ECSTASY, an adjustable membrane-tethered/soluble protein expression system for the directed evolution of mammalian proteins

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Introduction

Recombinant proteins are increasingly employed for industrial and biomedical applications, including new therapies for cancer, cardiovascular and genetic diseases. Manipulation of activity, stability and substrate specificity by directed evolution may greatly expand the therapeutic spectrum of recombinant proteins. Most directed evolution approaches have focused on bacterial screening methods due to low cost and convenience (Turner, 2009). Several powerful high-throughput methods utilizing fluorescence-activated cell sorting of enzymes expressed on bacteria have been developed (Varadarajan et al., 2009; Tu et al., 2011; Yoo et al., 2012). However, the majority of proteins found in nature are glycosylated and ≏70% of therapeutic proteins under development are glycoproteins (Apweiler et al., 1999; Durocher and Butler, 2009). Microbial expression is usually not suitable for these proteins due to lack of glycosylation, inefficient disulfide-bridge formation and frequent formation of protein aggregates.

Several elegant methods for functional library screening have been developed, including phage display (Fernandez-Gacio et al., 2003), fluorescence-activated sorting of double emulsions (Mastrobattista et al., 2005) and yeast surface display (Lipovsky et al., 2007). However, it is difficult to adopt the first two methods to proteins that require eukaryotic post-translation modifications. Yeast display has been successfully employed for evolving eukaryotic proteins, but has not been widely used for expressing large, complex proteins. Yeast glycosylation also differs from mammalian cells (Kukuruzinska et al., 1987). We recently described an approach to directed evolution which utilized stable expression of recombinant proteins on mammalian cells (Chen et al., 2008). This method, however, is difficult to generally apply to many recombinant proteins without first performing time-consuming cloning, transformation and transduction steps to produce soluble proteins for functional screening assays.

Here, we describe a new screening methodology for mammalian proteins that combines advantages of both cell surface display and soluble protein assays (Fig. 1). We investigated the utility of this methodology for identification of human β-glucuronidase variants that display enhanced catalytic activity at neutral pH. Human β-glucuronidase is a lysosomal acid exoglycosidase that cleaves β-D-glucuronic acid residues from glucuronide conjugates (Jain et al., 1996). Human β-glucuronidase is a typical glycoprotein that requires N-linked glycosylation for proper folding and catalytic activity (Shipley et al., 1993). It is also a large tetrameric protein (~320 kDa) that is not easily expressed in other
systems (i.e. we were unable to detect β-galactosidase secretion from transformed *Saccharomyces cerevisiae*).

Human β-glucuronidase is a candidate for cancer treatment by antibody-directed and gene-directed enzyme prodrug therapies (de Graaf et al., 2004; Chen et al., 2007, 2011). Here, we used ECSTASY (enzyme cleavable surface tethered all-purpose screening system) to identify human β-glucuronidase variants that display enhanced activity for the hydrolysis of a glucuronide metabolite to a clinically used anticancer drug.

**Materials and methods**

**Chemicals**

ELF-97 alcohol, ELF-97 β-D-glucuronide (ELF-97G) and phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* were from Molecular Probes (Eugene, OR, USA). 4-methylumbelliferol β-D-glucuronide (4-MUG), fluorescein diacetate and propidium iodide were from Sigma-Aldrich (St Louis, MO, USA). To generate SN-38G, 50 mg/kg CPT-11 was injected intraperitoneally into BALB/c mice and urine was collected over 16 h. SN-38G was purified from urine by high-performance liquid chromatography as described (Prijovich et al., 2009).

