Studies on the novel bioactive peptide screening systems for G-protein coupled receptors and neuraminidase

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Contents

Abbreviations

cAMP	Cyclic adenosine monophosphate
CRE	cAMP-responsive element
CHO	Chinese hamster ovary
DMEM	Dulbecco’s modified eagle medium
dPTP	2'-Deoxy-P-nucleoside-5'-triphosphate
Ex4	Exendin-4
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
GLP1	Glucagon-like peptide1
GPCR	G-protein coupled receptors
HEK293	Human embryonic kidney 293
IgG	Immunoglobulin G
IL6ss	Interleukin-6 secretion signal
MES	2-Morpholinoethanesulfonic acid
MU	Methylumbelliferone
MUNANA	4-Methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid
NA	Neuraminidase
NMR	Nuclear magnetic resonance
8-oxo-dGTP	8-Oxo-2'-deoxy-guanosine-5'-triphosphate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RFU	Relative fluorescence units
PtGFP	Ptilosarcus green fluorescent protein
SDC	Synthetic dextrose
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
WHO	World health organization

Introduction

The discovery and development of antibiotics, such as penicillin discovered by Alexander Fleming in 1928, is one of the greatest inventions in human history, and has contributed greatly to prosperity of humankind. Since 1950, the pharmaceutical industry has delivered over a thousand new drugs that have played an important part in improving public health and extending life expectancy [1] . However, the industry is now facing the reduction of drug research and development (R&D) efficiency partly due to the continuous emergence of competitive generic drugs and the progressive lowering of risk tolerance of drug regulatory agencies [2,3], which requires further innovative drugs that can cure rare and previously neglected diseases.

In contrast to small molecule drugs such as acetylsalicylic acid, biotechnology-based drugs (called ‘biologics’) like therapeutic monoclonal antibodies have recently been used to effectively treat conditions particularly in the area of autoimmune diseases that small molecule drugs are unable to address. In fact, the top three drugs in terms of global sales in 2012 were antibody or fusion protein products prescribed for the treatment of rheumatoid arthritis [4]. Unfortunately, these monoclonal antibodies have difficulties targeting intracellular molecules due to their large size. In addition, they are expensive to manufacture, resulting in high medication cost for patients [5].

Peptides are polymeric molecules composed of amino acids with around 50 residues in length. They are classified between small molecules and antibodies in molecular size. Considering that they are able to target intracellular molecules with good specificity, peptides should be the next major molecules to be used as medicines to address intractable disease and improve R&D efficiency. In this paper, we focus on a novel discovery method of therapeutic peptides acting on G-protein coupled receptors (GPCRs) and enzymes. To do so we applied and engineered budding yeast as a host for the production of agonistic peptides. Furthermore, we genetically grafted virus neuraminidase onto the yeast cell surface for use as drug screening targets that are easily prepared.

Drug research and development

The drug discovery and development process (Fig. 1) is one of the most challenging and difficult human endeavors, integrating various sciences and technologies as it balances efficacy in health benefits with safety at an appropriate therapeutic index. It is an indispensable campaign for prevention, management, and cure of disease, injuries, and other disorders for patients. Since the emergence of organic synthesis chemistry in the late nineteenth century, which was marked by the introduction of aspirin, and the use of rationale exploitation strategy, which led to the discovery of penicillin – the first antibiotic – it is indisputable that modern medicine has changed the world [6-8]. In fact, from 1950 to 2008 in United States, the pharmaceutical industry has delivered 1,222 new drugs that are composed of 1,103 small molecules and 119 biologics [2], leading to improved public health and extended life expectancy by an average of 2 months each year [1].

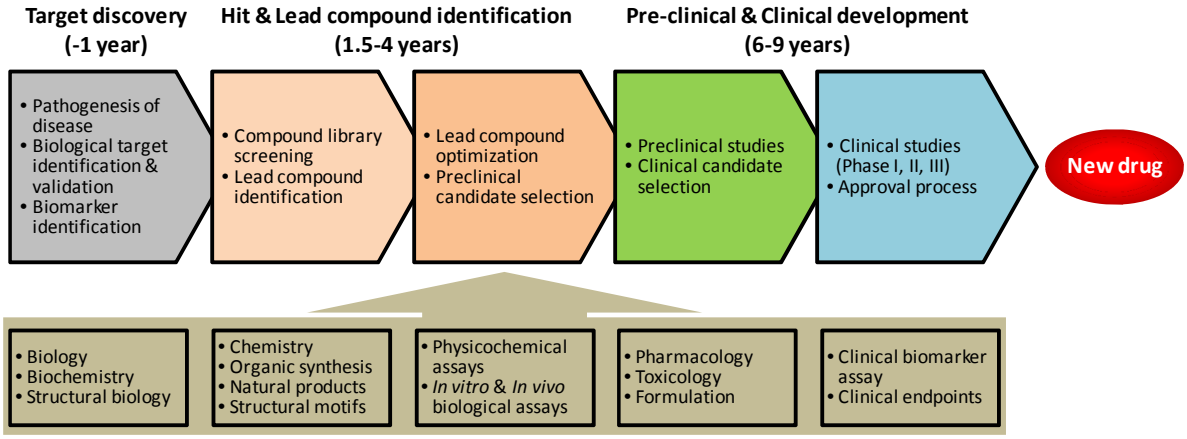


Fig. 1. Drug development process [9]

The industry, however, is now encountering reduced efficiency in drug research and development (R&D). Presently it takes nearly nine years from clinical testing to regulatory approval, which does not include the preclinical, animal testing phase nor the discovery and research phases. In addition to such a long lead time, the average cost for one biopharmaceutical product is $1.32 billion (in 2005 dollars). Although the number of new drugs marketed has never reduced in any year, R&D costs have been dramatically increasing [10] (Fig. 2). This is due to a number of reasons. One is the

extensive exploitation of drugs for diseases with a large number of patients like hypertension and hyperlipidemia, which are comparatively easy to deal with. Another is the concomitant emergence of generic drugs with evidence of long-term safety and efficacy. In addition, the increasing requirements imposed by regulatory authorities, especially on safety, increase costs. Finally, the molecular targets such as protein-protein interactions that are difficult to regulate with small molecules are becoming key for treatment of previously neglected diseases. These challenges mean that pharmaceutical companies have to address residual intractable diseases with smaller number of patients while targeting non-classical molecules.

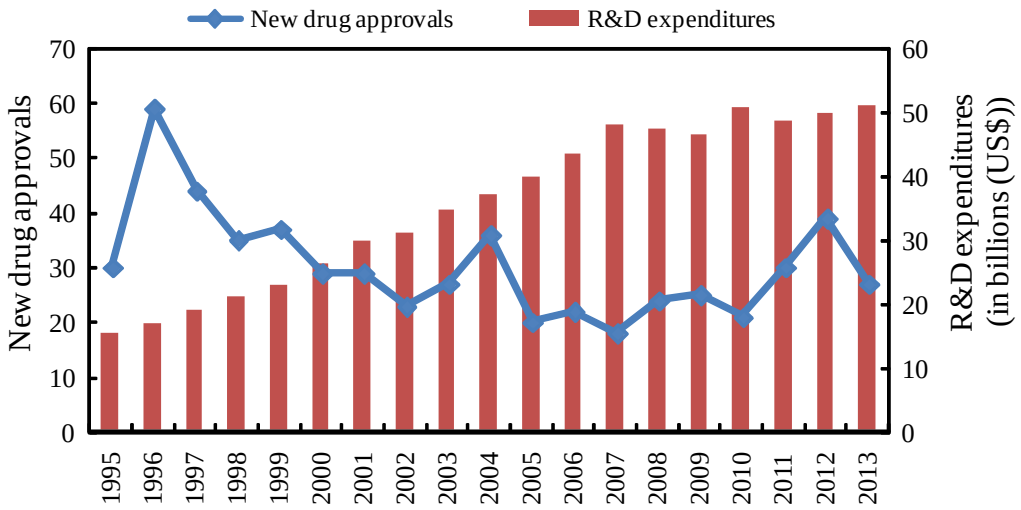


Fig. 2. New drug approvals and R&D expenditures in the United States from 1995 to 2013 [11,12]

Since the establishment of recombinant DNA technology, as typified by human insulin production using bacteria in the 1980s, pharmaceutical companies have developed protein-based drugs such as erythropoietin for anemia and interferon for virus hepatitis [13]. Both were diseases that small molecule drugs found difficult to access. More recently, humanized monoclonal antibodies were developed and dramatically improved clinical outcome of patients with autoimmune diseases such as rheumatoid arthritis and inflammatory bowel diseases. Indeed, the advent of these biopharmaceuticals changed the industry's business model to incorporate the therapeutic area of diseases, as seen in Table 1. In 2003, 9 of the top 10 drugs in terms of sales were

almost all small molecule drugs that targeted life style-related diseases with huge number of patients. But, in 2012, the lineup drastically changed; half are monoclonal antibodies that target autoimmune diseases and cancer. This innovation is undeniably a great success, but unfortunately, these monoclonal antibodies have difficulty targeting intracellular molecules, plus they impose high medication costs on patients around $25,000 U.S. annually [5]. Both are attributed to their huge molecular size, comparatively low productivity, and high dose required of monoclonal antibodies.

Table 1 Top 10 drugs by global sales in 2003 and 2012 [4]

2003			
Rank	Brand name (Generic name)	Indication	Sales (Bln$)
1	Lipitor (atorvastatin)	Lipidemia	9.96
2	Epogen (epoetin)	Anemia	6.89
3	Takepron (lansoprazole)	Gastric ulcer	5.14
4	Zocor (simvastatin)	Lipidemia	5.01
5	Norvaskc(amlodipine)	Hypertension	4.77
6	Mevalotin (pravastatin)	Lipidemia	4.75
7	Zyprexa (olanzapin)	Schizophrenia	4.28
8	Plavix (clopidogrel)	Thrombosis	4.13
9	Advair (salmel../flutica..)	Asthma	3.94
10	Paxil (paroxetine)	Depression	3.34

2012			
Rank	Brand name (Generic name)	Indication	Sales (Bln$)
1	Humira (adalimumab	Reumatoid arthritis	9.60
2	Remicade (infliximab)		9.07
3	Enbrel (etanercept)		8.47
4	Advair (salmel../flutica..)	Asthma	8.21
5	Crestor (rosuvastatin)	Lipidemia	7.43
6	Rituxan (rituximab)	Blood cancer	7.22
7	Lantus (insulin glargine)	Diabetes	6.55
8	Herceptin (trasutsuzumab)	Breast cancer	6.44
9	Avastin (vebacizumab)	Colon cancer	6.30
10	Januvia (sitagliptin)	Diabetes	6.20

*Yellow filling indicates biologics.

Peptides as drugs

Traditional small molecule drugs with typical molecular weights less than 500 Da have advantages such as low cost and low price, oral availability, ready synthesis, membrane-penetrating ability, and stability in the body. However, due to their small size, they may suffer from reduced target selectivity that often ultimately manifests in human side effects. On the other hand, larger biologics of more than 10,000 Da in molecular size tend to be exquisitely specific for their targets because of many more interactions with targets, but they are associated with high production costs, lack of oral bioavailability, poor membrane permeability, and metabolic instability, requiring injection. Thus there is a significant gap in molecular size between small molecule drugs and therapeutic antibodies, and peptides offer the potential of filling this gap while possessing advantages from small molecules and antibodies (Table 2) [14].

In nature, peptides are involved in a variety of physiological and pathological processes and play very important roles in modulating various cell functions. For instance, insulin is a 51-amino acid peptide hormone consisting of two chains, called A- and B-chain respectively, and is produced and secreted by pancreatic β cells in response to increased blood glucose levels, and regulates the metabolism of carbohydrates and fats by promoting the absorption of glucose from the blood to skeletal muscles and fat tissues [15].

Therapeutic peptides have traditionally been designed in endogenous form or analogues with some substitution in amino acids. Humulin® is simply human insulin produced by genetically-modified bacteria, whereas Lantus® (Insulin grargin) replaces Asp to Gly at position 21 in the human insulin A-chain and adds the two additional Arg residues to the C-terminus in B-chain for achieving sustainable absorption from injection site to blood. Because peptides are accessible to chemical synthesis, unnatural amino acids such as α-aminoisobutyric acid (dimethyl-alanine) and D-amino acid can also be introduced especially to increase proteolytic stability.

There are various types of molecular targets that peptides can interact and modulate. While insulin targets the insulin receptor expressed on the plasma membrane that belongs to tyrosine kinase receptors, octreotide (Sandostatin®), a synthetic somatostatin analog with a D-amino acid in its circular structure, activates the somatostatin receptor that is a class A GPCRs with seven-transmembrane structure, and is one of the most common drugs used in treating neuroendocrine tumors for which conventional chemotherapy and radiotherapy have very limited effects [16]. Zinocotide (Prialt®) act as a selective N-type voltage gated calcium ion channel blocker for the amelioration of severe and chronic pain [17]. In addition to these membrane proteins as targets by peptides, ecallantide (Kalbitor®) suppresses fluid leakage from blood vessels causing swelling of tissues typical of hereditary angioedema by inhibiting the protease activity of plasma kallikrein [18]. Eptifibatide (Integrilin®) interferes with the interaction of glycoprotein IIb/IIIa on platelets with fibrinogen and von Willebrand factor, suppressing platelet aggregation and thrombus formation for the treatment of unstable angina [19].

