Directed Evolution of a Lysosomal Enzyme with Enhanced Activity at Neutral pH by Mammalian Cell-Surface Display

Kai-Chuan Chen,1,2,5 Chia-Hung Wu,1,5 Chuan-Yuan Chang,1 Wei-Cheng Lu,1 Qingzong Tseng,1 Zeljko M. Prijovich,1 Wolfgang Schechinger,1,6 Yen-Chywan Liaw,3 Yu-Lin Leu,4 and Steve R. Roffler1,*

1Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan
2Department of Microbiology and Immunology, National Yang-Ming University, Taipei 11221, Taiwan
3Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan
4Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan
5These authors contributed equally to this work
6Present address: Bioavid Diagnostics GmbH & Co. KG, 64293 Darmstadt, Germany
*Correspondence: sroff@ibms.sinica.edu.tw
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SUMMARY

Human β-glucuronidase, due to low intrinsic immunogenicity in humans, is an attractive enzyme for tumor-specific prodrug activation, but its utility is hindered by low activity at physiological pH. Here we describe the development of a high-throughput screening procedure for enzymatic activity based on the stable retention of fluorescent reaction product in mammalian cells expressing properly folded glycoproteins on their surface. We utilized this procedure on error-prone PCR and saturation mutagenesis libraries to isolate β-glucuronidase tetramers that were up to 60-fold more active (kcat/Km) at pH 7.0 and were up to an order of magnitude more effective at catalyzing the conversion of two structurally disparate glucuronide prodrugs to anticancer agents. The screening procedure described here can facilitate investigation of eukaryotic enzymes requiring posttranslational modifications for biological activity.

INTRODUCTION

Chemotherapy is an important treatment modality for advanced cancers, but systemic toxicity typically limits therapeutic efficacy. Antibody- (Bagshawe et al., 1988; Senter et al., 1988) and gene-directed enzyme prodrug therapy (Chen et al., 1994) (ADEPT and GDEPT) are approaches to increase the therapeutic index of cancer chemotherapy. ADEPT and GDEPT employ antibody-targeted or gene-expressed enzymes to preferentially activate nontoxic antineoplastic drugs at cancer cells, thereby sparing normal tissues from high concentrations of toxic drug. Although microbial and viral enzymes are largely utilized for targeted prodrug activation, the development of strong immune responses against foreign proteins can rapidly hinder therapeutic effectiveness (Napier et al., 2000; Sharma et al., 1992). The utilization of human enzymes is therefore highly desirable to minimize immune responses and allow multiple rounds of treatment.

β-glucuronidase (βG, EC 3.2.1.31) is an attractive enzyme for ADEPT and GDEPT because both mammalian and bacterial homologs exist. A variety of glucuronide prodrugs have demonstrated impressive antitumor activity in diverse tumor models (de Graaf et al., 2002). Although many studies have employed Escherichia coli β-glucuronidase (εβG) to activate glucuronide prodrugs at cancer cells because of the high specific activity of the bacterial enzyme (Chen et al., 2001, 2007; Cheng et al., 1999b; Wang, 1992), development of human β-glucuronidase (hβG) therapies is desirable to minimize immune responses in patients. hβG is sequestered in lysosomes and is therefore largely inaccessible to membrane-impermeable glucuronides, minimizing the problem of systemic activation of prodrugs by endogenous enzymes (Cheng et al., 1999a). As an acid hydrolase, hβG displays maximum activity at pH 4.0, but relatively low activity at neutral pH (Chen et al., 2007). Translation of glucuronide prodrug therapy to the clinic could be facilitated by generating hβG variants that more efficiently hydrolyze prodrugs under the physiological conditions present in tumors.

The pH profile of enzymes has been altered by substituting specific amino acids that directly (Kim et al., 2006) or indirectly (Makde et al., 2006) contact substrates as well as by changing amino acids on the enzyme surface (Russell and Fersht, 1987). However, it is difficult to rationally alter the pH profile of an enzyme with retention of catalytic activity. Directed evolution, on the other hand, is an extremely versatile and powerful method to modify protein properties without a priori detailed knowledge of protein structure-function relationships (Kaur and Sharma, 2006).

Whereas most directed evolution has been performed in bacteria, many eukaryotic proteins require posttranslational modifications such as glycosylation for proper folding and catalytic activity. For example, hβG is expressed as a tetramer composed of four identical monomeric subunits of 629 amino acids, each possessing four N-linked oligosaccharides that are required for proper protein folding (Shipley et al., 1993). Furthermore, assay of hβG activity at defined pH values is difficult due to cell-to-cell variations in lysosomal pH (Anderson and Orci, 1988). In the present study, we solved these problems by tethering hβG on the surface of mammalian cells to allow assay of properly glycosylated and active enzyme under defined conditions. We
show that combination of mammalian cell-surface display with high-throughput flow cytometric sorting allows successful isolation of hβG variants with enhanced activity at neutral pH.