**Construction of the glycosyl phosphatidylinositol-linker cassettes**

pHook-hβG-DAF was generated by subcloning the human β-glucuronidase gene present in pLNCX-hβG-eB7 (Chen et al., 2007) into the HindIII and Sall sites in pHook-2C11-DAF (Liao et al., 2000). The transgene was cut from pHook-hβG-DAF with HindIII and XhoI and inserted into the HindIII and Hpal sites in pLNCX to generate the retroviral vector pLNCX-hβG-DAF, which codes for an immunoglobulin kappa chain signal peptide, an HA epitope tag, the mature human β-glucuronidase gene flanked by SfII and Sall restriction sites and the C-terminal portion of human decay-accelerating factor (DAF) responsible for attachment of a glycosyl phosphatidylinositol (GPI) anchor. Specific DNA primers were used to introduce SfII and Sall restriction sites at the 5′ and 3′ ends of cDNA for human carboxylesterase 2 (CE2), reverse transcriptase-polymerase chain reaction (RT-PCR) amplified from human EJ cells, human β-galactosidase, RT-PCR amplified from human embryonic kidney 293 cells, enhanced green fluorescence protein (eGFP), amplified from pTY-EFeGFP (generously provided by Dr Lung-Ji Chang, University of Florida, USA) and a single-chain human MHC class I molecule presenting a peptide derived from cytomegalovirus (HLAA2-CMV),
constructed based on a previously reported design (Yu et al., 2002). Mouse β-glucuronidase (Wu et al., 2004), mouse antidanysyl single-chain antibody (Cheng et al., 2004), anti-CD3 single-chain antibody fused to the Fc domain of human IgG1 (Liao et al., 2000) and human α-fetoprotein (AFP; Chou et al., 1999) genes were directly subcloned into pLNCX-hβG-DAF in place of the β-glucuronidase gene.

**Synthetic human β-glucuronidase library construction**
A DNA fragment containing either bacterial or mammalian amino acids at 13 positions of human β-glucuronidase was assembled by nine forward and nine reverse oligonucleotides as described (Stemmer et al., 1995). The 420-bp synthetic region was then assembled with a 620-bp N-terminal fragment and a 320-bp C-terminal fragment by overlap-extension PCR to create a 1.2-kb fragment which contained a 5′ BglII site and a 320-bp C-terminal fragment by overlap-extension PCR to create a 1.2-kb fragment which contained a 5′ BglII site and a 3′ Sall site. The synthetic library was inserted into BglII and Sall restriction sites in pLNCX-hβG-DAF to generate pLNCX-lib-DAF.

**Cells**
3T3 fibroblasts (CCL-163, ATCC, Manassas, VA, USA), 3T3/hβG-eB7 (Chen et al., 2007), 3T3/hβG-secrete (Wu et al., 2004), HEK-293 cells (CRL-1573, ATCC) and EJ human bladder cancer cells (Marshall et al., 1977) were used in this study. pLNCX-lib-DAF was cotransfected with pVSV-G into GP293V cells (generously provided by Dr Andre Lieber, University of Washington, Seattle, WA, USA) to generate recombinant retroviral particles for subsequent infection of 3T3 fibroblasts at a multiplicity of infection (MOI) of ~0.1 as previously described (Chen et al., 2008) to generate 3T3/lib-DAF cells. 3T3 cells were also infected with recombinant retroviral particles at a MOI of ~10 to generate all other stable cell lines used in this study.

**Immunoblotting**
Cells expressing GPI-tethered β-glucuronidase were incubated with 0.05 U/ml PI-PLC for defined times before the soluble proteins were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel, transferred to a poly(vinylidene difluoride) membrane and sequentially incubated with rabbit anti-β-glucuronidase antibody (Chen et al., 2008) and horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA). SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, MA, USA) was used to detect chemiluminescence on a Fuji LAS-3000 imager (Fujifilm, Tokyo, Japan).

**Cell survival assay**
3T3 and 3T3/hβG-DAF cells were incubated with 0.05 U/ml PI-PLC for defined times before trypsinized cells (10⁶) were incubated with 5 μg/ml fluorescein diacetate and 5 μg/ml propidium iodide for 15 min at room temperature. The cells were analyzed on a FACS Canto with an excitation wavelength of 488 nm and emission wavelengths of 530 and 670 nm for fluorescein and propidium iodide, respectively.

**Expression of GPI-anchored proteins on 3T3 cells**
3T3 cells that stably expressed various GPI-anchored recombinant proteins were incubated with phosphate-buffered saline (PBS) or PBS containing 0.05 U/ml PI-PLC for 1 h before the cells were stained with biotin-labeled goat anti-HA antibody (Vector Laboratories, Burlingame, CA, USA) followed by streptavidin-DyLight 649 (Jackson ImmunoResearch). The cells were washed and 5 μg/ml propidium iodide was added to label dead cells before the fluorescence of 10,000 viable cells was measured on a FACSAvant SE (BD Biosciences).