Peptides have been considered difficult to access intracellular targets due to their low membrane permeability, but a family of cell-penetrating peptides (CPPs) such as penetratin, M918, and TP10 are able to pass through cell membranes with high efficiency and less membrane damage [20,21]. Fusion of CPPs to therapeutic peptides could deliver them through cell membrane to improve poor oral bioavailability and intracellular targeting. Another drawback of peptides is the short half-life in the body caused by enzymatic degradation and renal clearance. However, short peptides that can bind to serum albumin have been discovered and demonstrated to extend the half-life of antibody fused to the albumin binding peptide in experimental animals [22]. More recently, circular peptides (called cyclotides) from plants have been found to be highly stable against proteolytic enzymes, which suggests that such circular scaffolding enables peptides to be orally administered [23]. In summary, peptides can be designed with natural and unnatural amino acids, and can target various types of molecules from membrane proteins to enzymes, interactions between proteins, and even intracellular molecules. These characteristics of peptides should attract the pharmaceutical industry to peptide-based drug discovery.

Table 2 Comparison of potential druggability among small molecules, peptides and antibodies

	Small molecules (0.5 kDa)	Peptides (5 kDa)	Monoclonal antibody (150 kDa)
Drug design	Needs large manpower	DNA randomization	DNA randomization
Safety	Needs many cares	Immunogenicity	Immunogenicity
Protein-Protein interaction	Difficult	Possible	Easy
Intracellular targeting	Easy	Possible	Difficult
In vivo stability	Low to High	Low	High
Production cost	Low	Middle	High

G-protein coupled receptors (GPCRs) and GLP1 receptor

G-protein coupled receptors (GPCRs) are critical eukaryotic signal transduction gatekeepers and represent the largest protein family in the human proteome, with more than 800 members. They share a common architecture of seven transmembrane helices, and can be classified into five major classes by sequence similarities (Fig. 3) [24]: rhodopsin receptor family (class A); secretine-like receptor family (class B); glutamate receptor family (class C); and frizzled/taste 2 receptor family and adhesion receptor family (other class).

Class A is by far the largest subgroup and contains the opsins, olfactory GPCRs, and small-molecule/peptide hormone GPCRs. Class A GPCRs are characterized by several highly conserved amino acids in the 7 TM bundle. Most of the class A receptors have a palmitoylated cysteine residue in the intracellular C-terminal tail. The ligand binding sites vary depending on the type of ligand. For endogenous small-molecule hormone ligands it is within the 7 TM bundle (Fig. 3, upper left). For peptide ligands it is within the N-terminus and superior parts of the TM helices (Fig. 3, upper middle). And for glycoprotein hormone ligands it is within the extracellular loop segments and superior parts of the TM helices (Fig. 3, upper right).

Class B comprises 50 GPCRs for peptides such as secretin and calcitonin. Class B GPCRs are characterized by a relatively long N-terminal tail, which forms ligand binding sites together with the extracellular surface of TM regions and contains a network of three conserved disulfide bridges defining a globular domain structure. (Fig. 3 bottom left)

Class C GPCRs include 17 members in the human genome such as the mGluR, γ-aminobutyric acid type B ($GABA_B$), and Ca^{-2}-sensing (CaR) receptors. The majority of class C receptors are characterized by very large N- and C-terminal tails. The ligand binding site is located in the large N-terminal domain (Fig. 3, bottom right).

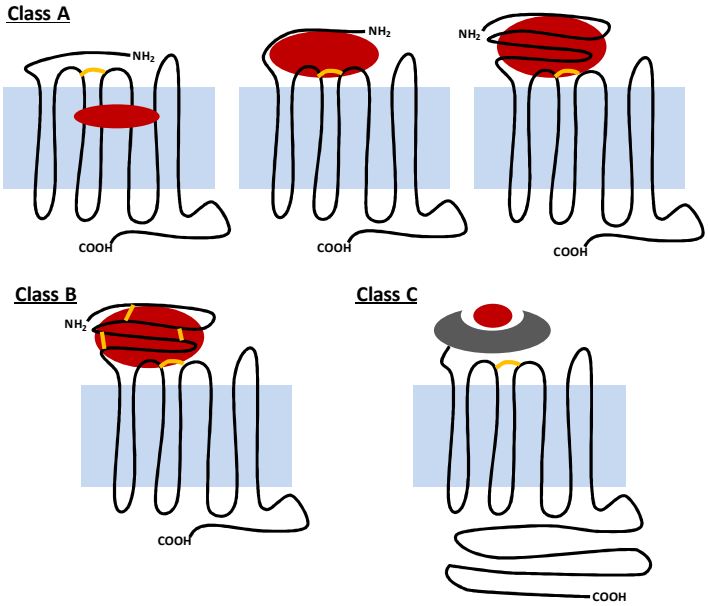


Fig. 3. Classification of human GPCRs [24]
Red: ligand, Yellow: disulfide bond, sky blue: membrane lipid bilayer

GPCRs recognize a variety of extracellular stimuli, including photons, ions, small molecules, peptides, and proteins, and transmit the resulting extracellular signals across the membrane to elicit intracellular responses. Signal transmission occurs through coupling to different intracellular proteins such as heterotrimeric G proteins and kinases, which then activate downstream effectors and trigger cascade of cellular and physiological responses (Fig. 4). The key role played by GPCRs in many physiological or disease-related processes has made them one of the favorite targets of the pharmaceutical industry. This therapeutic relevance is understood by the fact that those drugs target only up to 35 of the approximately 350 druggable, nonsensory GPCRs and that ligands or function are unknown for around 130 orphan GPCRs. Clearly their pharmacological potential remains largely untapped [25,26].

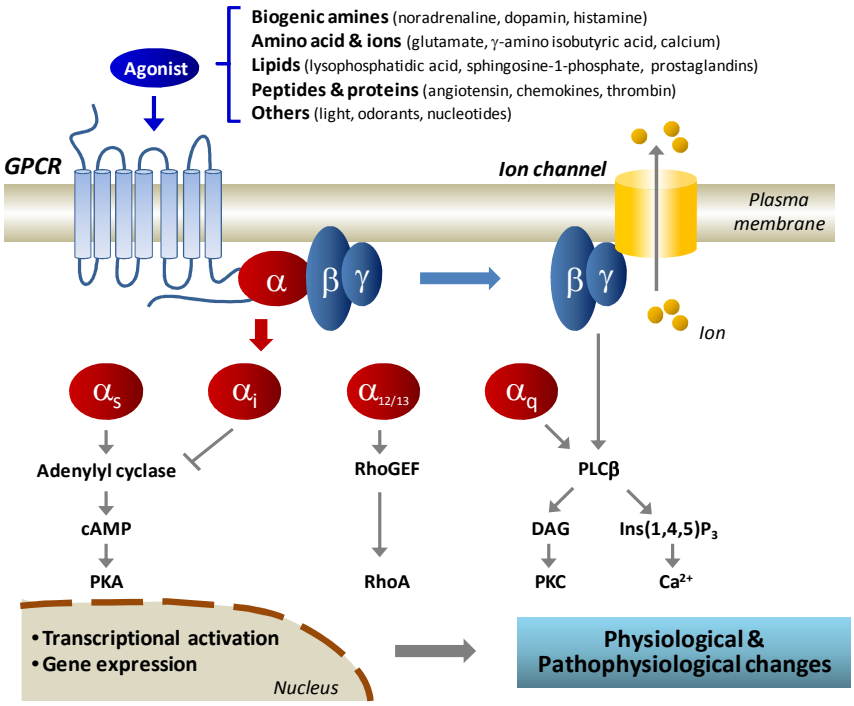


Fig. 4. GPCR activation by extracellular stimuli and intracellular signaling pathways [27]

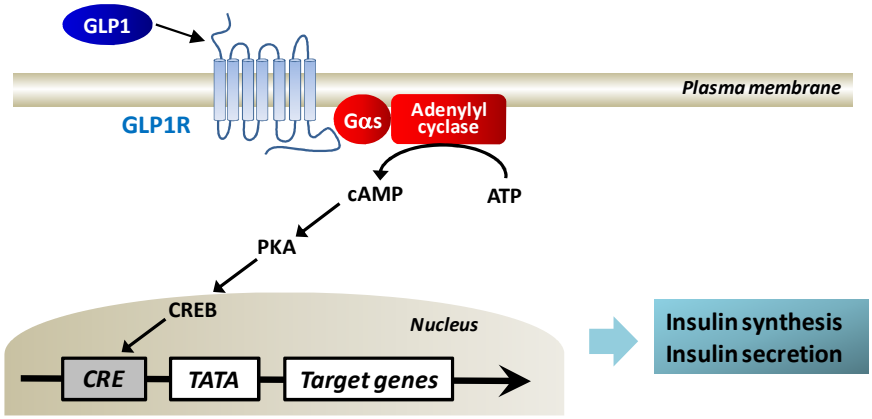
Glucagon-like peptide-1 (GLP-1) is a 30-amino acid peptide hormone produced and secreted in intestinal L-cells. GLP-1 is secreted at low basal rates in the fasting state, and its secretion is increased following nutrient ingestion. GLP-1 exerts its actions through binding to GLP-1 receptor (GLP1R), which is one of the class B GPCRs expressed on pancreatic β-cells (Fig. 5) [28]. GLP-1R activation promotes insulin secretion in pancreatic β-cells depending on blood glucose level, protects the β-cells from apoptotic cell death, and triggers proliferative pathways that lead to expansion of the β-cell mass in animal experiments. Accordingly, drugs activating GLP1R should be promising for the treatment of diabetes. Because native GLP-1 is rapidly degraded by dipeptidyl peptidase-4 after secretion to the blood stream, higher stable exenatide (synthetic exendin-4, Byetta®) [29], a naturally occurring GLP-1 analogue discovered in the venom of *Heloderma suspectum*, was developed and approved as the first peptide-based GLP1R agonists for human clinical use in 2005. However, exenatide was found to have antigenecity resulting in anti-drug antibodies in up to around 30% of patients receiving exenatide [27].

Presently, the development of novel GLP1R agonists with safer profiles, as well as the screening system for novel active peptides, need to be explored and identified [30].

A) Amino acid sequence of GLP1R agonists

GLP1 :	HAEGTFTSDV SSYLEGQAAK EFIAWLVKGR	EC_{50}=5.8 nM
S^2-GLP1 :	HSEGTFTSDV SSYLEGQAAK EFIAWLVKGR	EC_{50}=22 nM
Exendin4 (Ex4) :	HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS	EC_{50}=1.4 nM

B) GLP1R signaling upon activation in pancreas β cells



*CRE: cAMP response element, CREB: CRE-binding protein

Fig. 5. Amino acid sequence of GLP1R agonists and GLP1R signaling in pancreas β cells

Influenza virus and neuraminidase

Influenza is an infectious disease that predominantly affects higher eukaryotic hosts and is caused by an RNA virus of the family *Orthomyxoviridae*. Human seasonal influenza is responsible annually for an estimated 250,000–500,000 deaths and 3–5 million cases of severe illness worldwide [31]. Recently, the novel swine-origin virus (Influenza A H1N1 2009) entered the human population and spread rapidly around the globe, prompting the World Health Organization (WHO) to declare a pandemic in 2009 [32]. In addition, the highly pathogenic avian influenza A virus H5N1 has sporadically been epidemic since 1997 with a high mortality rate of 60% in humans and continues to pose a serious pandemic threat [33].

The two glycoproteins of the influenza virus membrane, hemagglutinin (HA) and neuraminidase (NA), recognize sialic acids on the host cell membrane surfaces. Initiation of virus infection involves multiple HAs binding to sialic acids on

carbohydrate side chains of cell surface glycoproteins and glycolipids. Following virus replication, the receptor-destroying enzyme, NA, removes its substrate, sialic acid, from the infected cell surface so that newly-made viruses are released to infect other cells (Fig. 6). Oseltamivir, one of the first-developed NA inhibitors, has been most widely used for the treatment of patients with influenza virus infection. However, an oseltamivir-resistant H1N1 virus that harbors a specific mutation (H274Y) in the NA protein has been recently reported [34]. It has also been reported that the avian influenza H5N1 virus with the same mutation has emerged, though to a lesser extent [35]. These findings raise public health concerns and therefore there is a need to develop a novel, efficient, and a rapid screening system for the identification of novel inhibitors.

