domain 9 of human CI-M6PR was examined using a BIAcore X100 biosensor system (Citiva, Tokyo, Japan). The SPR measurements were performed at 25 °C. Domain 9 of human CI-M6PR (ligand) was coupled to a certified grade CM5 chip (Citiva) using the amine coupling kit supplied by the manufacturer, and then the unreacted species on the surface of the chip were blocked with ethanolamine [12]. Then, determination of the binding-kinetics parameters was performed according to the manufacturer's method. For the formation of the complex, the solution of each LE (analyte: 15–120 ng) was passed over the chip at a flow rate of 30 μL/min. For dissociation of the complex, HBS-EP buffer, pH 7.4, was passed over the chip on which the ligand/analyte complex was coated.

2.4. Examination of cellular uptake of fluorescence-labeled recombinant LEs

To examine cellular uptake of the therapeutic recombinant LEs by cultured cells, the enzyme proteins were fluorescence-labeled using a HiLyte Fluor™ 555 labeling kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

Cultured mouse fibroblasts [13] were cultured in DMEM medium containing 10% (*v*/v) heat-inactivated fetal calf serum and antibiotics at 37 °C in a humidified incubator continuously flushed with a mixture of 5% CO_2–95% air. Then, the cells were spread on a 6-well plate at a density of 3×10^5 cells/well, and cultured for 24 h. After discarding the culture medium, the cells were washed with phosphate-buffered saline (PBS), pH 7.4, and further cultured in 2 mL of Ham's F10 medium containing 10 pmol of each HiLyte Fluor 555-labeled enzyme and 10% (*v*/v) heat-inactivated fetal calf serum. After 24 h incubation, the culture medium was discarded and the cells were washed with PBS. Next, the cells were harvested and sonicated in 20 mmol/L MES buffer, pH 6.0, containing protease inhibitors. Then, a sample was centrifuged and the supernatant was obtained. Finally, the fluorescence intensity of the supernatant was measured with an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA) at excitation and emission wavelengths of 555 and 570 nm, respectively. Cellular uptake of each fluorescence-labeled enzyme was calculated from the results of a calibration test.

2.5. Statistical analysis

The experimental value for each group is basically expressed as the mean ± SD [n: number of samples]. The correlation coefficient (r) between the targeted groups was calculated using Excel 2016 (Microsoft, Redmond, WA).

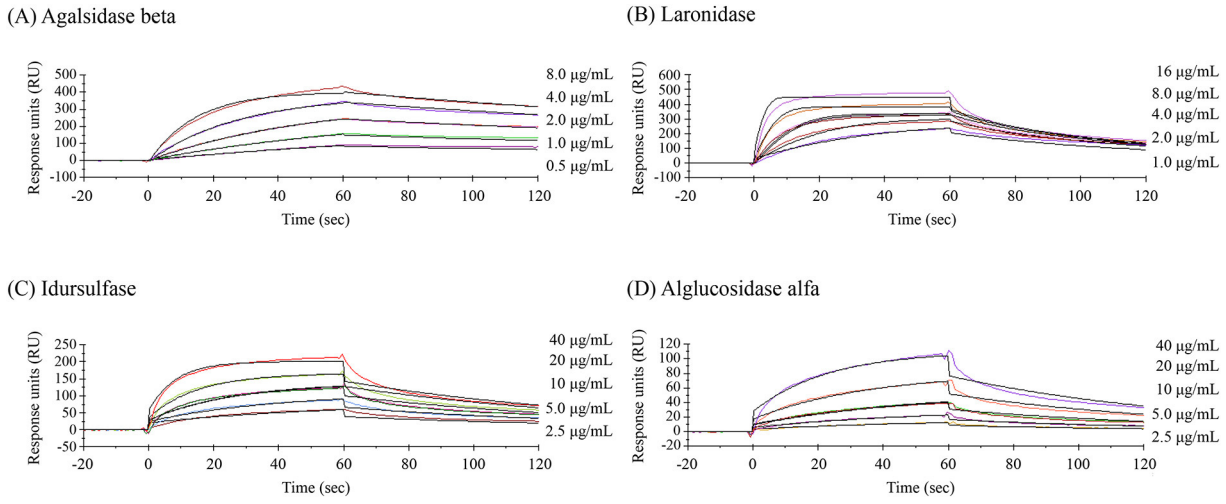

Fig. 1. Interaction between domain 9 of CI-M6PR and the LEs.
Representative sensorgrams obtained on SPR analyses showing response units (RUs) with increasing concentrations of the LEs are shown. (A) agalsidase beta, (B) laronidase, (C) idursulfase, and (D) alglucosidase alfa.

Table 1
Kinetics parameters for recombinant LEs/domain 9 of human CI-M6PR complex formation determined by means of SPR biosensor assays.