**ECSTASY procedure**
3T3/lib-DAF cells were incubated with ELF-97G for 3 min at pH 6.0 as described (Chen et al., 2008). The cells were washed and then stained with FITC-labeled anti-β-glucuronidase antibody 7G8-FITC (Chen et al., 2008) for 30 min at 4°C followed by propidium iodide staining. Dead cells (propidium iodide positive, FL3) were gated out on a FACS Vantage DiVa. Cells positive for 7G8-FITC (excitation/emission wavelengths of 488/515 nm, FL1) and ELF-97 alcohol (excitation/emission wavelengths of 351/530 nm, FL4) fluorescence were collected and cultured for 2 weeks. The cells were stained with 7G8-FITC and ELF-97G at pH 7.0 and individual double-positive cells were arrayed using a FACS Vantage DiVa into 96-well culture plates. Once the cells reached near confluence, GPI-anchored β-glucuronidase was released by incubation of 0.05 U/ml PI-PLC in 100 μl PBS in each well at 37°C for 2 h.

To measure enzyme activity, 30 μl samples from each well were dispensed to 96-well microtiter fluorescence plates (NUNC, Roskilde, Denmark) and incubated at 37°C with 200 nM SN-38G in 200 μl reaction buffer (100 mM acetic acid, 50 mM Bis-Tris, 50 mM triethanolamine, pH 7.0) for 16 h. Relative fluorescence was detected at excitation/emission wavelengths of 375/470 nm on a SpectraMax Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA). SN-38G conversion was estimated by the Beer Lambert’s law (Okui and Okada, 2005). Enzyme activity against other substrates was determined as described (Chen et al., 2008).

The amount of soluble human β-glucuronidase variant in each sample was estimated by enzyme-linked immunosorbent assay (ELISA). Anti-human β-glucuronidase antibody 7GB (0.1 μg) in 50 μl bicarbonate buffer, pH 8.0 was coated in the wells of 96-well ELISA plates at room temperature for 1 h. The plates were washed and blocked with 5% skimmed milk in PBS before 20 μl samples were added for 1 h at room temperature. 20 ng 7GB-biotin and 50 ng streptavidin-HRP (Jackson ImmunoResearch) in 50 μl PBS/2.5% skimmed milk were each subsequently added at room temperature for 1 h. After each step, the plates were washed three times with PBS containing 0.05% Tween 20. Freshly prepared 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid, 150 μl) ABTS substrate was added at room temperature for 30 min and the absorbance of each well was read at 405 nm on a microplate reader (Molecular Devices, CA, USA).

**Enzyme activity and stability**
Enzyme activity profiles were determined by incubating 30 ng β-glucuronidase or variant enzymes with 250 μM 4-MUG in 150 μl reaction buffer at defined pH values at 37°C for 30 min. To assess enzyme stability, β-glucuronidase or variants were immediately assayed or incubated at 37°C for 1 week before measuring βG activity using 4-MUG as
substrate. The reactions were terminated by adding 150 µl 1 M glycine, 0.5 M sodium carbonate, pH 11 and fluorescence was measured as described (Chen et al., 2008).

**Cell viability**
Graded concentrations of β-glucuronidase variants and SN-38G were incubated with EJ human bladder cancer cells at pH 7.0 for 48 h. The cells were washed and then stained with 0.5% methylene blue in 50% ethanol or pulsed for 16 h with [3H] thymidine before the cells were harvested and the radioactivity was measured in a TopCount microplate scintillation counter.

**Statistical analysis**
The Student t-test (unpaired) was used to calculate the significance of differences in the activity of hβG and variants by GraphPad Prism 5.0.

**Results**
To facilitate identification and isolation of mammalian proteins with altered properties, we designed a system in which collections of recombinant protein variants can be efficiently and stably expressed on mammalian cells for high-throughput enrichment of properly folded and/or active proteins, but that allows controllable solubilization of membrane-tethered proteins for accurate measurement of individual activities and concentrations (Fig. 1).