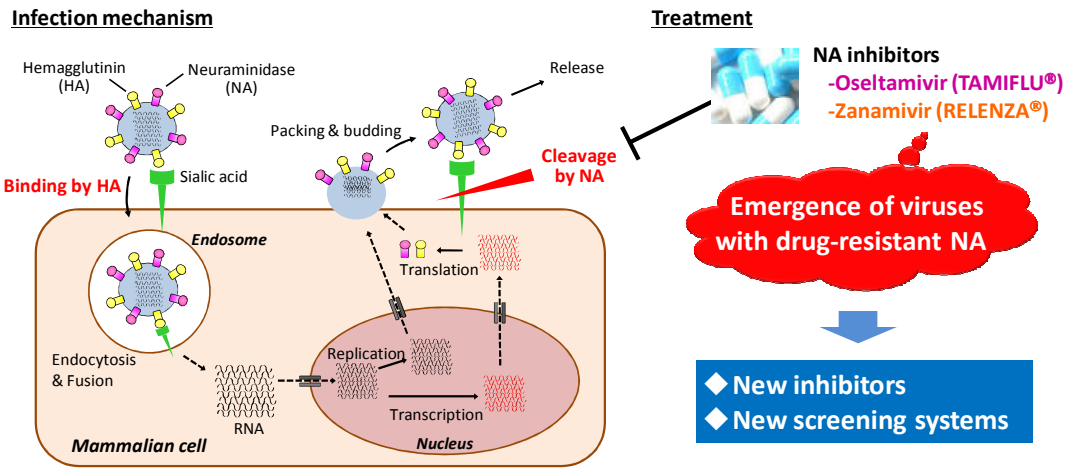


Fig. 6. Infection and expansion mechanisms of influenza virus and neuraminidase inhibition by small molecules

Discovery technologies for bioactive peptides acting on GPCRs and enzymes

There are two major ways to design novel bioactive peptides acting on GPCRs and enzymes: chemical optimization strategy and biotechnological strategy. In a classical way, using a chemical-based approach, structure-activity relationship experiments of newly synthesized peptide derivatives have been conducted for sequenced peptides. For example, N- and/or C-terminal truncation was performed to search for the minimum active sequence. On the other hand, alanine-scanning that replaces one-by-one all residues with alanine was conducted to identify which residue is important for bioactivity. These approaches are effective for bioactive peptides with known sequence, but are less beneficial for cases where peptides with novel sequence need to be designed. To discover novel bioactive peptides, a combinatorial peptide library that can be prepared through chemical and biological methods will be exploited as a source of peptides. However, the chemically-prepared peptide library is difficult to assay as single compounds because of synthesis principles, whereas with the biologically-prepared peptide library is easier to purify each unique peptide through colony formation when using phage, bacteria, and yeast.

Phage display was the first innovative technology established by Smith [36] in order to prepare and screen a large polypeptide library. Phage display delivers unique peptides that are displayed as fusions to a phage coat protein, and the phage particles propagated in *E.coli* are isolated by panning against a target molecule such as GPCRs or enzymes bound to a solid-phase support. However, this methodology is not as effective in discovering functional peptides such as activators for GPCRs or inhibitors for enzymes because phage-displayed peptides are screened based only on binding ability to targets, resulting frequently in simple binders without any bioactivity.

For these reasons, a novel functional screening system of bioactive peptides acting on GPCRs and enzymes is highly in demand. In addition, it would be even more beneficial if a secretion form of the peptide library is directly assayed because there may be dissociation in biological activity between peptides tied up on the phage as a fusion protein and the secretion form of peptides.

The present study has been carried out to develop a novel bioactive peptide screening system for GPCR agonists and influenza neuraminidase inhibitors toward further extension of peptide versatility as medicines. In Chapter I, we established a novel functional screening system for peptides acting on GLP1R without any purification and condensation of the peptides. In Chapter II, we identified novel modified GLP-1R agonists through the screening of random mutagenesis library of GLP1 by using an integrated yeast-mammalian assay system. In Chapter III, we attempted to reconstitute the head domain of wild-type or drug-resistant neuraminidase from influenza H5N1 virus on a yeast cell surface.

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Chapter I Novel functional screening system for yeast-secreted peptides acting on G-protein coupled receptors

G-protein coupled receptors (GPCRs) are one of critical eukaryotic signal transduction gatekeepers and represent the largest protein family in the human proteome with more than 800 members. They share a common architecture of seven transmembrane helices, and can be classified into five major classes of sequence similarities [1]: rhodopsin receptor family (class A), secretin-like receptor family (class B), glutamate receptor family (class C), frizzled/taste 2 receptor family, and adhesion receptor family. GPCRs recognize a variety of extracellular stimuli, including photons, ions, small molecules, peptides, and proteins; they transmit the resulting extracellular signals across the membrane to elicit intracellular responses. Consequently, they cause physiological changes such as regulations of blood pressure, pain, allergies and so on. Therefore, pharmacological modulation of GPCRs has been an effective means to treat various diseases.

Peptides are involved in a variety of physiological and pathological processes, and play very important roles in modulating various cell functions such as the absorption of blood glucose into the body through the promotion of insulin secretion in pancreatic β-cells by glucagon-like peptide-1 (GLP1), a peptide hormone that are postprandially secreted in intestinal L-cells and activates one of class B GPCRs GLP1 receptor (GLP1R) [2]. Because of their intermediate molecular size between that of small-molecule drugs and therapeutic monoclonal antibodies, peptides potentially have advantages of easy drug design, high safety, accessibility for protein-protein interactions, targeting of intracellular molecules, and low production cost [3].

Phage display is the first innovative technology established by Smith [4] that allows researchers to prepare and screen a large polypeptide library. However, this methodology is not as effective at discovering functional peptides such as activators for GPCRs. This is due to the nature of phage display, whose peptides are screened based only on binding ability to targets, resulting frequently in simple binding peptides without any bioactivity [5]. Furthermore, the identified peptides on the phage are never

in the functional, soluble form, potentially leading to dissociation in activity between peptides tied up on the phage as a fusion protein and the secretion form of peptides.

The yeast *Saccharomyces cerevisiae* are very suitable as host for production of peptide library; this is due to their abundance in gene manipulation tools, fast growth in a low-cost medium, and protein folding and secretory machinery homologous to that of mammalian cells, enabling production of complex peptides with disulfide bonds.

In this chapter, we study a novel functional screening method for bioactive peptides acting on GPCRs, which integrated a yeast secretion system and a functional detection system using GPCR-producing mammalian cells. GLP1R produced on mammalian cells was successfully activated by various GLP1R agonistic peptides that were secreted from yeast. We were also able to identify GLP1R agonist-secreting yeasts based on GLP1R activation from a number of background yeasts, which produced non-active control peptides, suggesting the effectiveness of our functional screening system to discover novel peptide-based drugs acting on GPCRs.

Materials and Methods

Strains and media

Esherichia coli DH5α [F^-, *ΔlacU169 (φ80lacZΔM15), hsdR17* (r_K^-, m_K^+), *recA1, endA1, deoR, thi-1, supE44, gyrA96, relA1,* λ^-] (TOYOBO, Osaka, Japan) was used as a host for DNA manipulation. *E. coli* transformants were grown in Luria-Bertani (LB) medium [1% (*w*/*v*) tryptone, 0.5% (*w*/*v*) yeast extract, 1% (*w*/*v*) sodium chloride, and 2% (*w*/*v*) agarose] containing 100 μg/mL ampicillin or kanamycin depending on the plasmids introduced.

Saccharomyces cerevisiae BY4742 (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*; EUROSCARF, Frankfurt, Germany) was used to construct the yeasts secreting GLP1 receptor (GLP1R) agonists, including GLP1, S^2-GLP1 substituted with serine at the position 2 of GLP1, and exendin-4 (Ex4), a naturally occurring peptide found in the saliva of the Gila monster [6]. Yeast transformants were selected on synthetic dextrose (SDC) solid medium [0.67% (*w*/*v*) yeast nitrogen base without amino acids, 2% (*w*/*v*)

glucose, 1% (*w*/*v*) casamino acids, 0.002% (*w*/*v*) adenine, 0.002% (*w*/*v*) L-tryptophan, and 2% (*w*/*v*) agar], and then, the resultant colonies were cultivated in 6-well plate (353046; Thermo Fisher Scientific, Waltham, MA, USA) or 96-well plate (353072; Thermo Fisher Scientific) containing a liquid SDC medium or Dulbecco's modified Eagle Medium (DMEM) (Nacalai Tesque, Kyoto, Japan) at 30°C.

Chinese hamster ovary (CHO) cells (85050302; European Collection of Cell Cultures, Salisbury, UK) were used as a host cell stably expressing human GLP1R and cultivated in Ham-F12 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Thermo Fisher Scientific) and 400 μg/mL G418 (Nacalai Tesque).

Construction of peptide-secreting yeast

All the primers used in plasmid construction are listed in Table 1. For peptide secretion in yeast, pULS harboring the engineered secretion signal of yeast α-factor, appS4 [7], in the downstream of GAPDH promoter, was constructed as follows. The DNA fragment encoding the appS4 (FASMAC, Kanagawa, Japan) in pUC19 was amplified using primer 1 and 2, and was inserted into pULI1 [8], which was digested with *EcoR*I and *Xba*I by using In-Fusion (Clontech Laboratories, Inc., Mountain View, CA, USA) to obtain pULS. The GLP1, S^2-GLP1 and exendin-4 gene, with or without a FLAG-encoding sequence at the 3' terminus, were synthesized from oligonucleotide mutually with the complementary region and the homologous region of pULS, and double-stranded with DNA polymerase KOD-FX-Neo (Toyobo). The double-stranded DNA encoding those GLP1 analogues were introduced into the multiple cloning sites of pULS by In-Fusion (Clontech) and named pULS-GLP1, -GLP1FLAG, -S^2-GLP1, -S^2-GLP1FLAG, -Ex4 and -Ex4FLAG, respectively. Yeasts were transformed with those plasmids using Frozen-EZ Yeast Transformation-II kit (Zymo Research, Orange, CA, USA), resulting in GLP1-yeast, GLP1F-yeast, S^2-GLP1-yeast, S^2-GLP1F-yeast, Ex4-yeast, and Ex4F-yeast, respectively. Yeast transformed with pULS (Ctrl-yeast) was used as control.

Construction of human GLP1R-producing CHO

Human GLP1R gene was PCR-amplified from the human brain cDNA library (BioChain, Newark, CA, USA) using DNA polymerase KOD-PLUS-Neo (Toyobo) with primer 3 and 4 (Table 1). The DNA fragment coding human GLP1R was inserted into pIRES (Clontech) digested with *Eco*RV and *Bam*HI by using In-Fusion (Clontech), resulting in pIRES-hGLP1R. CHO cells were transfected with pIRES-hGLP1R using Xfect (Clontech), and then, selected with G418 for about 2 weeks to construct a stable cell line expressing hGLP1R. Single cell cloning of the resistant cells was conducted by limiting dilution, resulting in GLP1R-CHO.

GLP1R activation assay using GLP1R-CHO

GLP1R-CHO was seeded onto a 96-well plate at 5×10^4 cells and cultured at 37°C for 24 h. After the cells were washed with HANKS buffer (Thermo Fisher Scientific), synthetic GLP1R agonists (GLP1 and Ex4; Peptide Institute, Osak, Japan. S^2-GLP1; Bachem, Bubendorf, Switzerland.) or culture supernatant of GLP1R agonists-secreting yeast were added and incubated at 37°C for 45 min. Then, the cells were lysed with Assay/Lysis buffer (Thermo Fisher Scientific) and the level of cyclic AMP in the cell lysate was determined by using the cAMP-screen® assay (Thermo Fisher Scientific) according to the manufacturer's instructions.

Model screening of Ex4-secreting yeast

Ten yeast cells comprised of Ctrl-yeast and Ex4-yeast in the theoretical respective ratio of 9:1 were seeded into 16 wells in 96-well plate containing 300 μL of SDC medium and grown for 48 h. Then, the medium was exchanged into 250 μL of DMEM and yeasts were additionally cultivated for 12 h at 30°C. After that, the supernatant was subjected to the GLP1R activation assay mentioned above. Yeasts included in three wells showing or not showing activity were seeded on SDC solid medium to form single colonies. Then, 48 colonies were subjected to colony-direct PCR with primers 5 and 6 (Table 1), and the resultant PCR products were analyzed by agarose gel electrophoresis to identify yeasts with the Ex4 gene.

Table 1 Primers used in this study

	Sequence
Primer 1	5'-AAACACACATAAACACCCGGGATG
Primer 2	5'-CAGTCTAGAGGATCCGAATTCTCTTTTATCCAAAGATACCCCTTCTTC
Primer 3	5'-CGAGCTCGGATCGATCGCCACCATGGCCGGCGCCC
Primer 4	5'-TATCTATGCGGCCGCTCAGCTGCAGGAGGCCTG
Primer 5	5'-GAAGAAGGGGTATCTTTGGATAAAAG
Primer 6	5'-CTTGTCATCGTCATCCTTGTAATC

*Underline indicates homologous region to the corresponding plasmids.

Table 2 Amino acid sequence of GLP1R agonists used in this study

Agonists	Amino acid sequence	EC_{50}
GLP1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR	5.8 nM
S^2-GLP1	HSEGTFTSDVSSYLEGQAAKEFIAWLVKGR	22 nM
Exendin4	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS	1.4 nM

*Underline indicates amino acids different from GLP1.

Results

Construction of stable CHO cells producing human GLP1R

CHO cells producing human GLP1R (GLP1R-CHO) were constructed to detect functional activity of synthetic- or yeast-secreted GLP1R agonists. CHO cells were transfected with pIRES-hGLP1R by lipofection and selected using the neomycin resistance gene on the pIRES vector. The cells established showed cAMP production upon GLP1R activation by three kinds of synthetic GLP1R agonists, GLP1, S^2-GLP1, and Ex4 in a dose-dependent manner, with EC_{50} value of 5.8, 21.5 and 1.4 nM, respectively (Fig. 1, Table 2). Therefore, functional human GLP1R was successfully produced in CHO cells.

