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Yeast-hybrid based high-throughput assay for identification of anthrax lethal factor inhibitors

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ABSTRACT

Inhibitors of anthrax lethal factor (LF) are currently being sought as effective therapeutics for the treatment of anthrax. Here we report a novel screening approach for inhibitors of LF, a yeast-hybrid-based assay system in which the expression of reporter genes from a Gal4 promoter is repressed by LF proteolytic activity. Yeast cells were co-transformed with LF and a chimeric transcription factor that contains an LF substrate sequence inserted between the DNA-binding and activation domains of Gal4. In the resulting yeast cells, LF cleaves the substrate, thus inactivating the chimeric Gal4 and resulting in lack of expression of reporter genes. Compounds that inhibit LF cleavage of its substrate are identified by changes in reporter gene activity. Relative to *in vitro* screens for inhibitors of LF proteolytic activity, this screen has the advantage of excluding compounds that are toxic or non-permeable to eukaryotic cells. Additionally, the screen has the advantage of being fast, easy and cheap because exogenous LF and substrate are not needed. An initial chemical library screen with this system has identified four candidate inhibitors which were confirmed to inhibit LF protease activity in an *in vitro* assay. Furthermore, FBS-00831, one of the compounds identified, protects Raw 264.7 macrophages from anthrax lethal toxin and the possible binding site on LF was also evaluated by molecular docking.

1. Introduction

Anthrax is a zoonotic disease whose etiologic agent is a Gram-positive sporulating bacterium, *Bacillus anthracis* [1,2]. Virulence of this bacterium relies on an antiphagocytic capsular antigen, which is unique among bacterial capsules consisting of poly-D-glutamic acid, and the tripartite anthrax toxin (protective antigen, PA; lethal factor, LF; edema factor, EF). LF is a Zn^{2+}-dependent metalloprotease, which specifically cleaves mitogen-activated protein kinase kinase (MAPKK) family members, leading to macrophage lysis of toxin-challenged cells [3; review within, 4]. Current FDA-approved treatments for anthrax infection involve antibiotics that kill the bacteria, but antibiotics cannot neutralize the toxins already released by the bacteria into the body. Therefore, LF is an attractive therapeutic target.

A wide variety of approaches have been used to identify LF inhibitors including library screening and optimization [5,6], MS-based screening [7], scaffold-based NMR screening [8], and using synthetic peptides as LF substrates [9]. Although synthetic peptide LF-substrates are convenient to screen for LF inhibitors, screens utilizing native MEK1 could identify chemicals with different inhibitory mechanisms because other regions distal to the cleavage site also play important roles in LF-mediated proteolysis [10] and in LF-substrate interaction [11]. High-throughput screening (HTS) assays that reveal the ability of a compound to inhibit cleavage of MAPKK1 could indicate the blockage of toxin internalization or the proteolytic activity of LF. In this respect, cell-based assays are an increasingly attractive alternative to *in vitro* biochemical assays for HTS since LF functions in the cytosol. In addition, cell-based screens can, in principle, eliminate many compounds with undesirable toxicity, biological instability, or poor availability.

Here we report a cell-based assay in *Saccharomyces cerevisiae* (yeast) that is applicable to monitor LF protease activity and to screen for potent inhibitors in a high-throughput format. We screened a compound collection to evaluate this assay and identified putative LF inhibitors followed by molecular docking to identify the LF binding site. The resulting molecular scaffolds can be further used to design therapeutically novel inhibitors of LF.

Abbreviations: LF, lethal factor; PA, protective antigen; LeTx, lethal toxin consisting of PA and LF; DNA-BD, DNA-binding domain; AD, transcription activation domain; cGAL4-LFS, minimal Gal4 transcription factor containing an optimized LF peptide substrate between the core Gal4 DNA-BD and AD; cGAL4-MEK1, minimal Gal4 chimera containing the full-length native LF substrate, MEK1.

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2. Materials and methods

2.1. Construction of LF and LF-substrate expression plasmids

The pGBKT vector was used as a bait expression vector in a yeast-hybrid-based screen. This pGBKT ΔBD vector encodes the *Trp1* gene, which acts as a selectable marker gene in tryptophan deficient medium. The LF gene was then subcloned into this vector (pGBKT ΔBD/LF). To prepare LF-substrate expression plasmids, the Gal4 DNA-BD was first subcloned into a pGADT 7 (LEU2, a selection marker on leucine deficient medium) vector at the C-terminus of the Gal4 transactivation domain. The genes encoding LF substrates, either peptide substrate LFS or full-length native substrate MEK1, were added between the Gal4 transcription activation domain (AD) and the Gal4 DNA-BD. The peptide substrate LFS consists of 11 amino acids, RRKKVYPYPME, which is an optimized LF cleavable sequence with a cleavage rate approximately 100-fold higher than the native substrate (MEK2) [5]. This peptide is linked with the Gal4 transactivation domain and DNA-BD using glycine linkers. Another LF-substrate expression vector contains full-length rat MEK1, of which Lys97 was replaced with Arg to inactive kinase activity.