GPI-anchored β-glucuronidase can be controllably released from 3T3 cells
A vector was designed to express recombinant proteins with an N-terminal signal peptide to direct protein expression to the secretory pathway and a C-terminal 37 amino acid fragment of DAF to direct attachment of a GPI anchor to the recombinant protein. The GPI anchor allowed display of human β-glucuronidase on 3T3 fibroblasts (3T3/hβG-DAF) at similar levels as fusion of β-glucuronidase to a previously optimized type I transmembrane domain (3T3/hβG-eB7) (Fig. 2a). Fluorescence-activated cell sorting analysis showed that ∼94% of surface β-glucuronidase could be cleaved from 3T3/hβG-DAF cells by addition of PI-PLC, which can cleave the phospho-glycerol bond in the GPI anchor (Brewis et al., 1994), whereas β-glucuronidase tethered with a type I transmembrane domain on 3T3/hβG-eB7 cells was stably retained on cells under the same conditions (Fig. 2a). Western blot analysis demonstrated that soluble β-glucuronidase was released into the culture medium from 3T3/hβG-DAF cells incubated with PI-PLC for 1 or 6 h (Fig. 2b). As expected, no soluble β-glucuronidase was released by addition of PI-PLC to 3T3/hβG-eB7 cells whereas soluble β-glucuronidase was present under all conditions in the culture medium of 3T3/hβG cells, which constitutively secrete β-glucuronidase (Wu et al., 2004). To directly visualize the cleavage of β-glucuronidase, cells seeded on coverslips were exposed to PI-PLC for 0 or 1 h before they were immunofluorescence stained for β-glucuronidase. β-Glucuronidase was visualized on 3T3/hβG-eB7 cells regardless of the addition of PI-PLC whereas the rhodamine signal was greatly reduced on 3T3/hβG-DAF cells after treatment with PI-PLC (Fig. 2c), confirming that β-glucuronidase was cleaved from the cells by PI-PLC treatment.

To evaluate the number of cells required to release sufficient β-glucuronidase for assay of enzymatic activity, defined numbers of 3T3/hβG-DAF cells were seeded into a 96-well plate overnight and the activity of β-glucuronidase released after the addition of PI-PLC for 1 h was determined by measuring the hydrolysis of the soluble substrate 4-MUG to the fluorescent product 4-methylumbelliferone. Cleavage of β-glucuronidase from as few as 2000 3T3/hβG-DAF cells generated sufficient soluble enzyme to produce significantly higher fluorescence than 3T3 cells (Fig. 2d). To determine if more β-glucuronidase could be harvested by extending the cleavage time, 10^5 3T3 or 3T3/hβG-DAF cells were incubated with PI-PLC for 0.5, 1, 2, 4, 6 or 16 h at 37°C. PI-PLC was dissolved in Dulbecco’s modified Eagle’s medium containing 0.5% serum for the 16-h cleavage time to maintain cell viability, whereas PBS was used for the other cleavage times. Enzyme activities were assayed at the non-optimal pH of 7 to simulate screening of pH shift variants. Increasing the exposure time of 3T3/hβG-DAF cells to PI-PLC resulted in progressively higher concentrations of β-glucuronidase activity in the culture medium, indicating that newly synthesized hβG-DAF could be continuously cleaved from the cells (Fig. 2e). About 10 times more soluble β-glucuronidase was released from cells treated with PI-PLC for 16 h as compared with 0.5 h. Determination of the viability of PI-PLC-treated 3T3 and 3T3/hβG-DAF cells demonstrated that <5% of the cells were stained by propidium iodide over the entire incubation time (Fig. 2f), indicating that PI-PLC treatment did not damage the cells. Thus, the GPI-anchor allowed effective attachment of β-glucuronidase to mammalian cells and the controllable release of assayable amounts of soluble enzyme by PI-PLC cleavage in a non-destructive manner.

**A variety of GPI-anchored recombinant proteins can be selectively released from cells**
We examined if other GPI-anchored proteins could be expressed on stably transfected 3T3 cells. Measurement of immunofluorescence showed good expression of GPI-anchored human CE2 on cells (Fig. 3a). PI-PLC treatment of the cells resulted in cleavage of 96% of CE2 from the cell surface (Fig. 3a). Likewise, GPI-anchored human β-galactosidase, mouse β-glucuronidase, a single-chain antibody (anti-DNS scFv), a single-chain antibody fused to a human IgG1 Fc domain (anti-CD3 scFv-Fc), eGFP, human AFP and a human single-chain MHC molecule were well expressed on 3T3 cells and could be cleaved with PI-PLC with efficiencies ranging from 74 to 95% (Fig. 3b). We conclude that a variety of recombinant proteins can be expressed on 3T3 cells in an adjustable membrane-tethered/soluble protein fashion.

**ECSTASY selection of β-glucuronidase variants**
We evaluated ECSTASY for identifying human β-glucuronidase variants that can more effectively convert SN-38G to the active antineoplastic agent SN-38 (Fig. 4a). SN-38G is a major glucuronide metabolite of the clinically used anticancer drug CPT-11 (Irinotecan). We recently demonstrated that tumor-located expression of β-glucuronidase can hydrolyze in vivo generated SN-38G to
SN-38, thereby enhancing the anticancer efficacy of CPT-11 in animal xenograft models (Prijovich et al., 2009; Huang et al., 2011). Human β-glucuronidase variants that effectively hydrolyze SN-38G therefore represent great potential for the targeted therapy of cancer.