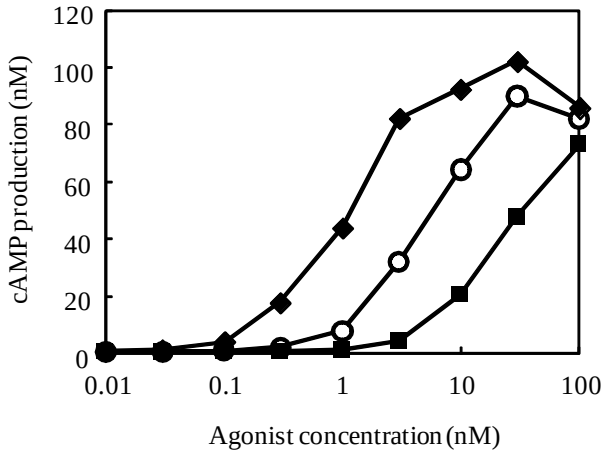


Fig. 1. Dose-response curve of GLP1R activation in GLP1R-CHO upon treatment of synthetic GLP1R agonists

GLP1R-CHO cells were exposed to three kinds of GLP1R agonists, GLP1 (white circle), S^2-GLP1 (black square) and Ex4 (black diamond), at increasing concentrations. After 45 min incubation, cells were lysed and cAMP production was determined by ELISA as indicated in Materials and Methods. Data represent the results of one experiment.

Medium optimization for GLP1R activation by yeast-secreted peptides and establishment of assay system

We first tested which medium was suitable for yeast growth and evaluation of GLP1R activation in GLP1R-CHO in 6-well plates. Ctrl-yeast and Ex4-yeast were inoculated in a 6-well plate containing SDC medium or DMEM at the initial optical density (OD) which was 600 nm of 0.1 and incubated for 40 h. Then, the growth rates of yeasts and the GLP1R activation in GLP1R-CHO by culture supernatant containing yeast-secreted peptides were evaluated (Fig. 2). As a result, SDC gave a higher growth reaching stationary phase at 20 h, showing about 90-fold expansion. On the other hand, yeasts cultivated in DMEM showed much lower growth, reaching only the maximal OD_{600} of 0.75 at 20 h. For GLP1R activation potency after the 40-h cultivation, the culture supernatant of Ex4-yeast grown in SDC showed no activity, although exogenously added Ex4 activated GLP1R by 15.7-fold, compared with the control. In contrast, Ex4-yeast grown in DMEM strongly induced GLP1R activation by 50-fold compared to the Ctrl-yeast. These results indicate that SDC medium is suitable for yeast proliferation, while DMEM is excellent for GLP1R activation.

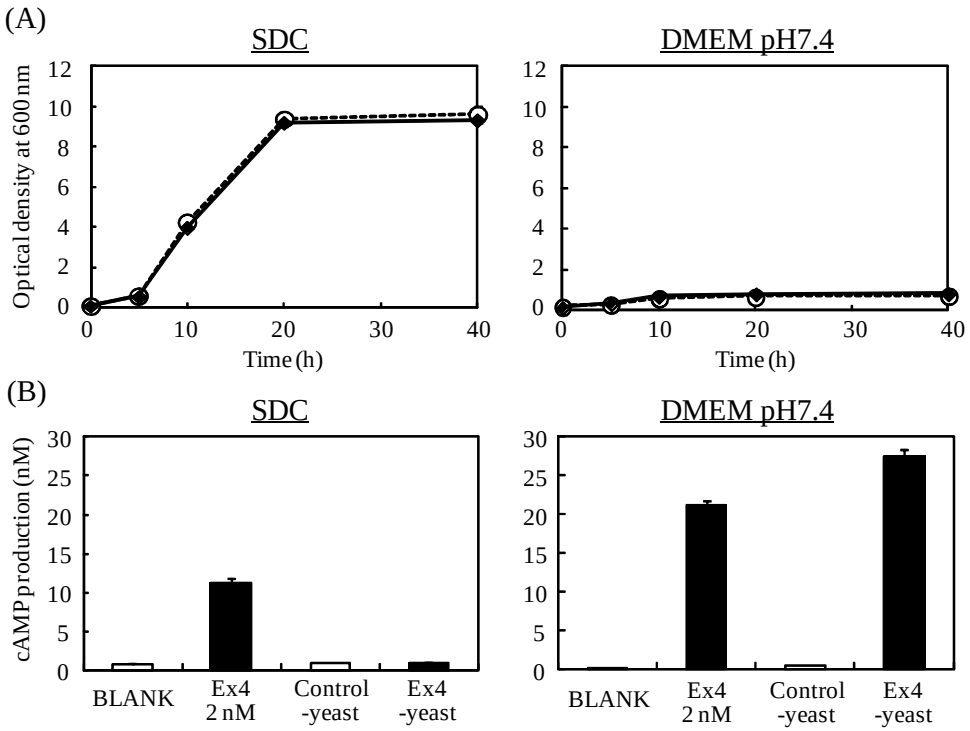


Fig. 2. Medium optimization for GLP1R activation assay of yeast-secreted peptides
Yeasts were grown in SDC and DMEM on 6-well plate for 40 h, and then, growth rate (A) and GLP1R activation potential of their culture supernatant (B) were evaluated by measurement of absorbance at 600 nm and ELISA for cAMP, respectively. For growth curve, white circle indicates Ctrl-yeast and black diamond shows Ex4-yeast. The data represent the average ± SEM. of 3 independent experiments.

Next, we tried to establish a GLP1R activation assay system combined with peptide-secreting yeast in a 96-well plate format. We first determined the number of Ex4-yeasts required for larger dynamic range in the GLP1R activation. Yeast cells suspended in DMEM were prepared at 4×10^4 to 5×10^6 cells in 96-well plates, and after a 12 h-cultivation at 30°C, a GLP1R activation assay using the culture supernatants was carried out (Fig. 3A). The results showed that 4×10^4 yeast cells were enough to detect GLP1R activation, showing 73-fold activation, compared with the control, and 1×10^6 yeast cells provided the largest dynamic range with 224-fold activation of GLP1R. Further, we investigated the cultivation time required for the yeast

cell number of 1 × 10^6 (Fig. 3B). Yeast preparations with the initial cell number of 3 were cultivated in SDC medium that showed the highest growth as in Fig. 1, and then, yeast cell number was counted at 24, 48, and 72 h cultivation. As results, the cell number per initial cell number exceeded 1 × 10^6 cells after 48 h cultivation. These results suggested that a single yeast cell can reach 1 × 10^6 cells, showing the highest dynamic range in GLP1R activation assay when cultivated in SDC for 48 h.

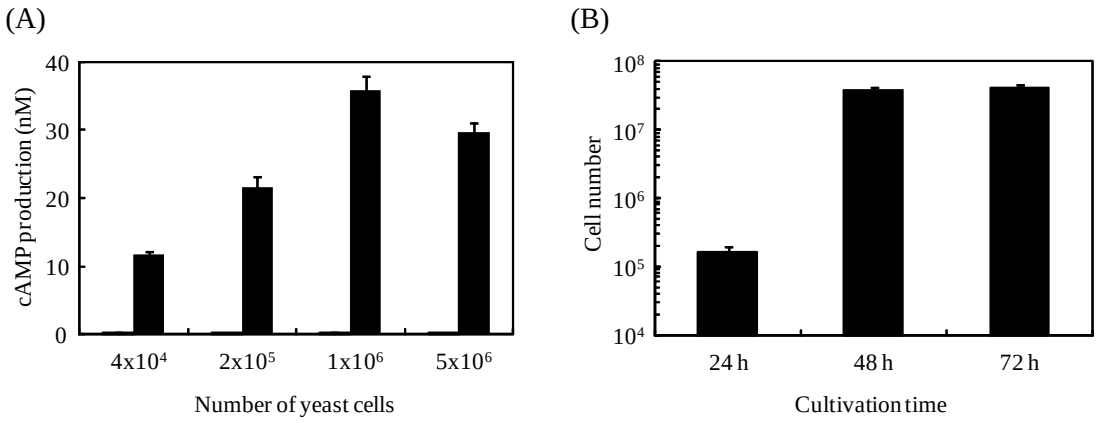


Fig. 3. Scale-down assay for GLP1R activation by yeast-secreted peptides

(A) Indicated number of yeast cells (Ctrl-yeast or Ex4-yeast, shown by white and black bar, respectively) were seeded onto 96-well plate containing DMEM and incubated for 12 h. Then, GLP1R activation assay was conducted to find the yeast cell number providing wider dynamic range. The data represent the average ± SEM. of 3 independent experiments. (B) Yeast cultivation time to achieve the target yeast cell number, 1 x 10^6, was evaluated. Yeasts were seeded onto 96-well plate containing SDC at the initial number of 3. After 24-, 48-, and 72-h cultivation, the number of yeasts was determined using hematometer. The data represent the average ± SEM. of 3 independent experiments.

Direct functional assay of various yeast-secreted GLP1R peptide agonists

Based on these optimizations, we constructed a series of workflow for direct functional assay of yeast-secreted peptides on GLP1R, composed of 3 steps (Fig. 4A): cultivation for yeast growth, peptide secretion in yeasts, and GLP1R activation assay using GLP1R-CHO. To demonstrate the effectiveness of the direct functional assay system, we attempted to detect the GLP1R activation by native agonist GLP1, its analogue S^2-GLP1, and Ex4 secreted by yeast. Single colony of the yeasts secreting GLP1, S^2-GLP1, and Ex4 were inoculated and incubated in a 96-well plate containing SDC for 48 h, and then, the medium was exchanged into DMEM with additional incubation of 12 h, followed by the GLP1R activation assay (Fig. 4B). While yeast-secreted Ex4 showed the highest activation by 59.1-fold, GLP1 and S^2-GLP1 secreted by yeasts provided only 5.8 and 1.7-fold activation, respectively. Therefore, GLP1 and S^2-GLP1 were considered insufficient given those EC_{50} values are a quarter and one sixteenth of the Ex4 values, respectively. Whereas the N-terminal two residues (His-Ala) in GLP1 are reported to be critical for its biological activity, the C-terminal part is tolerable to various modifications such as fatty acid conjugation or albumin fusion [9,10]. Thus, we fused FLAG tag to the C-terminus of GLP1, S^2-GLP1, and Ex4 initially for affinity purification and Western blotting, if needed. Remarkably, when we evaluated the GLP1R activation potency for such yeast-secreted GLP1 agonists with the FLAG tag, GLP1 and S^2-GLP1 were activated GLP1R about 10-times higher than those without the FLAG tag, respectively. These results demonstrated that yeast-secreted GLP1 and S^2-GLP1 with the addition of C-terminal FLAG tag were successfully and directly detected without any purification and condensation by using the novel functional detection system established here.

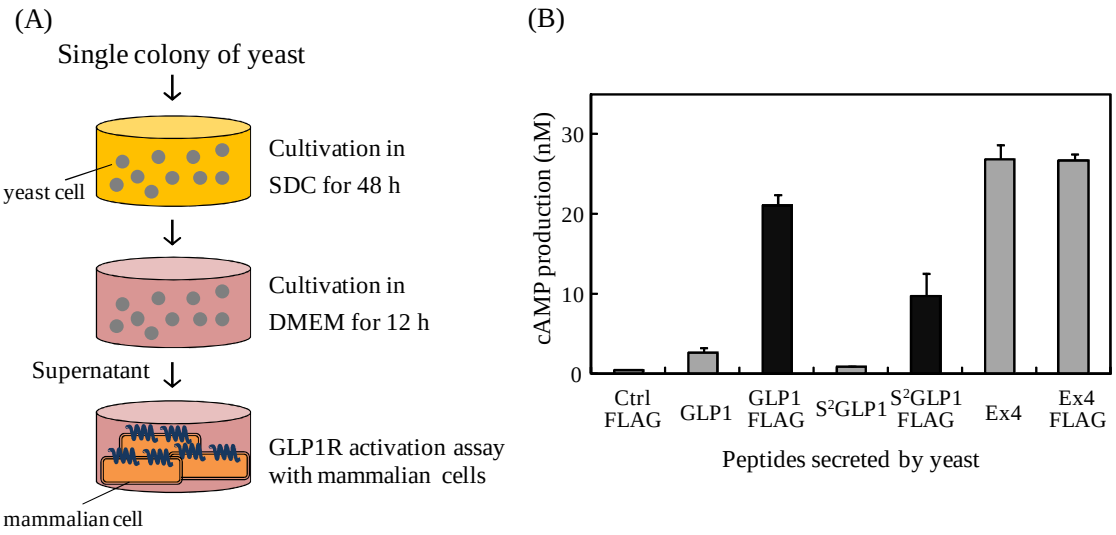
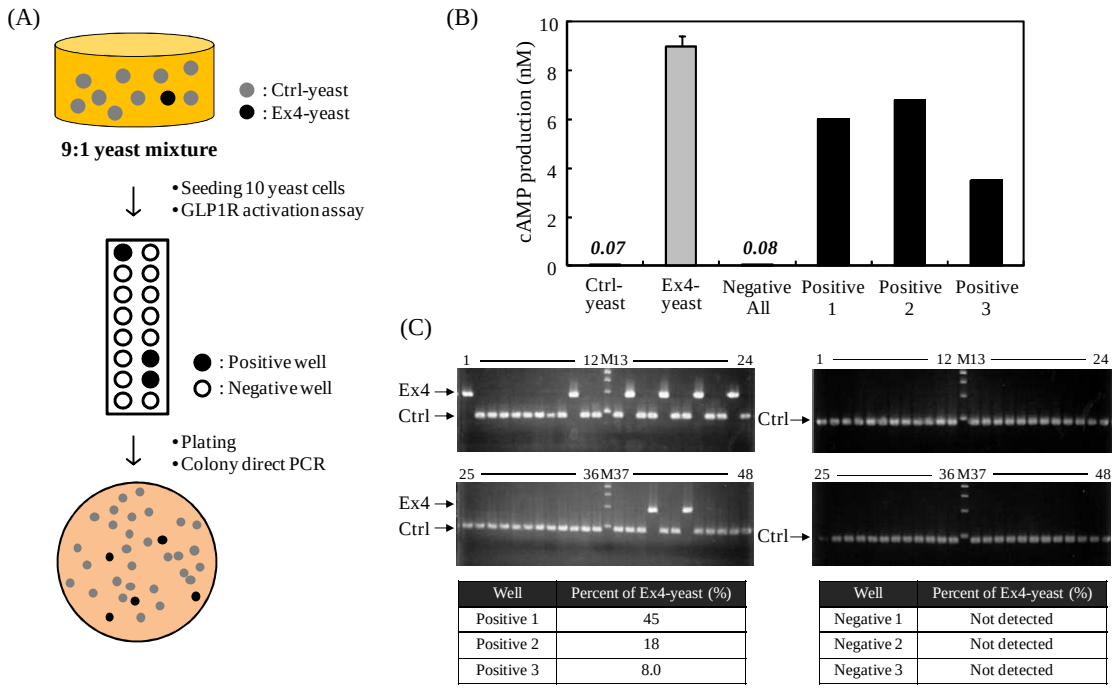


Fig. 4. Direct activity detection of various yeast-secreted GLP1R agonists

(A) Schematic illustration for direct activity detection assay was depicted. Single colony of yeasts was inoculated in 96-well plate containing SDC and cultivated for 48 h. After removal of SDC, DMEM was added and incubated for additional 12 h. Then, the culture was subjected to GLP1R activation assay using GLP1R-CHO. (B) Seven kinds of yeasts were tested for GLP1R activation potency according to the assay flow mentioned above. The data represent the average ± SEM. of 3 independent experiments.