Disease	Recombinant eyzyme		Binding-kinetics parameters			M6P (mol/mol)[c,e]
	Generic name	Brand name	*ka* (L/mol·s × 10^5)[c]	*kd* (1/s × 10^{-3})[c]	K_D (mol/L × 10^{-8})[d]	
Fabry disease	Agalsidase beta	Fabrazyme®	5.5 ± 0.4 (3)	9.2 ± 0.3 (3)	1.7	2.9 ± 0.1 (5)
MPS I[a]	Laronidase	Aldurazyme®	31 ± 2 (3)	27 ± 1 (3)	0.87	2.5 ± 0.2 (5)
MPS II[b]	Idursulfase	Elaprase®	1.7 ± 0.1 (3)	12 ± 1 (3)	7.2	3.2 ± 0.2 (5)
Pompe disease	Alglucosidase alfa	Myozyme®	0.84 ± 0.03 (3)	13 ± 0 (3)	15	0.7 ± 0.1 (5)

[a] MPS I: mucopolysaccharidosis type 1.
[b] MPS II: mucopolysaccharidosis type 2.
[c] Values are presented as means ± SD (n).
[d] The K_D value was calculated as kd/ka.
[e] Described in Ref. [8].

3. Results

3.1. Kinetics of complex formation between LEs and domain 9 of human CI-M6PR

The binding reaction between each recombinant LE and domain 9 of human CI-M6PR was biophysically examined by means of SPR biosensor assays. Sensorgrams obtained on SPR analysis of these enzymes are shown in Fig. 1, and the results of the examination are summarized in the (Table 1). The rate constants of complex formation and dissociation (*ka* and *kd*) for the enzymes in the reaction were determined. From the results, the equilibrium constant (K_D) values for laronidase, agalsidase beta, idursulfase, and alglucosidase alfa in the binding reaction were calculated to be 0.87, 1.7, 7.2, and 15 mol/L × 10^{-8}, respectively. These results revealed that laronidase had the highest affinity for domain 9 of human CI-M6PR although the M6P content of laronidase was a little less than those of agalsidase beta and idursulfase, and alglucosidase alfa most weakly bound out of the four recombinant LEs examined. The results further suggested that the strong binding of laronidase with domain 9 of the receptor was due to fast complex formation rather than dissociation of the complex, considering the *ka* and *kd* values.

3.2. Uptake of therapeutic recombinant LEs by cultured mouse fibroblasts

The uptake of each fluorescence-labeled therapeutic recombinant LE by cultured mouse fibroblasts was examined, and the results are summarized in Fig. 2. The enzymes were efficiently incorporated into the fibroblasts in the following order: laronidase > idursulfase ≃ agalsidase beta > alglucosidase alfa. The cellular uptake of the LEs was compared with their K_D values determined on SPR analysis, and the results are shown in Fig. 3. As the results, negative correlation was found between the K_D values of the LEs and the amounts of fluorescence-labeled enzyme proteins incorporated into the cells ($r = -0.76$), suggesting that the affinity of LEs for domain 9 of CI-M6PR was associated with their cellular uptake. On the other hand, the calculated correlation coefficient values between the M6P contents of the LEs and the amounts of enzyme proteins incorporated into the cells were not so high ($r = 0.44$).

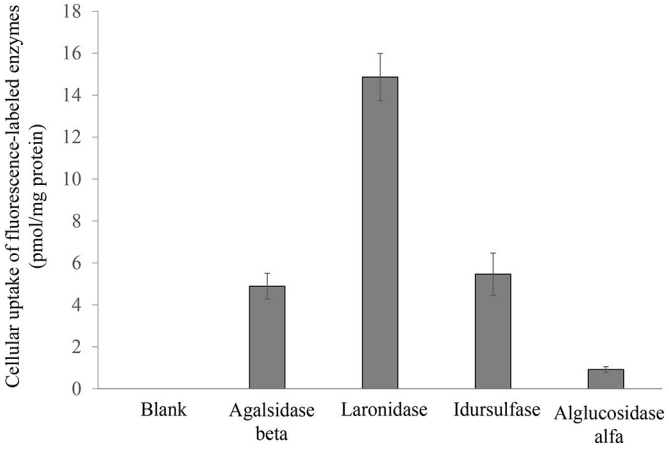

Fig. 2. Uptake of the LEs by cultured mouse fibroblasts.
The uptake of enzyme proteins is represented as the amounts of fluorescence-labeled enzymes incorporated into the cells based on the calibration test, and each value is exhibited as the mean ± SD [$n = 4$]. A "blank" experiment was performed without a fluorescence-labeled enzyme.