RESULTS

Development of a Mammalian Cell-Surface-Tethered Enzyme Screening System

We tethered human βG (hβG) and mouse βG (mβG) on the surface of mammalian cells by fusing the cDNA for the respective βG genes to the juxtamembrane Ig-like extracellular domain, transmembrane domain, and cytoplasmic tail of murine B7-1 (Chen et al., 2007; Cheng and Roffler, 2008). mβG was employed for some assays because it displays about 3-fold greater enzymatic activity than hβG at pH 7.0, allowing better sensitivity for initial method development. hβG was stably expressed with retention of enzymatic activity on 3T3 fibroblasts, as determined by surface immunofluorescence staining with a FITC-labeled anti-hβG antibody (7G8-FITC) and by hydrolysis of the substrate ELF-97 β-D-glucuronide to ELF-97 alcohol (Telford et al., 2001), a fluorescent product that remained associated with the cells (Figure 1A). Trypsin proteolysis of cell-surface proteins reduced hβG expression and enzymatic activity in parallel (Figure 1A), demonstrating that membrane-tethered hβG was responsible for hydrolysis of ELF-97 β-D-glucuronide. The enzymatic activity of membrane-tethered mβG depended on the extracellular pH, allowing convenient control of the enzyme environment (Figure 1B). Cells expressing membrane-tethered mβG were clearly distinguishable from wild-type (WT) cells under fluorescence illumination after staining mixed-cell populations with ELF-97 β-D-glucuronide (Figure 1C). To determine whether high-throughput flow cytometric sorting of cells based on relative βG activity was feasible, a mixture of WT cells and cells expressing membrane-tethered mβG was first stained with rat anti-mβG antibody followed by goat anti-rat FITC conjugate and then incubated with ELF-97 β-D-glucuronide. Two discrete populations of cells could be clearly separated (Figure 1D), demonstrating that enzymatically generated ELF-97 alcohol remained associated with cells expressing membrane-tethered βG during the staining and analysis procedure.
Selection of h\(\beta\)G Variants with Enhanced Activity at Neutral pH

Based on these results, we developed a robust high-throughput fluorescence-activated cell-sorting method to isolate h\(\beta\)G mutants with enhanced activity at neutral pH (Figure 2A). We employed error-prone PCR to generate a cDNA library (EP1 library) with a diversity of \(\sim 5 \times 10^6\) containing an average of 4.5 amino acid mutations per h\(\beta\)G gene. 3T3 fibroblasts were infected at low MOI (multiplicity of infection; \(\sim 0.6\) cfu/cell) with a VSV-G pseudotyped retroviral virus library to generate \(\sim 10^7\) independent 3T3 clones. After selection in G418, viable cells were stained with 7G8-FITC (anti-h\(\beta\)G-FITC conjugate) and incubated with ELF-97 \(\beta\)-D-glucuronide before they were sorted on a flow cytometer for enhanced h\(\beta\)G activity relative to h\(\beta\)G expression levels. The first round of screening was performed at pH 5.0 to enrich for rare cells displaying enhanced h\(\beta\)G enzymatic activity at slightly elevated pH (Figure 2B, left panel). Selected cells (~0.5% of the total cell population) were cultured to expand their numbers and then resorted two additional times after immunofluorescence staining for h\(\beta\)G expression with 7G8-FITC and labeling h\(\beta\)G activity with ELF-97 \(\beta\)-D-glucuronide at pH 6.5 to isolate cells displaying enhanced surface enzymatic activity at elevated pH. The sorted population displayed increased enzymatic activity as compared to cells expressing membrane-tethered WT h\(\beta\)G (Figure 2B, right panel). The sorted population also exhibited enhanced enzyme activity at both pH 5.0 and pH 6.5, indicating that enzyme variants with a broader pH profile can be isolated by applying selection pressure based on reaction pH (Figure 2C). Sequencing the h\(\beta\)G gene in 12 individual cell clones revealed multiple mutations. However, all h\(\beta\)G variants had mutations at position 545 (50% T \(\rightarrow\) A and 50% T \(\rightarrow\) S). To determine the effect of amino acid substitutions at position 545 on enzyme activity, the single amino acid mutants T545A (E1-A), T545G (E1-G), T545S (E1-S), and T545Y (E1-Y) were generated and the corresponding enzymes were purified from the culture medium of stably transfected fibroblasts. Assay for enzymatic activity at pH 7.0 with the substrate \(p\)-nitrophenol \(\beta\)-D-glucuronide revealed that E1-G and E1-A displayed about 2.5-fold greater activity than WT h\(\beta\)G, E1-S exhibited about 1.5-fold greater activity, and E1-Y was almost inactive (data not shown). Thus, single amino acid changes can affect h\(\beta\)G activity at neutral pH. We employed the E1-G (50%) and E1-A (50%) variants as starting material to generate an error-prone cDNA library (EP2) with 5 \(\times\) 10^6 members containing an average of 2.6 amino acid mutations per h\(\beta\)G gene. Independent 3T3 clones (5 \(\times\) 10^5) were immunofluorescence stained with 7G8-FITC and reacted with ELF-97 \(\beta\)-D-glucuronide at pH 7.0. Sixteen individual cell clones were isolated after three rounds of sorting and the h\(\beta\)G genes were sequenced. All of the isolated h\(\beta\)G genes possessed identical amino acid substitutions at position 255 (L \(\rightarrow\) Q) and 545 (T \(\rightarrow\) G) with variable substitutions at other positions. One high-activity variant, termed E2-20, was selected for further examination (Table 1).