Examination of model screening using our established functional assay system

Finally, we performed a model screening of yeast by applying the direct functional assay system, for example, a screening of Ex4-yeast in the co-presence of excess Ctrl-yeast. A yeast cell mixture including Ctrl-yeast and Ex4-yeast at a theoretical ratio of 9:1 was prepared, and a GLP1R activation assay was conducted according to the determined workflow (Fig. 5A). As a result, we identified three positive wells showing GLP1R activation. We next investigated the existence of Ex4-yeast in the positive and negative wells by colony-direct PCR after the colony formation of yeasts from both wells. The results indicated the existence of 23.8% of Ex4-yeast in the positive wells, whereas Ex4-yeast was not identified at all in the negative wells. These results clearly suggest that our direct functional assay system established was effective in the model screening.



Well	Percent of Ex4-yeast (%)
Positive 1	45
Positive 2	18
Positive 3	8.0

Well	Percent of Ex4-yeast (%)
Negative 1	Not detected
Negative 2	Not detected
Negative 3	Not detected

Fig. 5. Model screening of Ex4-secreting yeast using the direct activity detection system

(A) Schematic illustration for model screening of Ex4-yeast was indicated. (B) We prepared 16 wells including 9:1 yeast mixture (10 cells in total per well) in the ratio of Ctrl-yeast to Ex4-yeast and conducted GLP1R activation assay according to the workflow indicated above. Only Ctrl-yeast or Ex4-yeast was tested as a control. (C) The existence of Ex4-yeast in the positive and negative wells was investigated by colony-direct PCR after the colony formation of yeasts from both wells. The representative electrophoresis data are shown for colonies from a positive and a negative well, respectively. M indicates band size marker (New England Biolabs, Ipswich, MA, USA).

Discussion

In this chapter, we established the novel functional screening system of yeast-secreted peptides acting on GLP1R. The system directly detected the functional activity of yeast secreted-GLP1R agonists based on activation of GLP1R produced on mammalian cells, without any purification and condensation of yeast-produced peptides. In addition, application of the system enabled identification of agonist-secreting yeast in the model screening.

Binding-based peptide screening strategy as represented by phage display has been the most common method used to discover novel peptide-ligands for certain drug targets, including GPCRs [11,12]. However, with peptides discovered through this methodology, it is uncertain whether they are biologically active or not. In addition, phage-displayed peptides are distinct from functional peptides in their soluble form. These challenges result in additional chemical synthesis of the identified peptides in soluble form to evaluate their true biological activity, which are time-consuming and quite expensive.

In the first optimization step using Ex4-yeasts as a model for our novel functional assay system, we remarkably found that DMEM buffered at neutral pH designed for mammalian cell culture was very suitable for yeast peptide secretion and GLP1R activation in mammalian cells, even though yeast cells could not grow in the medium. Another remarkable point in this culture system is a protection of the target peptides from degradation by yeast-derived proteases. Because heterologous proteins produced in yeasts could be degraded during several steps, including the intracellular secretory pathways and the post-secreted extracellular environment [13], yeast-derived proteases such as Yps1p and Kex2p, which are most active at a mild acidic condition around pH 5.0, are considered accessible to heterologous proteins, especially when yeasts are incubated in SDC medium, which generally has pH 4.5 to 5.5 [14,15]. In addition, yeasts were a good tool for producing peptides because yeasts swiftly grew in SDC medium; they even in a static 96-well plate setting which would be compatible with high-throughput screening and could easily reach the targeted yeast cell number

providing high dynamic range in GLP1R activation assay (Fig. 2).

These findings prompted us to conduct the direct functional detection of other GLP1R agonists with weaker activity than Ex4, GLP1, and S^2-GLP1 to be secreted by yeasts. It was difficult to detect their GLP1R activation potency at the high level as expected from the difference of the EC_{50} value of Ex4. Surprisingly, the C-terminus fusion of the FLAG tag with DYKDDDDK in GLP1 and S^2-GLP1 much increased the GLP1R activation potency by about 10-fold compared to those without the FLAG tag (Fig. 4B). In spite of the fact that the precise mechanisms of the FLAG are still uncertain, the beneficial effect of the FLAG fusion was also observed in somatostatin that is endogenous circular peptide agonist acting on class A GPCR SST receptor. Accordingly, the FLAG fusion to isolated peptides would be a promising way to increase their activity.

As shown in Fig. 5, we successfully identified the wells including Ex4-yeast by evaluating the GLP1R activation potency, and importantly, the wells that did not show activity did not possess any Ex4-yeast, as confirmed by colony-direct PCR detecting Ex4 gene. This successful model screening of yeasts secreting agonist acting on GLP1R encourages us to carry out an actual screening for novel bioactive peptides through our direct functional detection system.

In conclusion, we successfully established a novel system for direct functional assay for yeast-secreted peptides on GLP1R. This system will be applied not only for biological activity assay of sequenced peptides instead of their chemical synthesis but also discovery of novel bioactive peptides.

Summary

We established a novel functional screening system for peptides acting on G-protein coupled receptors (GPCRs). Peptides are a promising drug scaffold because of their intermediate molecular size between that of therapeutic small molecules and antibodies. They also offer potential advantages of targeting not only membrane proteins but also intracellular protein-protein interactions. Although peptides acting on GPCRs have been explored based on binding affinity to targets by using phage display, it is unclear whether the identified peptides could functionally modulate the targets. In a novel screening system that we established, yeast cells were utilized as a peptide producer while mammalian cells stably expressing the receptor for glucagon-like peptide 1 (GLP1R) were used as a biosensor for receptor activation. Three kinds of GLP1R agonists secreted by yeasts were successfully detected for their functional activity without any purification and condensation of the peptides. By applying the functional screening system, we were able to identify GLP1R agonist-secreting yeasts based on GLP1R activation from a number of background yeasts that produced non-active control peptides. Further applications of this system would include not only activity evaluation of bioactive peptides without chemical synthesis but also discovery of novel peptides activating druggable GPCRs.

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Chapter II Screening of randomly mutagenized GLP1 library by using an integrated yeast-mammalian assay system

Glucagon-like peptide-1 (GLP-1) is a 30-amino acid peptide hormone produced in intestinal L-cells at low basal rates in the fasting state, whereas its secretion increases following nutrient ingestion [1]. GLP-1 exhibits its actions through binding to GLP-1 receptor (GLP1R), a class B G-protein coupled receptors (GPCRs) expressed on various organs including pancreatic β-cells. GLP1R activation promotes insulin secretion in pancreatic β-cells depending on blood glucose levels, protects the β-cells from apoptotic cell death, and triggers proliferative pathways that lead to expansion of the β-cell mass in animal experiments [2]. Therefore, drugs (agonists) activating GLP1R should be promising for the treatment of diabetes. However, therapeutic use of native GLP-1 has been limited due to its very short half-life in the body based on enzymatic degradation and/or renal clearance [3].

One of the most important limitations of therapeutic peptides, including GLP-1, is low bioavailability due in part to high biodegradability by gastrointestinal, plasma and tissue peptidases. Therefore, these peptides often receive chemical modifications with long fatty acid chains and/or polyethylene glycol that are conjugated onto amino acid residues less affecting their biological activity [4,5]. Alanine-scanning is one strategy to determine the relationship between amino acid sequences and functions of peptides, whereby, one by one, all amino acid residues are substituted by alanine with a relatively small neutral side-chain [6]. Such alanine-substituted peptides are analyzed in terms of biological activity, revealing which residue is crucial and which could be chemically modified [7]. However, this chemical approach requires a cumbersome process that includes chemical synthesis, purification with a separation column, and condensation of peptides of interest.

In this chapter, we study a novel biological method to screen GLP1R agonists, integrating a yeast secretion system and a functional detection system with mammalian cells harboring GLP1R and inducible secretory luciferase as a reporter (Fig. 1). By using this functional assay system, we found GLP1R agonists with various substitutions through screening of a randomly mutagenized GLP1 library secreted by yeast.

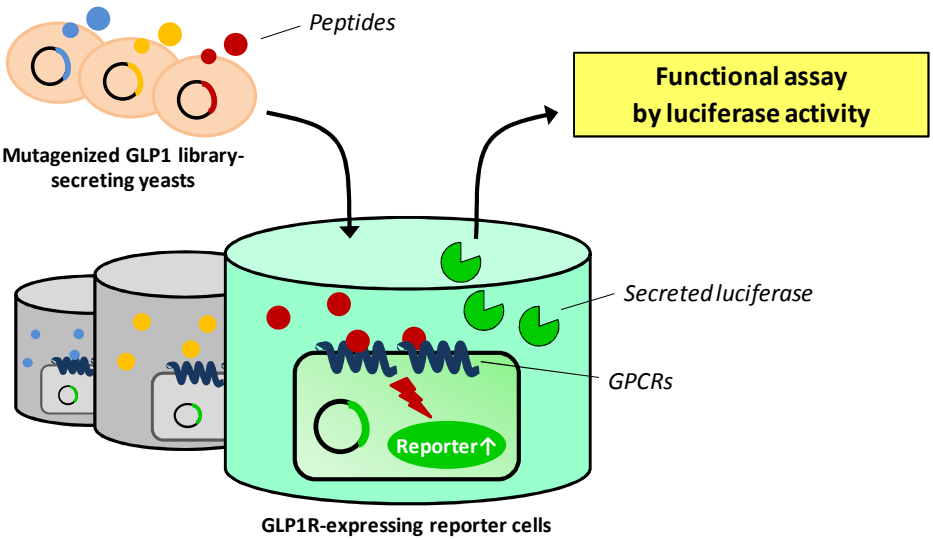

Fig. 1. Novel functional assay system integrating peptide-secreting yeasts and GLP1R-producing reporter cells

A novel functional assay system integrating peptide-secreting yeasts and GLP1R-producing reporter cells was constructed. Unique peptides secreted by yeasts were directly assayed with GLP1R-producing mammalian cells designed to induce secretory luciferase upon receptor activation.

Materials and Methods

Strains and media

Escherichia coli DH5α [F^-, *ΔlacU169 (φ80lacZΔM15), hsdR17* (r_K^-, m_K^+), *recA1, endA1, deoR, thi-1, supE44, gyrA96, relA1,* λ^-] (Toyobo, Osaka, Japan) was used as a host for DNA manipulation. *E. coli* transformants were grown in Luria-Bertani (LB) medium [1% (*w*/*v*) tryptone, 0.5% (*w*/*v*) yeast extract, 1% (*w*/*v*) sodium chloride, and 2% (*w*/*v*) agarose] containing 100 μg/mL ampicillin or kanamycin depending on plasmids introduced.

Saccharomyces cerevisiae BY4742 (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*; EUROSCARF, Frankfurt, Germany) was used to construct the yeasts secreting GLP1, S^2-GLP1 substituted with serine at amino acid position 2, C-terminally FLAG-fused GLP1 (GLP1F) and S^2-GLP1 (S^2-GLP1F), and a randomly-mutagenized GLP1 library.

Yeast transformants were selected on a synthetic dextrose (SDC) solid medium [0.67% (*w*/*v*) yeast nitrogen base without amino acids, 2% (*w*/*v*) glucose, 1% (*w*/*v*) casamino acids, 0.002% (*w*/*v*) adenine, 0.002% (*w*/*v*) L-tryptophan, and 2% (*w*/*v*) agar], and then the resultant colonies were cultivated in 6-well plates (353046; Thermo Fisher Scientific, Waltham, MA, USA) or 96-well plates (353072; Thermo Fisher Scientific) containing liquid SDC medium or Dulbecco's Modified Eagle Medium (DMEM) (Nacalai Tesque, Kyoto, Japan), at 30°C.

HEK293 cells (85120602; European Collection of Cell Cultures, Salisbury, UK) were used to construct reporter cells that stably possess genes for human GLP1R and a secretory luciferase, NanoLuc (Promega, Fitchburg, WI, USA). Reporter cells were cultivated in DMEM containing 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), 400 μg/mL G418 (Nacalai Tesque), and 0.1 μg/mL puromycin (Wako Pure Chemical Industries, Osaka, Japan).