A synthetic library was constructed based on comparison of the amino acid sequences of six mammalian (dog, cat, rat, mouse, African green monkey and human) β-glucuronidase and Escherichia coli β-glucuronidase enzymes based on the observation that E. coli β-glucuronidase displays much greater activity than mammalian β-glucuronidases at pH 7.0 (Chen et al., 2007). We hypothesized that conserved amino acids in mammalian β-glucuronidase that differed at the homologous positions in E. coli β-glucuronidase would be good candidates for developing human β-glucuronidase variants with enhanced activity at the neutral pH values found in the tumor microenvironment (Martin and Jain, 1994; Helminger et al., 1997; Garcia-Martin et al., 2001). The library contained two or 4-fold degenerate mutations at 13 positions in the catalytic domain of β-glucuronidase and had a predicted diversity of ~130 000 (Supplementary Table S1).
We expanded 13 cell clones displaying enhanced activity against SN-38G in 15-cm petri dishes and then generated soluble β-glucuronidase variants by addition of PI-PLC. The enzyme concentrations were quantified by ELISA and enzyme activities were measured by incubation with 200 nM SN-38G at 37°C for 16 h. All variants displayed enhanced hydrolysis of SN-38G as compared with β-glucuronidase or S2 (Fig. 4c). To rule out possible artifacts due to quantification of β-glucuronidase concentrations by ELISA, we also visualized the concentration of β-glucuronidase variants by western blot analysis. One μg of human β-glucuronidase, S2 or newly identified enzyme variants 7A8, 7F5, 8C9, 13G2, 14F12 and 16A3 were immunoblotted with either an anti-β-glucuronidase antibody or an anti-HA antibody against the HA epitope tag located on the N-terminus of the recombinant enzymes. The band intensity of each sample was similar (Fig. 4d), verifying the ELISA results.

Functional characterization of selected human β-glucuronidase variants

Three human β-glucuronidase variants (7A8, 7F5 and 16A3) displaying high activities against SN-38G were further characterized. The pH for maximal enzymatic activity for wild-type β-glucuronidase and S2, 7A8, 7F5 and 16A3 variants were ~4, 4.5, 4.5, 5 and 5, respectively (Fig. 5a), representing a one-unit pH shift for the 7F5 and 16A3 variants. 7F5 and 16A3 possessed six identical amino acid substitutions whereas 7A8 had seven amino acid substitutions (Supplementary Table S2). The amino acid mutations in the most active 7F5 and 16A3 variants were grouped in three positions near the active site of β-glucuronidase in positions that may affect substrate interactions or the properties of the catalytic groups (Supplementary Fig. S1).

The enzyme variants demonstrated excellent stability at 37°C for 1 week (Fig. 5b). The 7F5 and 16A3 variants displayed over an order of magnitude greater $k_{cat}/K_M$ values for the hydrolysis of SN-38G as compared with wild-type human β-glucuronidase (Supplementary Table S3). The ability of wild-type β-glucuronidase and selected variants to generate a cytotoxic anticancer drug was examined by incubating EJ human cancer bladder cells with 200 nM SN-38G and graded concentrations of enzyme for 48 h. Observation of the surviving cells showed that 16A3, 7F5 and 7A8 variants displayed markedly better ability to activate SN-38G to SN38 and kill cancer cells as compared with wild-type human β-glucuronidase (Fig. 5c). To quantify the ability of the variants to sensitize cancer cells to SN-38G, we measured $[^{3}H]$thymidine incorporation into cellular DNA after exposure of EJ cancer cells to 200 nM SN-38G and graded concentration of β-glucuronidase variants. $EC_{50}$ values were 26.9, 4.1, 2.3, 1.0 and 0.87 ng/ml for wild-type enzyme, S2, 7A8, 7F5 and 16A3 variants, respectively. The 7F5 and 16A3 variants displayed about a 25–30-fold lower $EC_{50}$ values as compared with human β-glucuronidase (Fig. 5d), suggesting that we would need to target ~25 to 30 times less 7F5 or 16A3 variant to the tumor microenvironment to produce similar effects as wild-type β-glucuronidase.
Discussion

High-throughput screening methods for identification of mammalian proteins with enhanced properties will facilitate the development of novel biotechnological and medical applications. Here, we describe an adjustable membrane-tethered/soluble protein expression system in which recombinant protein variants are attached to the surface of mammalian cells via a GPI anchor. This allows high-throughput fluorescence-activated cell sorting to enrich cells expressing properly folded and/or active proteins. An array of individual cells into microtiter plates allows generation of soluble protein variants by PI-PLC cleavage for multiplex measurement of both the concentration and activity of individual protein variants.