To help differentiate between beneficial and null mutations in the h\(\beta\)G variants, h\(\beta\)G cDNA isolated from the sorted EP1 library (30%) was shuffled with the WT h\(\beta\)G gene (70%) to breed out null mutations. The shuffled backcross library was then expressed on 3T3 fibroblasts and screened with ELF-97

\[\text{\(\beta\)-D-glucuronide at pH 7.0.}\]

After one round of flow cytometric sorting, individual cell clones were isolated and the h\(\beta\)G gene was sequenced. Among 13 single-cell clones, amino acid changes at positions 545, 595, and 599 were consistently associated with increased h\(\beta\)G activity at neutral pH values.
Table 1. Amino Acid Substitutions in Selected hG Variants

<table>
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<tr>
<th>Clone</th>
<th>Amino Acid Residue</th>
<th>Relative Activitya</th>
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<tr>
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<td>159</td>
<td>243</td>
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<tr>
<td>Wild-type</td>
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<td>E1-S</td>
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<td>E1-G</td>
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<td>S2</td>
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<tr>
<td>S28</td>
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<td>E2-20</td>
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ND, not determined.

a 3T3 fibroblasts expressing membrane-tethered forms of the indicated hG variants were assayed for hG surface expression and G enzymatic activity. Relative activity of membrane-tethered hG variants was calculated as the ratio of FL4 (hG activity)/FL1 (surface hG expression) at pH 7.0 and normalized to the FL4/FL1 of WT hG. The substrate was ELF-97 β-D-glucuronide.

Saturation Mutagenesis

To further examine the influence of varying the amino acids at positions 545, 595, and 599 on the catalytic activity of hG, we employed saturation mutagenesis to generate a small library (~10^5 cell clones) covering all possible combinations of amino acids at these three sites. The library was screened by two rounds of cell sorting at pH 7.0. Among 14 single-cell clones displaying high activity, seven different amino acids (all nonnegatively charged) appeared at position 599. Furthermore, 79% of the hG variants had serine at position 545, and 13 of 14 clones contained amino acids with hydrophobic side groups (28.5% leucine, 28.5% isoleucine, and 43% phenylalanine) at position 599. Two variants (S2 and S28 in Table 1) were selected for further analysis.

Characterization of βG Variants

Table 1 summarizes the mutations present in five membrane-tethered hG variants which displayed 20- to 115-fold greater enzyme activity than WT hG for ELF-97 β-D-glucuronidase at pH 7.0. The amount of hG protein expressed by each cell line varied as determined by immunoblot analysis (see Figure S1A available online). There was a strong linear correlation between total hG expression level (as determined by hG band intensity on the immunoblot in Figure S1A) and surface hG levels, as determined by immunofluorescence staining of hG followed by flow cytometric analysis (Figure S1B). This confirms that immunofluorescence staining allows convenient normalization of enzyme activities for differences in hG expression levels.