Construction of GLP1R agonist-secreting yeast

Primers used in plasmid construction are listed in Table I. For peptide secretion in yeast, pULS, encoding the engineered secretion signal of yeast α-factor appS4 [8] downstream of the GAPDH promoter, was constructed as follows. The DNA fragment encoding appS4 (FASMAC, Kanagawa, Japan) in pUC19 was amplified with primers 1 and 2, inserted into pULI1 [9], and then digested with *Eco*RI and *Xba*I, using In-Fusion® HD Cloning Kit (Clontech, Mountain View, CA, USA), resulting in pULS. DNA fragments encoding GLP1 or S^2-GLP1 with or without a FLAG tag at the 3' terminus were synthesized from oligonucleotide mutually with the complementary region and the homologous region with pULS, and double-stranded with DNA polymerase KOD-Fx-Neo (Toyobo). Double-stranded DNA encoding the GLP1 analogs was introduced into the multiple cloning sites of pULS by In-Fusion® and named pULS-GLP1, -GLP1F, -S^2-GLP1, and -S^2-GLP1F, respectively. Yeasts were transformed with these plasmids, using Frozen-EZ Yeast Transformation-II™ Kit (Zymo Research, CA, USA), resulting in GLP1-yeast, GLP1F-yeast, S^2-GLP1-yeast, and S^2-GLP1F-yeast, respectively. Yeast transformed with pULS (Ctrl-yeast) was used as a negative control.

Table I Primers used in this study

Name	Sequence
Primer 1	5'-AAACACACATAAACACCCGGGATG
Primer 2	5'-CAGTCTAGAGGATCCGAATTCTCTTTTATCCAAAGATACCCCTTCTTC
Primer 3	5'-CGAGCTCGGATCGATCGCCACCATGGCCGGCGCCC
Primer 4	5'-TATCTATGCGGCCGCTCAGCTGCAGGAGGCCTG
Primer 5	5'-AGCCGCTTAAAAGTTGGTACCGCCACCATGAACTCCTTCTC
Primer 6	5'-GCGTACGTCTCATGCGCATTACGCCAGAATGCGTTCG
Primer 7	5'-GAAGAAGGGGTATCTTTGGATAAAAG
Primer 8	5'-GTAATCAGATCCTCCACCACC
Primer 9	5'-CATTGCTGCTAAAGAAGAAGGGGTATCTTTGGATAAAAG
Primer 10	5'-ACTTGTCATCGTCATCCTTGTAATCAGATCCTCCACCACC

Construction of GLP1R/NanoLuc gene-harboring reporter cells

Human GLP1R gene was PCR-amplified from a human brain cDNA library (BioChain, Newark, CA, USA), using DNA polymerase KOD-Plus-Neo (Toyobo) with primers 3 and 4. The DNA fragment coding human GLP1R was inserted into pIRES (Clontech) digested with *Eco*RV and *Bam*HI, using In-Fusion™, resulting in pIRES-hGLP1R.

The gene encoding NanoLuc luciferase with an IL-6 secretion signal sequence at the N-terminus was PCR-amplified from pNL2.3[secNluc/Hygro] vector (Promega) with primers 5 and 6. The DNA fragment including the luciferase gene was replaced with the green fluorescent protein coding region on pCRE-PtGFP (Xactagen, Shoreline, WA, USA), a plasmid vector possessing cyclic AMP-responsive element (CRE) upstream of a TATA box, resulting in pCRE-NLuc. HEK293 cells were co-transfected with pIRES-hGLP1R and pCRE-NLuc by using Xfect™ (Clontech), and then selected with G418 and puromycin-containing medium to establish a stable cell line harboring the GLP1R and secretory NanoLuc genes (GLP1R/Luc-293).

Construction of a randomly mutagenized GLP1 library

DNA encoding wild-type GLP1 was first randomly mutagenized by PCR, using mutagenic dNTP analogs (8-oxo-dGTP and dPTP) (dNTP-mutagenesis Kit; Jena bioscience, Jena, Germany) with primers 7 and 8, and 10 or 20 PCR cycles. After column purification by using MinElute PCR Purification Kit (Qiagen, Venlo, Netherlands), a second PCR was performed using KOD-Fx-Neo DNA polymerase with primers 9 and 10 to eliminate the mutagenic dNTP analogs. The randomly mutagenized GLP1 DNA library and pULS digested with *Eco*RI and *Pst*I were co-introduced into yeasts by using Gene Pulser® II Electroporation System (BioRad, Hercules, CA, USA), yielding the yeast library secreting randomly mutagenized GLP1.

GLP1R activation assay using luciferase reporter gene

GLP1R activation by synthetic GLP1 analogs [GLP1 (Peptide Institute, Osaka, Japan), S^2-GLP1 (Bachem, Bubendorf, Switzerland) and GLP1FLAG (Toray Research Center, Tokyo, Japan)], yeast-secreted GLP1 analogs, and the yeast-secreted random mutagenesis GLP1 library was evaluated by determining secreted luciferase activity in GLP1R/Luc-293. For assay of yeast-secreting GLP1 analogs and the random mutagenesis GLP1 library, each yeast colony was inoculated into a 96-well plate (353072; Thermo Fisher Scientific) containing 300 μL of SDC medium and grown for 48 h at 30°C. Then, the medium was changed to 250 μL of DMEM and yeasts were cultivated for another 12 h at 30°C. After this, culture supernatant (50 μL) was transferred to 96-well white plates (353296; Thermo Fisher Scientific) including GLP1R/Luc-293 at a cell concentration of 5×10^3/50 μL. After 12 h of incubation at 37°C, luciferase activity in the culture supernatant was determined by adding 50 μL of substrate solution, provided by Nano-Glo™ Luciferase Assay System (Promega), and measuring chemiluminescence intensity with a Fluoroskan Ascent™ Microplate Fluorometer (Thermo Fisher Scientific). In the first screening assay for the randomly-mutagenized library of GLP1, Ctrl-yeast culture supernatant was used as negative control (0% activity) and GLP1F-yeast culture supernatant was used as positive control (100% activity).

DNA sequencing of random GLP1-secreting yeast library

Yeasts selected from the first screening of the random GLP1 library were subjected to direct colony PCR, using KOD-Fx-Neo to amplify the introduced peptide-coding region, and then the amplified DNA fragment was purified using MinElute PCR Purification Kit. DNA fragment were verified following nucleotide sequencing by BigDye Terminator v3.1 Cycle Sequencing Kit and 310 genetic Analyzer (Thermo Fisher Scientific) according to the manufacturer's instructions.

Validation assay of identified peptides

Selected yeasts in the first screening of the random GLP1 library were grown in SDC medium in 24-well plates (353047; Thermo Fisher Scientific) for 24 h, and then their plasmids were purified using Zymoprep™ Yeast Plasmid Miniprep II (Zymo Research). Plasmids were amplified by introduction into competent *E.coli* DH5α, followed by purification using LaboPass™ Plasmid Mini Kit (Hokkaido System Science, Sapporo, Japan). Yeasts were retransformed with these plasmids and were then subjected to functional assays for GLP1R activation as outlined above. DNA sequences of the newly prepared plasmids were also analyzed as described above.

Results

Construction of stable GLP1R-producing cells with inducible-secretory luciferase

NanoLuc is an engineered, small, monomeric luciferase with high thermal stability and enzyme activity across a broad range of pH, and it catalyzes a novel chemiluminogenic substrate to yield higher and more durable brightness than that obtained using conventional luciferases from *Photinus pyralis* and *Renilla reniformis* [10]. These properties enable greater sensitivity and more robust detection of luciferase assay, and if it is secreted, enzyme activity can be determined without cell lysis. GLP1R-producing HEK293 cells with secretory luciferase gene (GLP1R/Luc-293), designed to be induced upon receptor activation, were constructed by co-transfection with plasmids harboring constitutive human GLP1R gene and inducible secretory luciferase gene under the control of cyclic AMP responsive element (Fig. 2A).

When GLP1R/Luc-293 was exposed to synthetic GLP1 agonists such as GLP1, S^2-GLP1, and GLP1 with FLAG tag at the C-terminus (GLP1F), luciferase activity indicated that GLP1R activation was driven in a dose-dependent manner for all agonists, with the highest induction by GLP1F and the lowest induction by S^2-GLP1 (Fig. 2B). These results suggest that the newly-constructed GLP1R/Luc-293 successfully responded to synthetic GLP1 agonists and that the functional assay system using GLP1R/Luc-293 worked well when adding only chemiluminogenic substrate and measuring its intensity, enabling high-throughput assay using 96-well plates.

Functional assay of GLP1R activation by yeast-secreted peptides

Next, we attempted to assay GLP1R activation using GLP1R/Luc-293 for yeast-secreted GLP1 analogs including GLP1, S^2-GLP1, GLP1F, and S^2-GLP1F. When the culture supernatants of yeasts transformed with plasmids harboring these GLP1 analogs were added to GLP1R/Luc-293, GLP1R on the reporter cells was successfully activated (Fig. 2C). The difference in receptor activation from yeast-secreted GLP1 was 7.5-fold for yeast-secreted GLP1F and 0.3-fold for yeast-secreted S^2-GLP1, showing a similar tendency to activation by synthetic GLP1 analogs. In addition, FLAG fusion to GLP1 analogs was likely to increase activity, not only in synthetic peptides but also in yeast-produced peptides, as shown in the difference between GLP1 and GLP1F (7.5-fold), and the difference between S^2-GLP1 and S^2-GLP1F (13-fold). These results indicate that yeast-secreted GLP1 analogs also activate GLP1R/Luc-293 to induce luciferase secretion depending on each activity of the analogs, and that FLAG tag fusion to GLP1 analogs is effective in increasing assay sensitivity.

Screening of the randomly-mutagenized GLP1 library using a yeast secretion system

We attempted to screen random mutations in a GLP1 library to be secreted by yeasts, using the established functional assay system. We prepared a DNA library for randomly mutagenized GLP1s by using PCR with mutagenic dNTPs, 8-oxo-dGTP, and dPTP [11]. With this method, mutation rates can be controlled by changing the number of PCR cycles. Yeasts were transformed with the DNA library encoding randomly mutagenized GLP1s and the linearized pULS, a plasmid for library peptide secretion, thereby creating a yeast library secreting GLP1s with random mutations. Functional assay of the library according to the indicated workflow (Fig. 3A) revealed GLP1R activation by the library peptides, ranging from 0% to 171% (Fig. 3B). Out of these, 13 yeasts showing a variety of activities were selected and subjected to DNA sequencing analysis to deduce the amino acid sequences of secreted peptides (Table II). The identified peptides had from one to six amino acid substitutions in comparison with wild type GLP1.

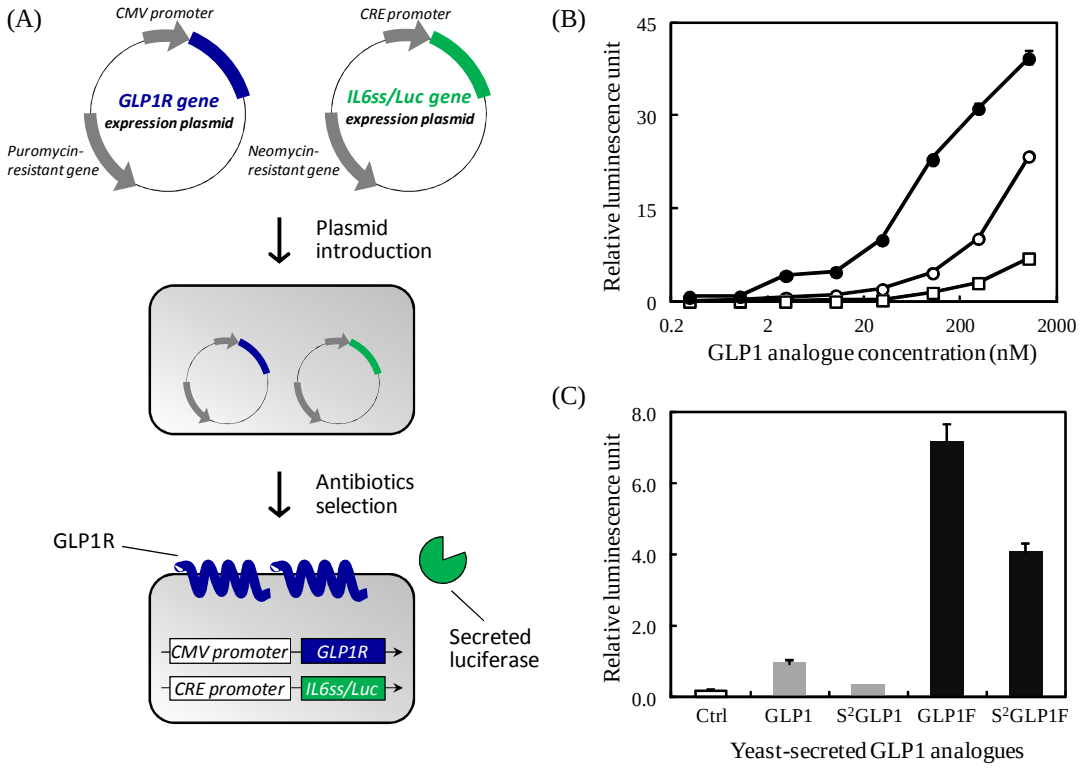

Fig. 2. Construction of stable GLP1R-producing cells with inducible-secretory luciferase

(A) HEK293 cells were co-transfected with pIRES-hGLP1R and pCRE-NLuc, and selected with G418 and puromycin-containing medium to establish a stable cell line harboring GLP1R gene and secretory luciferase gene (GLP1R/Luc-293). CMV; cytomegalovirus, CRE; cAMP response element, IL6ss; interleukin6 secretion signal. (B) Exposure of synthetic GLP1 agonists such as GLP1 (white circle), S^2-GLP1 (white square), and GLP1F (black circle) to GLP1R/Luc-293 induced luciferase secretion in a dose-dependent manner. Luciferase activity was determined by evaluating the chemiluminescence intensity with Nano-Glo™ luciferase assay system. Data were represented as mean ± standard error (n=3). (C) Culture supernatants of yeasts secreting GLP1 agonists including GLP1, S^2-GLP1, GLP1F, and S^2-GLP1 were subjected to GLP1R activation assay using GLP1R/Luc-293 as stated above. Data were represented as mean ± standard error (n=4).