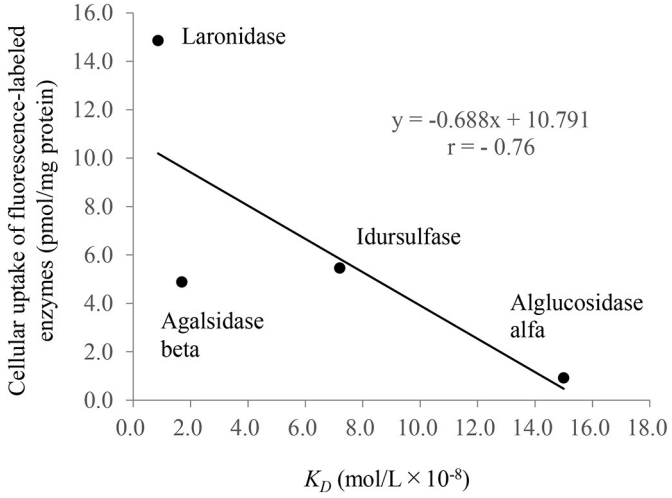

Fig. 3. Correlation between K_D values and amounts of LEs incorporated into the cells.
The correlation coefficient (r) between them was calculated to be −0.76.

4. Discussion

ERT is an effective treatment for many LSDs, and it reduces substrates accumulated in the affected organs and tissues [1,2]. The therapeutic effectiveness of ERT generally depends on the cellular uptake of recombinant LEs via widely distributed receptors which recognize M6P residues of the enzymes [3,4,14,15].

It is known that there are two kinds of specific receptors that recognize the M6P residues of glycoconjugates including LEs [3,4]. They are CI-M6PR and the cation-dependent M6P receptor (CD-M6PR), the former on the cell membrane being thought to predominantly bind extracellular LEs and mediate their cellular internalization [3]. On the other hand, CD-M6PR participates inefficiently in the uptake of LEs because it binds LEs poorly on the cell membrane [16,17].

CI-M6PR is a huge integral membrane protein composed of three structural regions: an extracellular region consisting of 15 homologous

domains, a single transmembrane one, and a cytoplasmic one [7]. Two M6P binding sites with high affinity were first mapped to domains 3 and 9, respectively, of the extracellular region [18,19], and then domain 5 exhibiting lower affinity than the two M6P-binding domains [20]. As to domain 3 of the receptor, there is a report that a construct encoding domain 3 alone exhibits about 1000 fold lower affinity for a LE than domains 1–3, suggesting that the affinity of domain 3 is enhanced by the presence of additional regions of the receptor [21]. Recently, Olson et al. reported that CI-M6PR contained a fourth carbohydrate-recognition site, domain 15, and that it possibly utilized another domain to enhance the affinity [10]. Considering the above, we used recombinant domain 9 of CI-M6P as a ligand for SPR analysis in this study.

It has been reported that not only the content of M6P residues but also the glycan structures are possibly associated with the binding with CI-M6PR and the cellular uptake of glycoconjugates; i.e., Yamaguchi et al. reported that a single M6P moiety located on the α-1, 3-branch of the oligomannose context was sufficient for high-affinity binding to CI-M6PR, while the presence of a M6P moiety on the α-1, 6-branch was dispensable. They further revealed that the close proximity of two M6P-oligosaccharide ligands was critical for the high affinity for the CI-M6PR [22]. Furthermore, Zhou et al. reported that the phosphorylated dimannose moiety appeared to be the minimal structure determinant for enhanced CI-M6PR binding and that the orientation of the glycan is critical for maximum receptor interaction [23].

Thus, we tried to determine the kinetics of complex formation between therapeutic recombinant LEs and domain 9 of CI-M6PR by means of SPR biosensor assays. This biophysical assay system is very sensitive and useful for analysis of complex formation. The results revealed that laronidase exhibited strong binding with domain 9 of human CI-M6PR, and then the other three enzymes examined in the following order: agalsidase beta, idursulfase, and alglucosidase alfa, the high affinity of laronidase for domain 9 of CI-M6PR being due to fast complex formation rather than slow dissociation of the complex. The affinity of the enzymes for domain 9 of CI-M6PR almost coincided with their cellular uptake. Such strength of the binding reaction may reflect the dosages of the enzymes clinically used: laronidase, 0.58 mg/kg (once per week); agalsidase beta, 1 mg/kg (every other week); idursulfase, 0.5 mg/kg (once per week); and alglucosidase alfa 20 mg/kg (every other week) [2].

This study has two limitations. As human CI-M6PR is a huge molecule and we could not prepare the whole protein by means of genetic engineering, we here used domain 9 of the receptor as a ligand. In the next study, usage of the whole CI-M6PR protein is expected. The cellular uptake of LEs may depend on the cell type and the biochemical characteristics of the enzymes.

5. Conclusion

We examined the biomolecular interaction between therapeutic recombinant LEs and domain 9 of human CI-M6PR by means of SPR biosensor assays. The results provided a lot of information for elucidating the mechanisms of the LEs/CI-M6PR complex formation. The SPR biosensor assay is sensitive, and it will be useful for finding new enzyme proteins that can be efficiently incorporated into the target cells for LSDs.

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Conflicts of interest

We declare that none of the authors have any competing interests.

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