To further characterize the hG variants, we purified soluble hG enzymes from 3T3 producer cells (Figure 3A). The proteins migrated slower than the predicted molecular weight of an hG monomer due to the presence of N-linked oligosaccharides in the enzyme (Shipley et al., 1993). hG displayed maximal activity at pH 4.0 but only 2% of maximal activity at pH 7.0 (Figure 3B). The hG variants exhibited maximal activity at pH 4.5 with relatively broad pH profiles. The kinetic properties of hG and variants were compared at pH 4.5 and pH 7.0 (Table 2). Consistent with enhanced enzymatic activity of membrane-tethered hG, the soluble hG variants displayed enhanced kcat/Km values at both pH 4.5 and 7.0 (Figure 3C). At pH 7.0, E1-S and E1-G displayed kcat/Km values 4- and 10-fold higher than WT hG, whereas S2, S28, and E2-20 variants displayed 20- to 60-fold enhancements in kcat/Km. Surprisingly, the kcat/Km values of the S2 and E2-20 enzymes at pH 7.0 were about 2-fold greater than the kcat/Km of WT hG at pH 4.5. The dramatic increases in substrate affinity at acidic and neutral pH explain the overall enhancement in the activities of the hG variants. Changes in amino acid sequence can adversely affect protein stability (Smith et al., 1997). However, the enzymatic activity of recombinant hG variants at pH 7.0 was fully retained for at least 2 weeks.
Evolution of Membrane-Tethered Human β-Glucuronidase

DISCUSSION

Alteration of the pH profile of mammalian enzymes by directed evolution has not been described to our knowledge, likely due to the difficulty in establishing an appropriate high-throughput screening system. By combining error-prone PCR with a high-throughput mammalian cell-surface-tethered screening system, we successfully generated hβG variants with enhanced activity over an extended pH range. Importantly, the hβG variants more effectively hydrolyzed two structurally distinct anticancer glucuronide prodrugs at neutral pH. Because previous studies have demonstrated that antibody-hβG immunoenzymes can selectively activate glucuronide prodrugs in tumor xenografts to produce strong antitumor activity (Bosslet et al., 1994), the hβG variants developed here are anticipated to further increase the therapeutic efficacy of glucuronide prodrug therapy.

A β-Glucuronidase can significantly improve the therapeutic index of cancer chemotherapy. In ADEPT, an antibody-enzyme conjugate or fusion protein is allowed to accumulate at cancer cells before a relatively nontoxic prodrug is administered. Selective enzymatic hydrolysis of prodrug at cancer cells provides high localized concentrations of cytotoxic agent in tumors (Svensson et al., 1995). GDEPT is analogous, except that the prodrug-activating enzyme is expressed at the tumor site after viral or nonviral gene transfer of the cancer cells. Most enzymes employed for ADEPT/GDEPT have been derived from microbial or viral sources to increase specificity and reduce systemic prodrug activation, including β-lactamase (Svensson et al., 1995), carboxypeptidase G2 (Marais et al., 1997), β-glucuronidase (Wang, 1992), thymidine kinase (Moelten, 1986), nitroreductase (Anlezark et al., 1995), and cytosine deaminase (Mullen et al., 1992). However, nonhuman enzymes can induce robust immune responses, limiting the number of treatment cycles that can be administered to patients (Napier et al., 2000). Several strategies have been investigated to reduce enzyme immunogenicity. Immunosuppressive drugs such as cyclosporine A, cyclophosphamide, and deoxyspergualin can decrease or delay the immune response against foreign proteins (Bagshawe and Sharma, 1996). Immunosuppression, however, besides producing toxicity in patients and hindering the development of antitumor immunity generated by prodrug therapy (Chen et al., 2001), has displayed rather limited suppression of strong antibody responses against bacterial enzymes (Napier et al., 2000; Sharma et al., 1996). Genetic engineering can be employed to remove immunodominant epitopes from foreign proteins (Spencer et al., 2002). For example, this technique allowed reduced binding of preexisting antibodies against an immunodominant epitope in carboxypeptidase G2 (Mayer et al., 2004). However, the large antibody repertoire and the heterogeneity of human MHC molecules make this approach technically challenging. Catalytic antibodies that can selectively hydrolyze anticancer prodrugs have
also been developed (Abraham et al., 2007). This elegant approach can utilize humanized catalytic antibodies to reduce immunogenicity. Highly active catalytic antibodies, however, are rare. Utilization of human enzyme variants with only a few amino acid changes represents an alternative approach for clinical applications.