Validation assay of the identified peptides

To validate the activity results from the screening assay, plasmids possessing in the identified yeasts were purified, amplified in *E.coli*, and introduced back into new, competent yeasts. Then, the new yeast transformants were again subjected to functional assay of GLP1R activation. Peptides mGLP1-1 and -2, with two and five mutations respectively, showed lack of activity, revealing a result similar to that obtained in the screening assay. All other peptides also reflected screening assay data.

For relationships between sequence and activity, mGLP1-1 and -2 with substitutions at the same positions (amino acids 20 and 22) lacked biological activity. Peptides from mGLP1-3 to -8 had substitutions at positions 5, 8, 10, 11, 12, 13, 18, 19, 20, 24, and 28, and showed biological activity that was 50% less than that of native GLP1. Peptides from mGLP1-9 to -12 had one or two substitutions at position 11, 20, 28, and 30, and their activities ranged from 71.1% to 149% of native GLP1. In spite of having six substitutions, mGLP1-13 had 1.7-fold higher activity than that of native GLP1.

These results demonstrate little disagreement between data from the first screening assay and the second validation assay, indicating that the screening assay may be sufficient to analyze the relationship between amino acid sequences and activities in mutated GLP1 analogs. In addition, we demonstrated successful use of our screening system in isolating functional GLP1R agonists.

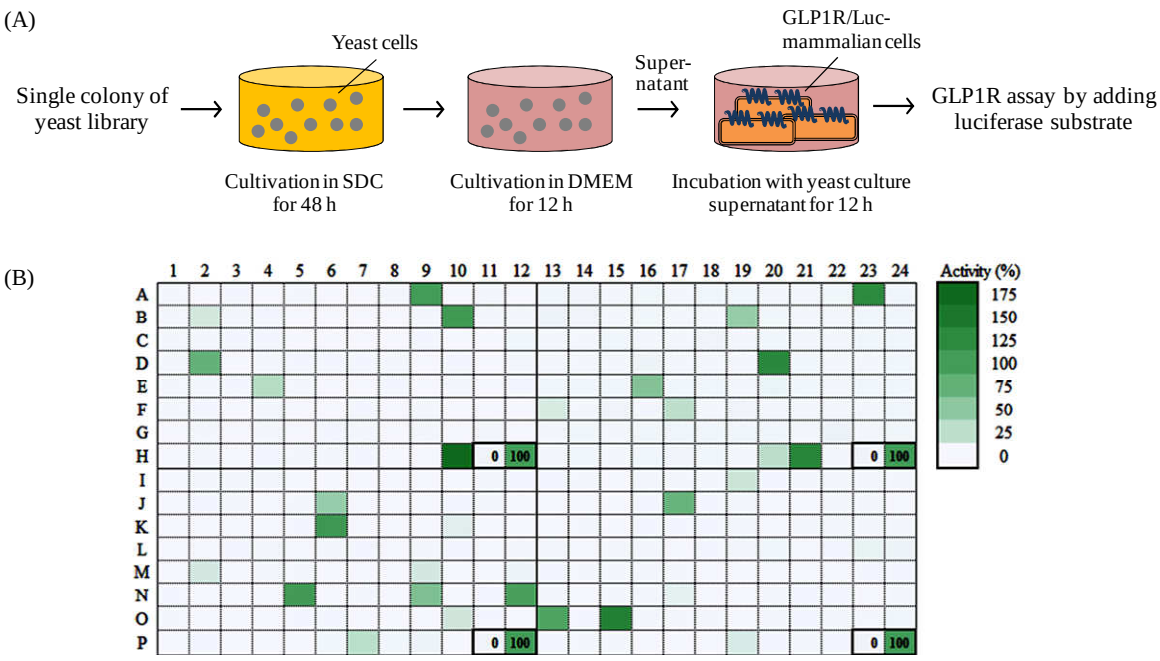

Fig. 3. Functional screening of yeast-secreted random GLP1 library

(A) Assay flow for functional screening of the yeast-secreted random GLP1 library. Randomly mutagenized GLP1 DNA library and pULS digested with *Eco*RI and *Pst*I were co-introduced into yeasts by electroporation, yielding the yeast library secreting randomly mutagenized GLP1. The yeast library was isolated by colony formation on solid selection medium, cultivated, and assayed. (B) Three hundred seventy-six library yeasts were screened based on luciferase activity reflecting GLP1R activation. The results are indicated in a heat map, with yellow showing lower activity and green showing higher activity. Ctrl-yeast culture supernatant was used as a negative control (0% activity indicated by “0”), whereas GLP1F-yeast culture supernatant was used as positive control (100% activity indicated by “100”).

Table II Amino acid sequence and biological activity of identified peptides

Peptides	Relative activity (%)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
	1st assay	2nd validation assay																														
Native	100	100 ± 3	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R
mGLP1-1	no activity	0.133 ± 0.235	H	A	E	G	T	F	T	S	D	V	S	S	H	L	E	G	Q	A	A	R	G	S	I	A	W	L	V	G	G	R
mGLP1-2	no activity	1.76 ± 0.37	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	Q	E	L	I	A	W	L	V	K	G	R
mGLP1-3	11.5	14.6 ± 0.6	H	A	E	G	A	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	R	K	G	R
mGLP1-4	24.7	14.9 ± 0.4	H	A	E	G	T	F	T	S	D	V	G	S	Y	L	E	G	Q	T	A	K	E	F	I	A	W	L	V	E	G	R
mGLP1-5	9.95	15.7 ± 0.4	H	A	E	G	T	F	T	S	D	I	S	S	C	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R
mGLP1-6	10.8	16.7 ± 0.7	H	A	E	G	T	F	T	S	D	V	S	P	Y	L	E	G	Q	A	V	K	E	F	I	A	W	L	V	K	G	R
mGLP1-7	40.2	22.2 ± 3.4	H	A	E	G	T	F	T	N	D	V	S	S	Y	L	E	G	Q	A	A	E	E	F	I	A	W	L	V	E	G	R
mGLP1-8	21.2	46.7 ± 2.9	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	T	W	L	V	R	G	R
mGLP1-9	51.3	71.1 ± 2.4	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	E	E	F	I	A	W	L	V	R	G	R
mGLP1-10	40.5	93.8 ± 1.9	H	A	E	G	T	F	T	S	D	V	G	S	Y	L	E	G	Q	A	A	R	E	F	I	A	W	L	V	K	G	R
mGLP1-11	99.0	125 ± 6	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	K
mGLP1-12	172	149 ± 5	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	R	G	G
mGLP1-13	129	171 ± 2	H	A	E	G	T	L	T	S	D	I	S	S	Y	L	E	S	Q	A	A	R	E	F	I	A	W	L	V	R	G	G

*Underlined red letters indicate mutated amino acids different from those of native GLP1.

Discussion

In this chapter, we established a novel, functional assay system for GPCR-acting peptides, which integrated a yeast secretion system for peptides to be tested and a functional detection system with mammalian cells expressing target GPCRs. Because secretory luciferase coupled with GPCR activation was introduced into the functional detection system, agonistic potency of yeast-secreted peptides was easily evaluated by simple addition of chemiluminogenic substrate and measurement with a plate reader. Using this simple assay system, we attempted to isolate functional GLP1R agonists from a randomly mutagenized GLP-1 library to be secreted by yeast. Our results were consistent with the alanine-scanning study of GLP1 previously-reported [7], and in addition, we discovered novel GLP1 analogs with multiple amino acid substitutions.

Our GLP1R-producing reporter mammalian cells (GLP1R/Luc-293) secreted NanoLuc upon receptor activation. GLP1R/Luc-293 dose-dependently produced luciferase enzyme outside the cells in response to synthetic GLP1 agonists including GLP1, serine-substituted S^2-GLP1, and FLAG tag-fused GLP1F (Fig. 2B). In addition to synthetic GLP1 agonists, GLP1R activation by yeast-secreted GLP1 agonists was directly determined without any purification or condensation of peptides of interest (Fig. 2C). We found a novel effect of FLAG tag, which was fused to the C-terminus of GLP1 and increased its agonistic activity, possibly by elevated stability of peptides or increased affinity/recognition of the receptor, thereby increasing sensitivity in our assay for GLP1R activation. Because we did not observe elevated activity of synthetic GLP1F over GLP1 based on intracellular cAMP production, which reflects earlier signaling events in receptor activation, protection of peptides from unknown degradation enzymes is likely to be associated.

NMR analysis of native GLP1 (Table II) in dodecylphosphocholine micelles revealed that, while the N-terminus residues 1–7 are unstructured, the rest of the peptide forms two helices from positions 7–14 and 18–29, separated by a linker region formed around Gly at position 16 [12]. In addition, it has been reported that Phe at position 22 in GLP1 is critical for receptor activation and that substitution for Ala led to a

significant activity loss with reduced helix contents [7]. In accordance with this finding, mGLP1-1 and -2 with substitution of Phe at position 22 for Ser and Leu, respectively, showed no activity. mGLP1-3, -4, -5, and -7 contained substitutions of residues important for receptor activation such as T5A, A18T, Y13C, and S8N/K20E, respectively, making them less active by 12－18% relative to native GLP1. Peptide mGLP1-8 did not have mutations leading to significant activity loss, but exchange of Ser at position 11 for a Pro that breaks helix structure is likely to cause activity reduction. According to the potential less effects of K28R which they are equivalent basic amino acid each other, A24T in mGLP1-8 and K20E in mGLP1-9 are considered to cause their reduced potency, respectively. In addition to these predictions based on previous reports [7] of sequence-activity relationships, mGLP1-13 showed novelty because of its 1.4-fold higher potency than native GLP1 in spite of having six substitutions, including Phe at position 6, a residue critical for receptor activation. One explanation would be the exchange of Phe with Leu but not Ala, with differences in their propyl side chains. Alternatively, replacement of Gly at position 16 by Ser may have increased its agonistic activity through an increase in helix content because positions 15–17 in GLP1 have no structure. This may have been due to incorporation of Gly, a helix breaker, although a more precise analysis using chemically synthesized peptides is needed to understand this effect.

In conclusion, we constructed a new, functional assay system for GPCR and GPCR-acting peptides, which were individually produced in mammalian reporter cells and engineered yeasts. Functional assay of a randomly mutagenized GLP1 library secreted by yeasts demonstrated a successful, streamlined method for analyzing modified GLP1s without chemical synthesis, purification, and condensation of peptides of interest. The assay system established here can be applied to other sets of peptide ligands against GPCRs.

Summary

Glucagon-like peptide1 (GLP1) is a 30-amino acid peptide hormone activating the GLP1 receptor (GLP1R), a class B G-protein coupled receptor, and is considered to be effective for treating diabetes and other metabolic diseases. In this chapter, we identified a novel and attractive GLP1R agonist from a random mutagenesis GLP1 library through a novel, integrated method combining peptide-secreting yeasts with mammalian cells expressing GLP1R, and inducible secretory luciferase as a reporter. Our biological screening system can be applied widely, including in the discovery of novel bioactive peptides acting on GPCRs.

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Chapter III Cell surface-engineered yeast for screening inhibitors of mutated influenza virus neuraminidase

Human seasonal influenza is responsible for an estimated 250,000–500,000 deaths and 3–5 million cases of severe illness annually worldwide [1]. Recently, the novel swine-origin virus (Influenza A H1N1 2009), which emerged potentially through the reassortment of triple North American H3N2 and H1N2 swine viruses with Eurasian avian-like swine viruses [2], entered the human population and spread rapidly around the globe, prompting the WHO to declare a pandemic in 2009 [3]. In addition, the highly pathogenic avian influenza A virus H5N1 has sporadically been epidemic since 1997 with a high mortality rate of 60% in humans and poses a serious pandemic threat [4].

Neuraminidase (NA) is a viral surface glycoprotein that plays a crucial role in the release of the newly formed virus from the infected cells, and has been the main target of current influenza antiviral treatment. Oseltamivir, one of the first-developed NA inhibitors, has been most widely used for the treatment of patients with influenza virus infection. However, an oseltamivir-resistant H1N1 virus that harbors a specific mutation (H274Y) in the NA protein has been recently reported [5] as has been, to a lesser extent, the emergence of the avian influenza H5N1 virus with the same mutation [6]. These findings raise public health concerns and therefore there is a need to develop a novel, efficient, and a rapid screening system for the identification of novel inhibitors.