We demonstrated that a variety of recombinant proteins, including enzymes, antibody fragments, a fluorescent protein and MHC molecules, can be expressed on 3T3 cells for subsequent release in soluble form by PI-PLC. We further showed that ECSTASY allowed rapid isolation of human β-glucuronidase variants that hydrolyzed an anti-neoplastic prodrug ~30-fold more effectively than wild-type human β-glucuronidase. This activity enhancement is likely to be highly relevant because an antibody fusion protein incorporating a less active human β-glucuronidase variant (S2) displayed greatly enhanced antitumor activity against human tumor xenografts as compared with the wild type β-glucuronidase fusion protein (Chen et al., 2011). The high frequency of human β-glucuronidase variants with enhanced enzymatic activity for SN-38G observed in the present study (Fig. 4b) is likely due to a combination of using a highly focused cDNA library containing specific amino acid substitutions at highly relevant positions in the catalytic domain of human β-glucuronidase coupled with the ability of ECSTASY to directly screen against the substrate of interest and to eliminate non-functional, poorly folded and nonsense mutants during the preliminary sorting steps.

ECSTASY possesses several important attributes that may promote efficient evolution of mammalian recombinant proteins. Post-translational modifications such as N-linked glycosylation and disulfide bridge formation or protein multimerization are normally processed because proteins are expressed on mammalian cells. Fluorescence-activated cell sorting facilitates selection of stable protein variants and rapid arraying of individual clones into microtiter plates in a single step. Immunofluorescence staining of surface variants can also substantially reduce screening by eliminating nonsense and poorly folded mutants and enriching stable and properly folded variants. For example, 87% of β-glucuronidase variants were not well expressed on cells and could be eliminated from downstream screening. Recombinant protein variants that fold improperly or are unstable are likely to be retained and degraded intracellularly by the rigorous quality control system of the endoplasmic reticulum of mammalian cells (Sitia and Braakman, 2003). Competency for surface display therefore likely correlates with protein stability and perhaps good secretion from

Fig. 4. Selection of β-glucuronidase variants for enhanced hydrolysis of SN-38G. (a) SN-38G can be hydrolyzed by β-glucuronidase to generate the active anticancer drug SN-38. (b) Wild-type human β-glucuronidase, and β-glucuronidase variants were released by PI-PLC treatment from arrayed 3T3/hBG-DAF (n = 6), 3T3/S2-DAF (n = 6) or 3T3-lib-DAF (n = 438) cells, respectively. The amount of soluble β-glucuronidase released from each clone and specific enzyme activities against SN-38G were determined to calculate specific enzyme activities against SN-38G. (c) 15 ng of the indicated enzymes were incubated with 200 nM SN-38G at pH 7.0 for 16 h at 37°C. Results show the relative conversion of SN-38G to SN-38 as measured as SN-38 fluorescence (n = 3). (d) 1 μg of each enzyme (as determined by ELISA) was electrophoresed on an 8% sodium dodecyl sulfate-polyacrylamide electrophoresis and then immunoblotted with anti-HA or anti-β-glucuronidase antibodies.
mammalian cells. This is an important advantage over non-mammalian expression systems since even a single amino acid substitution can lead to instability and loss of activity in vivo (Wolfe et al., 1999).

Efficient release of recombinant proteins from cells by treatment with PI-PLC facilitates screening by colorimetric, radiometric or fluorometric assays as well as by functional assays. This solves a major limitation of traditional mammalian display in which soluble substrates cannot be directly assayed since they diffuse away from the cells before fluorescence-activated cell sorting can be completed (Chen et al., 2008). ECSTASY is also compatible with multiplex assays; ELISA can be used to measure variant protein concentrations and multiple assays can be performed. For example, parallel assay of multiple substrates would allow simultaneous estimation of a spectrum of specific enzyme activities for each variant; information that should be invaluable for protein sequence activity relationship-based approaches to enzyme evolution (Fox et al., 2007). Our results showing successful expression of a variety of GPI-anchored recombinant proteins indicate that this approach should be applicable to a wide range of proteins. Based on these advantages, ECSTASY represents a robust and general platform to select altered mammalian proteins for diverse applications.

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