We successfully isolated hβG variants that displayed greatly enhanced activity at pH 7. Several factors were important for successful identification of mutant enzymes. First, high levels of hβG could be tethered to the surface of 3T3 fibroblasts using membrane-anchoring domains previously developed in our lab (Chou et al., 1999; Liao et al., 2001), allowing sensitive detection of variants with desirable properties. Second, the high infectability of fibroblasts allowed generation of large libraries (>10^9 clones). Third, expression of hβG on mammalian cells allowed proper glycosylation required for enzymatic activity (Shipley et al., 1993). Fourth, the reaction pH could be simply controlled by altering the buffer composition because hβG was tethered to the cell surface. Finally, utilization of ELF-97 β-D-glucuronide as the substrate resulted in good retention of fluorescent reaction product in individual clones with little cross contamination of neighboring cells. ELF-97 β-D-glucuronide appears to display similar attributes as ELF-97 phosphate, which possesses high photostability and sensitivity, an unusually large Stokes shift (~170 nm), and precipitates rapidly and remains highly localized to sites of enzyme activity (Cox and Singer, 1999; Paragas et al., 2002; Telford et al., 2001). By contrast, we observed high background and lack of specificity when screening cell libraries with fluorescein di-β-D-glucuronide (FDGlcU) or pentafluorobenzozylamino fluorescein di-β-D-glucuronide (PFBFDGlcU) substrates, as they yield a soluble product (fluorescein) which diffuses rapidly between cells and is therefore not suitable (Lorincz et al., 1999). Several ELF-97 substrates are commercially available, including ELF-97 N-acetylgalosaminide, ELF-97 palmitate, ELF-97 acetate, and ELF-97 phosphate (Molecular Probes). Simple synthesis of analogous substrates should allow screening by mammalian cell-surface display of almost any eukaryotic enzyme that requires secretory-pathway posttranslational modifications.

Besides allowing good control of reaction conditions, mammalian cell-based surface display combined with quantitative flow cytometric sorting has additional advantages such as (1) high-throughput (10^9 variants can be analyzed per hour), quantitative screening; (2) the presence of an endoplasmic reticulum quality control system to minimize protein-folding errors; (3) efficient surface localization of large oligomeric proteins, such as hβG (a 380 kDa tetramer); and (4) the opportunity to reduce host expression bias by using two-dimensional labeling. For example, we normalized enzyme activity to enzyme surface levels to ensure that observed enhancements were due to altered catalytic activity rather than to cell-to-cell differences in enzyme expression levels. Mammalian surface display thus couples genotype and phenotype while allowing control of selection conditions for evolution of even large oligomeric proteins within a living system. These attributes of mammalian surface display may complement high-throughput screening methods for selection of enzymes based on bacteria (Becker et al., 2007; Olsen et al., 2000; Varadarajan et al., 2005) and yeast (Lipovsek et al., 2007) surface display.

![Figure 5. Mutations Involved in Enhanced Enzyme Activity at Neutral pH Values](http://pymol.sourceforge.net/).

The hβG variants identified in our study include amino acid substitutions at positions 159, 243, 255, and 518 on the surface of hβG and positions 545, 595, and 599 near the catalytic pocket (Figure 5A) (Islam et al., 1999). The catalytic site of hβG is formed by an α/β TIM barrel consisting of eight alternating parallel β strands and α helices. The top of the barrel is formed by loops connecting the C termini of the β strands to the N termini of the α helices. Two of the loops (β4z4 and β7z7) harbor the catalytic residues of hβG (E451 and E540, respectively), in common with many enzymes possessing TIM barrels (Altamirano et al., 2000). In addition, the β7z7 and β8z8 loops are involved in direct interactions with the corresponding loops in a neighboring monomer that form a large cavity harboring two active sites (Figure 5B) (Jain et al., 1996). T599 in the β8z8 loop interacts with the same amino acid in the neighboring subunit. Single amino acid
substitutions in E1-S (T545S) and E1-G (T545G) resulted in substantially enhanced substrate affinity. Interestingly, eG variants that displayed enhanced catalytic activity against β-D-galactoside substrates also contained serine or alanine substitutions at the corresponding threonine (T509 in eG) (Matsumura and Ellington, 2001), suggesting that amino acids with smaller side chains might increase the conformational flexibility of the β7α7 loop and alter substrate affinity. Replacement of T599 with hydrophobic amino acids was associated with increased βG activity at neutral pH, suggesting that alterations in monomer interactions may alter substrate binding. The negatively charged glutamic acid residue at position 595 was replaced by neutral or positively charged amino acids in many βG variants, suggesting that removing a negative charge in the binding pocket might increase enzyme affinity for the negatively charged β-D-galacturonide (pKₐ ~2.8).