2.2. Yeast strains and manipulation

Yeast strain AH109, consists of the following genotype: *MAT*a, two selectable markers (deletion of trp1-901 and the double mutation of leu2-3, 112), and four reporter genes ($GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, $MEL1_{UAS}$-$MEL1_{TATA}$-lacZ, and $MEL1_{UAS}$-$MEL1_{TATA}$-MEL1). Yeast cells were maintained on YPDA medium containing 1% yeast extract, 2% peptone, 2% glucose, and 0.003% adenine hemisulfate. Synthetic dropout (SD) media are designated by the missing nutrient components (e.g., SD/-Trp, -Leu, -His, and -Ade medium that lacks tryptophan, leucine, histidine, and adenine, respectively). Growth and transformation of yeast were accompanied by standard yeast experimental techniques [12].

2.3. Establishment of yeast cell lines co-expressing LF and its substrate

The plasmid pGBKT ΔBD/LF was transformed into yeast cells, and cells harbouring this plasmid were selected on SD/-trp agar plates. Cells were then transformed with either LF-substrate expression vectors (pGADT 7/cGal4-LFS or pGADT 7/cGal4-MEK1) or control vectors (minimal cGal4 transcription factor consisting of the Gal4 AD and DNA-BD, pGADT 7/cGal4, or wild-type full-length Gal4 transcription factor-expression vector, pCL1 [13]). Transformants were maintained on SD/-Trp or SD/-Leu medium.

2.4. Chemical library screening

Screening plates were prepared as follows. Sterile, molten SD medium (SD agar/-Trp, -Leu, -His, and -Ade) cooled to 55 °C was added to 96-well plates containing a unique chemical compound in each well (~6500 represented scaffolds, Korea Research Institute of Chemical Technology) or an equal volume of DMSO to a total volume of 300 μl. Plates were incubated at room temperature with gentle shaking for 30 min. Then, cells co-expressing LF and cGal4/MEK1 were inoculated at a density of 3×10^4 cells in a chemical library plate and incubated at 37 °C for 48 h in a humidified incubator. Cells grown in the nutrient deficient medium containing chemical compounds were selected by comparing with cells expressing LF/cGal4-wt (LF/minimal cGal4 transcription factor consisting DNA-BD and AD) and LF/cGal4-MEK1 in the same minimal nutrient medium without chemical compounds. Selected cells were transferred onto a filter membrane and further analyzed for β-galactosidase activity to confirm the inhibition of LF-mediated cleavage of cGal4-MEK1. Briefly, filter membranes were frozen and thawed several times to lyse the cells and the membrane was placed on the filter paper containing the β-galactosidase reaction mixture (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 3.8 mM β-ME, and 0.82 mM X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside]). To quantify β-galactosidase activity, cells were inoculated in the minimal nutrient medium containing the same chemical compound and grown for 40 h. The cells were harvested by centrifugation and lysed by repeated freeze–thaw cycles. Cell free extract was added into the β-galactosidase reaction mixture and incubated at 30 °C for 3 h. The activity was then measured by recording optical density at 620 nm. The inhibition potency of hit compounds on cultured macrophage, Raw 264.7 was determined as reported previously [14].

2.5. In vitro assay of LF activity

The expression, purification and *in vitro* assay of LF was carried out as reported previously [14,15]. See Supplementary material for details.

2.6. Molecular docking

The hit compound, FBS-00831 was docked onto LF by using AutoDock 4.0 [16,17]. See Supplementary material for details.

3. Results

3.1. A cell-based protease assay to monitor LF activity

A cell-based LF activity assay system was developed in high-throughput format (Fig. 1) that exploits a yeast-hybrid-based assay [18]. In this assay, the proteolytic cleavage of LFS or MEK1 by LF cleaves the Gal4 transcription factor chimera to dissociate two functional domains, resulting in the inactivation of cGal4. Inactivation of cGal4 inhibits the expression of four reporter genes which have been placed under the control of the chimeric transcription factor in the genetically modified yeast strain AH109 (Fig. 1). The expression of this set of reporter genes produces a visible readout of cell growth on minimal medium (*HIS3* and *ADE2*) and of color development on X-gal reagent (*LacZ* and *MEL1*). As a control, the co-expression of LF and cGal4-LF substrate (LF/cGal4-LF substrate i.e. LF/cGal4-LFS and LF/cGal4-MEK1) in yeast cells significantly reduced cell growth in minimal medium (SD/-Trp, -Leu, -His, -Ade) (Fig. 2A). However, LF/cGal4-wt expressing cells grow normally in the same minimal medium and cells reached stationary phase following 42 h of incubation. The full-length substrate chimera (cGal4-MEK1) and a short peptide-substrate chimera (cGal4-LFS) led to reduced yeast growth. However, after 40 h of incubation, cells expressing cGal4-LFS and cGal4-MEK1 grow to approximately 50% and 20% of cGal4-wt cells, respectively.

cGal4-MEK1 was cleaved more efficiently by LF than cGal4-LFS within cells (Fig 2B) which suggests that MEK1 forms a more stable enzyme–substrate complex compared to LFS. All further experiments were therefore carried using cells co-expressing LF/cGal4-MEK1 as an assay model system. LF/cGal4-MEK1 expressing cells showed growth inhibition on solid culture medium as well as decreased β-galactosidase activity (Fig. 2C). These results indicate that the cGal4-MEK1 based assay system is suitable to monitor LF activity in yeast strain AH109. The reliability of the assay method was validated by measuring assay variation for cell growth and β-galactosidase activity. Cell growth and β-galactosidase activity in LF/cGal4-MEK1 expressing cells were significantly reduced com-