Clone E2-20, which includes two mutations on the enzyme surface (L243Q and L255Q), displayed about a 3-fold smaller Km as compared to E1-G, which shares a common mutation of surface (L243Q and L255Q), displayed about a 3-fold smaller Km as compared to E1-G, which shares a common mutation of surface (L243Q and L255Q), suggesting that amino acids with smaller side chains might increase the conformational flexibility of the β7α7 loop and alter substrate affinity. Replacement of T599 with hydrophobic amino acids was associated with increased βG activity at neutral pH, suggesting that alterations in monomer interactions may alter substrate binding. The negatively charged glutamic acid residue at position 595 was replaced by neutral or positively charged amino acids in many βG variants, suggesting that removing a negative charge in the binding pocket might increase enzyme affinity for the negatively charged β-D-galacturonide (pKₐ ~2.8).

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In summary, we developed a mammalian cell-surface display approach to evolve hG variants with enhanced capability to hydrolyze anticancer glucuronide prodrugs at the physiological conditions of the tumor. These hG variants are anticipated to improve the efficacy of ADEPT and GDEPT and may allow multiple treatment rounds due to their low immunogenicity. Moreover, this technology may be applied on other enzymes and other selection conditions, as mammalian expression libraries of membrane-tethered enzymes allow straightforward in vitro evolution of enzyme properties.

**SIGNIFICANCE**

Many proteins require posttranslational modifications, such as glycosylation, to correctly fold and display biological activity. Development of high-throughput screening methods for identification of glycoproteins with altered properties will facilitate elucidation of protein structure-function relationships as well as allow isolation of improved proteins for novel biotechnological and medical applications. Toward this goal, we developed a high-throughput screening procedure for enzymatic activity based on tethered enzyme expression and stable retention of fluorescent reaction products in mammalian cells. A key advantage of this methodology is the ability to precisely control screening conditions such as reaction pH. Furthermore, fluorescence-activated cell sorting allows screening of 10⁷ enzyme variants per hour and reduces protein expression bias by simple quantitative measurement of surface expression levels. We demonstrated the utility of this screening procedure by isolating variants of human β-glucuronidase, a tetrameric glycoprotein normally present in lysosomes, that displayed significantly enhanced enzymatic activity at pH 7.0. The human β-glucuronidase variants isolated in our study are anticipated to extend the utility of selective cancer therapy by glucuronide prodrug treatment due to low immunogenicity in humans and enhanced catalytic activity at the physiological pH present in human tumors. The screening methodology described here should greatly facilitate investigation and modification of eukaryotic enzymes requiring posttranslational modifications for biological activity.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**

ELF-97 alcohol and ELF-97 β-D-glucuronide were from Molecular Probes (Eugene, OR, USA). 4-methylumbelliferyl β-D-glucuronide and CPT-11 were from Sigma-Aldrich (St. Louis, MO, USA). Biotinylated anti-HA antibody was from Vector Laboratories (Burlingame, CA, USA). Rhodamine-conjugated streptavidin and goat anti-rat FITC conjugate were from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Mouse anti-hG mAb 7G8 and rat anti-mouse IgG (mIgG) mAb 7G7 have been described (Chen et al., 2007). 7G8 was directly labeled with FITC as described (Goding, 1976). HAMG was synthesized as described (Roffler et al., 1991). SN-38G was purified by HPLC from the urine of mice treated with CPT-11.

**Cells**

BALB/3T3 fibroblasts (CCL-163; ATCC, Manassas, VA, USA) and CT26 murine colon carcinoma cells (ATCC CRL-2638) were cultured in DMEM (4.5 g/l glucose) supplemented with 10% bovine serum, 2.98 g/l HEPES, 2 g/l NaHCO₃, 100 U/ml penicillin, and 100 μg/ml streptomycin. EJ human bladder carcinoma...
cells (Marshall et al., 1977) were cultured in RPMI containing the same supple-
ments. CT26 and EJ cell lines expressing membrane-tethered NlB on their surface have been described (Chen et al., 2007). All cells were free of myco-
plasma as determined by PCR.

Error-Prone PCR

A silent mutation was made to change cytosine to guanine at position 411 of the hJG gene in pLNCH-hJG-eB7 (Chen et al., 2007) to remove an internal Sall site. The resulting vector, pLNCH-hJG-eB7, was employed as a template for error-prone PCR (Cadwell and Joyce, 1992) using primer P1 (5′-TAT GCT GGG GCC CAG CGG GCC-3′), which contains part of the Ha epotope at the 5′ end and an SfiI restriction site at the 3′ end (underlined), and primer P2 (5′-CTG AGA TGA GTT TTT GTT CGA C3′), which contains part of the myc epi-
lope at the 5′ end and a Sall restriction site at the 3′ end (underlined). Mutagenic buffer (8 mM dCTP, 8 mM dTTP, 48 mM MgCl2, 5 mM MnCl2) (Matsu-
mura and Ellington, 2001) was added (1.25 to 2.5 μl) to each 50 μl PCR reaction using 5 units Taq polymerase (Takara, Shiga, Japan) for amplification.

The PCR product was digested with Sall and SfiI enzymes, ligated into the same sites in pLNCH-hJG-eB7, and transformed into DH5α competent cells by electroporation. Transformed bacteria were selected on 15 cm carbenicil-
in-containing LB agar plates for 16 hr at 37°C. Colonies from multiple plates were collected and expanded in carbenicillin-containing LB medium and then amplified by addition of 170 μl of medium/chloramphenicol. Plasmid DNA from each library was purified by centrifugation in a CsCl-ethidium bromide density gradient at 60,000 rpm in a Ti 70.1 rotor at 4°C. The resulting vector, pLNCX-hJG-eB7, was ligated into the SfiI and Sall sites of EJ, ligated into GP293 cells (Clontech) to produce recombinant retroviral parti-
cles. Two days after transfection, the culture medium was filtered, mixed with 8 mg/ml polybrene, and added to 3T3 fibroblasts. Stable cell lines were se-
clected in medium containing 0.5 mg/ml G418 (Calbiochem, San Diego, CA, USA) into GP293 cells (Clontech) to produce recombinant retroviral partic-
les. Two days after transfection, the culture medium was filtered, mixed with 8 mg/ml polybrene, and added to 3T3 fibroblasts. Stable cell lines were se-
clected in medium containing 0.5 mg/ml G418 (Calbiochem, San Diego, CA, USA).

Selection of High-Activity hJG Cells

3T3 cell libraries were screened by fluorescence-activated cell sorting (FACS). Typically, 10^5 3T3 cells expressing membrane-tethered hJG variants were washed and suspended in 0.4 ml BSA/BSBSS (5.4 mM KCl, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, 4.2 mM NaHCO3, 1.3 mM CaCl2, 0.5 mM MgCl2, 0.6 mM MgSO4, 137 mM NaCl, 5.6 mM D-glucose [pH 7.4]) containing 0.5% BSA and 20 μg/ml 7G8-FITC for 30 min at 4°C. Cells were then washed and incubated with 25, 50, or 100 μM ELF-97 [β-D-glucuronide at defined pH values in 50 mM bis-Tris, 25 mM glucose, 85.6 mM NaCl, 5.4 mM KCl, 0.6 mM MgSO4, 1.3 mM CaCl2 at 37°C for 10 min. The cells were washed with ice-cold 0.5% BSA/HBSS and suspended in 0.5% BSA/HBSS containing 5 μg/ml propidium iodide (Sigma). Cells were sorted on a FACS Vantage DIVA (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with an argon laser for dual excitation at 488 and 531–564 nm. Dead cells (propidi-
um iodide positive, high FL3 fluorescence) were gated out before 7G8-
FITC immunofluorescence was detected at excitation/emission wavelengths of 488/515 nm (FL1) and ELF-97 alcohol was detected at excitation/emission wavelengths of 355/530 nm (FL4). In later experiments, cells were sorted twice each round; cells exhibiting the highest 10% activity were collected and then immediately sorted again to collect the cells displaying the highest 5% activity (representing 0.5% of the total starting population). Double sorting greatly de-
creased contamination with low-activity cells. The sorted cells were cultured for 2–8 days for sequential rounds of cell sorting or RNA extraction. Flow cytometer data were analyzed using FlowJo (Tree Star, Ashland, OR, USA).

DNA Shuffling

Variant hJG genes were recovered by RT-PCR from the cells collected after three rounds of FACS. hJG DNA (1 μg) was digested with 0.025 U of Dnase I (Takara) in 25 μl of 50 mM Tris-HCl (pH 7.4), 1 mM MgCl2 for 11 min at 25°C. The reaction was quenched by adding 5 μl of 0.5 M EDTA. DNA frag-
ments ranging from 100 to 300 bases on a 1% agarose gel were reassembled in 35 cycles of polymeraseless PCR (Stemmer, 1994). The full-length recombinant

products, amplified in a standard PCR reaction with P1 and P2 primers, were cloned in pLNCH-hJG-eB7 to create a new 3T3 cell library.

Saturation Mutagenesis

To generate randomized mutations at amino acid positions 545, 595, and 599 in hJG, two rounds of site-directed mutagenesis were performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All possible amino acids were introduced at amino acid position 545 with primers P3 (5′-GAG GTA TGG AGC APA GNN SAT TGC AGG GTT CCA CGA GQA TCC-3′) and P4 (5′-GAGA TGG TCC TGG TGA AAC CCT GCA ATS NNT TCT GCT CCA TAC TG-3′), where N represents G, A, T, or C, and S represents G or C. A second round of site-directed mutagenesis was performed with primers P5 (5′-GAGA TTG CCG ATT TCA TGA CNS AGT CAC CGG NGS AGA TGC TGG GAA ATA AGG GG-3′) and P6 (5′-CCC TT TTT ATT CC CAG CAC TCT SNV CGG TGA CTG SNV AGT CAT GAA ATC GGC AAA ATT CC-3′) to further introduce all possible amino acids at positions 595 and 599 of hJG. The resulting saturation mutagenesis DNA library was ligated into the SfiI and Sall sites present in pLNCH-hJG-eB7 and employed to generate a 3T3 cell library.

Purification of Recombinant hJG Proteins

Recombinant His-tagged hJG was purified from stable 3T3 fibroblast lines as described (Wu et al., 2004).

Enzyme Assay

The pH-dependent enzyme activities of recombinant hJG variants were mea-
sured in triplicate with 0.1 mM ELF-97 [β-D-glucuronide at 37°C for 30 min at

defined pH ranging from 3 to 10 in iJG reaction buffer (50 mM bis-Tris, 50 mM triethanolamine, 100 mM acetic acid, 0.1% BSA). The reaction was quenched by adding an equal volume of stop buffer (2 M Tris-HCl, 0.8 M sodi-
um bicarbonate [pH 8]). The fluorescence of ELF-97 alcohol and 4-methyl-
umbelliferone was measured in a Gemini EM microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) at excitation/emission wavelengths of 355/555 nm. Kinetic values for hJG substrates hydrolysis were determined by diluting ELF-97 [β-D-glucuronide (2 mM) in pH 4.5 or pH 7.0 iJG reaction buffer 1:1 with defined concentrations of hJG in 200 μl. Fluorescence was immedi-
ately measured under thermal control at 37°C for 8–10 min. The measure-
ment was repeated using the same amount of enzyme for different concentrations of substrate in an optimal range. The acquired readings were converted to prod-
uct concentration by preestablished standard curves. Double reciprocal plots were used to determine Km and kcat. Kinetic assays were performed at least three times and mean values were calculated. The stability of recombinant hJG variants was assayed by measuring their enzymatic activity at time 0 and again after incubating 5 μg of purified enzyme in PBS containing 0.5 mg/ml BSA at 37°C for 14 days. The enzymatic activities of the hJG variants were measured in triplicate with 0.5 mM 4-methylumbelliferyl [β-D-glucu-
oride at 37°C for 15 min at pH 7.0 in iJG reaction buffer. The reaction was terminated by adding an equal volume of stop buffer (1 M glycine, 0.5 M sodi-
um bicarbonate [pH 11]) and the fluorescence was measured at excitation/ emision wavelengths of 355/460 nm in a microplate spectrofluorometer.

[^H]Thymidine Incorporation Assay

Defined concentrations of purified recombinant hJG were added with 10 μM p-hydroxyaniline mustard glucuronide (HAMG) or 100 mM SN-38 glucuronide (SN-38G) to 5000 EJ human bladder cancer cells in 200 μl of complete medium at pH 6.8 for 24 hr. The cells were washed and incubated in fresh medium for 24 hr and then pulsed for 16 hr with [^H]thymidine (1 μCi/well). The cells were harvested and the radioactive measurement was performed in a TopCount microplate scinti-
illation counter (Packard, Meriden, CT, USA). Results are expressed as per-
cent inhibition of [^H]thymidine incorporation into cellular DNA in comparison to untreated cells.

Molecular Modeling

To examine potential interactions of ELF-97 [β-D-glucuronide with mutated amino acids in hJG variants, the X-ray crystal structure of eJG with glucaro-
1,5-lactone in the active site (unpublished data) was employed as a template to position the glucuronide group of ELF-97 [β-D-glucuronide. ELF-97
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β-D-glucuronide was then docked into the active sites formed by the interaction of two hJG monomers using Molegro 2.2.0 (Aarhus, Denmark).

**Statistical Analysis**

Statistical significance of differences between mean values was estimated by Excel (Microsoft, Redmond, WA, USA) using the independent t test for unequal variances. P values of less than 0.05 were considered to be statistically significant.

**SUPPLEMENTAL DATA**

Supplemental Data include three figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00411-0/.

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**REFERENCES**