One of the first steps in drug discovery is the preparation of the target protein, which requires laborious processes including not only protein production and confirmation of activity but also purification, a most time-consuming process. As to influenza virus NAs, although their biotechnological production was achieved by the secretary system using insect, yeast and mammalian cells for the head domain of NA, all the researchers will needs several steps to purify it [7-9]. In this regard, the yeast cell surface display technology is promising; it has been used for screening binding proteins with specific affinity to a target molecule and endowing yeasts with heterogenous

abilities like glucoamylase activity [10,11]. In this system, a heterologous protein of interest can be displayed on the yeast cell surface as a fusion protein with agglutinins, a cell wall protein that is involved in mating. In fact, the yeast cell surface display system has been used in various fields such as bioconversion, antibody engineering, and the development of bio-adsorbents [12-17]. Besides versatile applications as described above, the cumbersome steps involved in the purification of the recombinant protein are avoided by using the yeast cell surface display system. Further, thanks to the ease of gene manipulation, the fast growth in a low-cost medium and the existence of the quality control system of endogenous protein production in the endoplasmic reticulum, though it is not special to yeast, we can easily establish yeasts displaying proteins of interest through construction of plasmids with mutations in the target protein-coding DNA (Fig. 1).

In this chapter, we constructed yeasts displaying the head domain of wild-type or oseltamivir-resistant NAs. Then, we evaluated the biochemical properties such as NA enzymatic activities at different cultivation times, sensitivity to NA inhibitors, and thermal stability of the cell surface-displayed NAs.

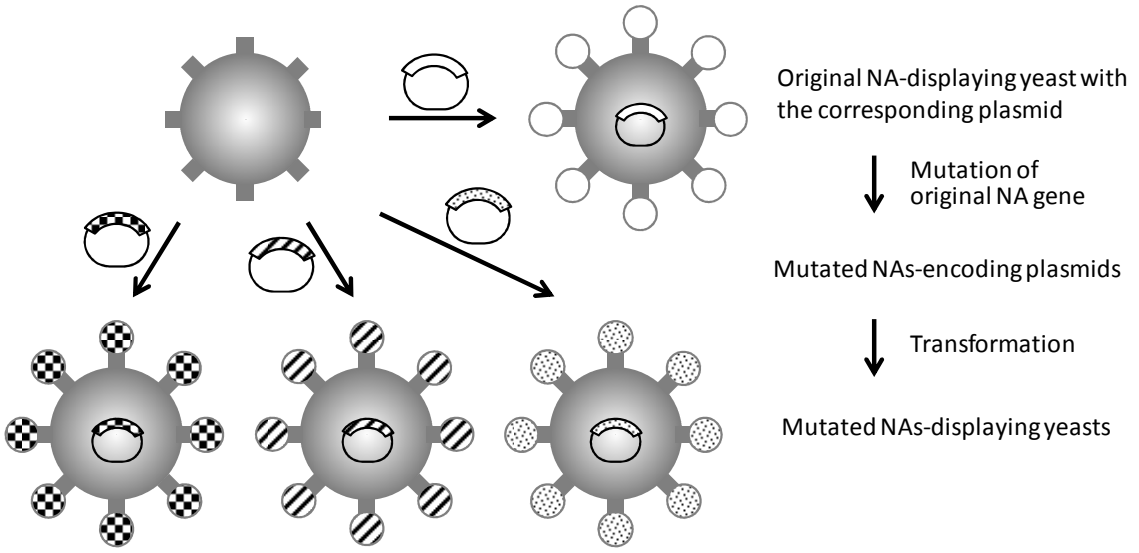

Fig. 1. An illustration of the steps involved in use of yeast cell surface display system to express the enzymes of interest. Once the original NA-displaying yeast cells are successfully constructed, it will take only within 3 ~ 4 days, including transformation and incubation, to obtain yeast cells displaying mutated NAs.

Materials and Methods

Strains and media

Escherichia coli DH5α [F^-, *ΔlacU169 (φ80lacZΔM15), hsdR17* (r_K^-, m_K^+), *recA1, endA1, deoR, thi-1, supE44, gyrA96, relA1,* λ^-] (TOYOBO, Osaka, Japan) was used as a host for DNA manipulation. *Saccharomyces cerevisiae* BY4742 (*MATa, his3-1, leu2, lys2, ura3*; EUROSCARF, Frankfurt, Germany) was used to construct yeasts displaying NAs. *E. coli* transformants were grown at 37°C in Luria–Bertani media [1% (*w*/*v*) tryptone, 0.5% (*w*/*v*) yeast extract, and 1% (*w*/*v*) sodium chloride] containing 100 μg/mL ampicillin. Yeast transformants were selected on a synthetic dextrose (SDC) solid medium [0.67% (*w*/*v*) yeast nitrogen base without amino acids, 2% (*w*/*v*) glucose, 1% (*w*/*v*) casamino acids, 0.002% (*w*/*v*) adenine, 0.002% (*w*/*v*) L-tryptophan, and 2% (*w*/*v*) agar], and then, the resultant colonies were aerobically cultivated at 30°C in a liquid SDC medium (pH 6.5) supplemented with 100 mM 2-morpholinoethanesulfonic acid (MES).

Vectors for expression

The DNA fragment encoding head domain neuraminidase (HNA, 387 amino acids in length) was PCR-amplified from the pTZ-NA plasmid that harbors the neuraminidase gene from the Fujian H5N1 virus, using a combination of sense [5′-CTTACTTTTCTTTGCTCGTTTCTGCCGTAACATTAGCGGGCAATTCATCTC-3′] antisense [5′-CATCCTTGTAATCAGATCCACCCTCGAGCTTGTCAATGGTGAATGGCAAC-3′] primers and the KOD-Plus-Neo DNA polymerase (TOYOBO) (Fig.2A). The DNA fragment was inserted into *Bgl*II/*Xho*I-digested pULD1, which codes for the C-terminal half of α-agglutinin downstream of the multiple cloning sites, using In-Fusion HD Cloning kit (Clontech, Mountain view, CA, USA). The resultant plasmid is referred to as pULD1-HNA/WT. The plasmid encoding the head domain of oseltamivir-resistant NA (H274Y) was constructed by mutating pULD1-HNA/WT using the primers 5′-GAATGCTCCTAATTATTACTATGAGGAATGCTCC-3′ and

5′-GGAGCATTCCTCATAGTAATAATTAGGAGCATTC-3′ (the underlines indicates mutation points) and the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA), and the plasmid was termed pULD1-HNA/H274Y. pULD1-Strep, which encodes a Strep-tag instead of a FLAG-tag, was constructed as the negative control plasmid for immunofluorescence staining.

Transformation of yeast

Yeasts were transformed using the Frozen-EZ Yeast Transformation-II kit (Zymo Research, CA, USA). After the introduction of plasmids, the yeast transformants were selected on a uracil-deficient SDC solid medium.

Neuraminidase activity assay

Yeast transformants were pre-cultivated in a buffered SDC medium for 36 h, and then the main cultivation was initiated at an initial optical density (OD) of 0.1 at 600 nm in 10 mL of buffered SDC medium. At the indicated times of cultivation, the yeast cells were collected and suspended in the NA assay buffer containing 5 mM $CaCl_2$ and 50 mM MES pH 6.5. The yeast suspension (50 μL) containing 10^7 cells was transferred onto a 96-well microplate (353072; BD Biosciences, CA, USA) and mixed with 50 μL of the NA assay buffer containing 100 μM 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (MUNANA) (Japan Food & Liquor Alliance, Kyoto, Japan) as a fluorogenic substrate. After incubation for 15 min at 37°C, 250 mM glycine pH 10.4 was added to stop the reaction and then the fluorescence was measured with a Fluoroskan Ascent Fluorometer (Labsystems, Helsinki, Finland) with an excitation at 355 nm and an emission at 460 nm. For the inhibition assay, 2 types of NA inhibitors, oseltamivir carboxylate (Shanghai Haoyuan Chemexpress, Shanghai, China) and zanamivir (LKT Laboratories, St. Paul, MN, USA), were used.

Microscopic observation and determination of the NA display efficiency

Yeasts were collected at 10^7 cells per tube in a 1.5 mL microtube and washed once with 1 mL of phosphate-buffered saline (PBS) pH 7.4. The pellets after

centrifugation were suspended in PBS containing 0.5% (*w*/*v*) bovine serum albumin and 2.5 μg/mL anti-FLAG M2 mouse IgG antibody (Sigma-Aldrich, ST. Louis, MO, USA) and incubated for 1 h with rotation. After washing with PBS, the pellets were suspended in PBS containing 0.5% (*w*/*v*) bovine serum albumin and 5 μg/mL Alexa Flour 488-conjugated anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA) and incubated for 1 h with rotation. After washing twice with PBS, the pellets were suspended in 300 μL of PBS and transferred onto a glass slide for fluorescence microscopic observation. To determine the display efficiency, 250 μL of the cell pellet was transferred into a 96-well microplate, and the fluorescence was measured with a Fluoroskan Ascent Fluorometer, with an excitation at 485 nm and an emission at 537 nm [17].

Evaluation of thermal stability

Yeast displaying HNA/WT was suspended in NA assay buffer at the concentration of 2 x 10^8 cells/mL and incubated at 37°C for 72 h, and the enzyme activity was assayed by the same method described above. The activity was indicated as a percentage, with the activity of control sample incubated at 4°C for 72 h considered to be 100%.

Results

Confirmation of NA display on yeast surface

To display the head domain of Fujian H5N1 virus-derived NA (HNA), we constructed expression plasmid, pULD1-HNA/WT (Fig. 2B) and introduced it into *S. cerevisiae* BY4742. After the cultivation, we detected the displayed NAs on the yeast cell surface by fluorescence immunostaining. These results indicated that HNA was successfully displayed on the yeast surface (Fig. 2C).

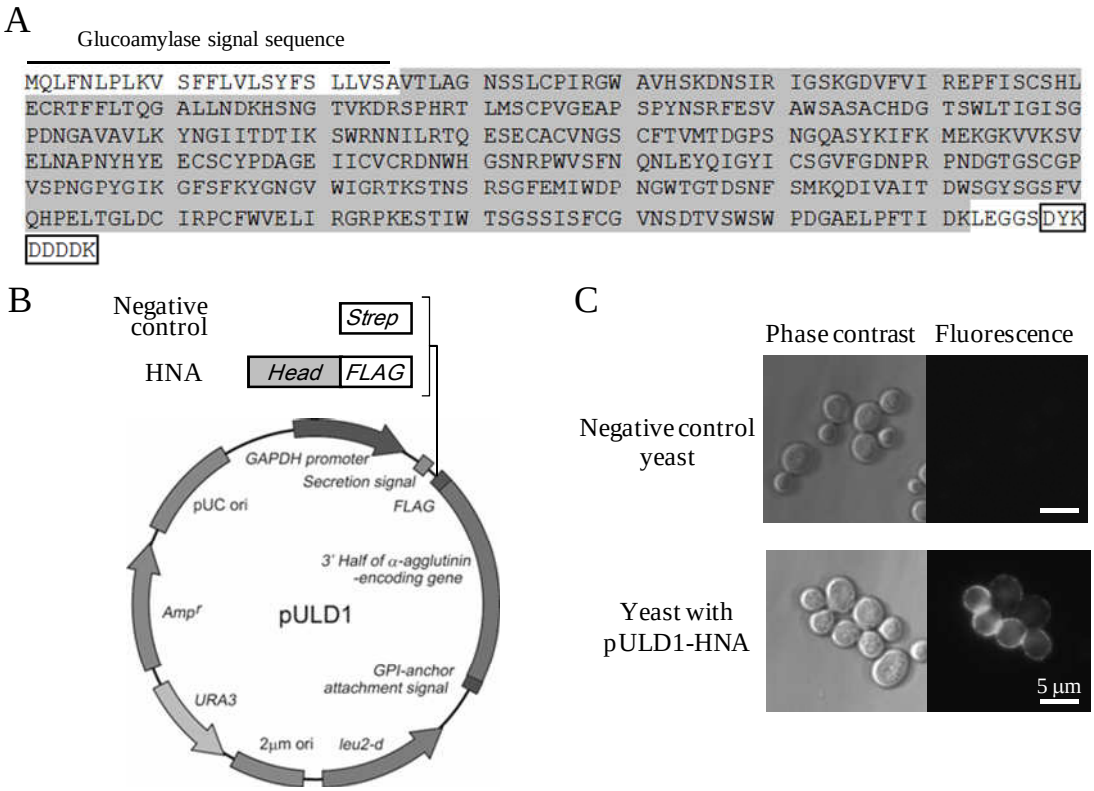

Fig. 2. Construction of NAs-displaying yeasts

(A) Sequence of HNA. The gray color shows the sequence of the HNA. The box indicates FLAG tag. (B) Plasmid constructed for yeast surface display of the head domain of neuraminidase (HNA). Head, Head domain. (B) A microscopic examination of surface display after immunofluorescence staining. Forty-eight hours after cultivation of the yeast, the display of NAs on yeast was examined by immunofluorescence staining using an anti-FLAG M2 mouse IgG antibody followed by an Alexa Flour 488-conjugated anti-mouse IgG antibody. The yeast suspension was transferred onto a glass slide and observed under a fluorescence microscope. Scale bars indicate 5 μm.

Enzyme activity and display efficiency of NAs on the yeast cell surface

We next examined the enzymatic activity of the displayed HNAs (both the wild-type and the mutant) by using a fluorogenic substrate under the condition without an interfering effect of yeasts on the fluorescence intensity (Fig. 3). The HNA mutant with H274Y substitution (HNA/H274Y) was chosen because this mutant is resistant to oseltamivir, one of the most widely used drugs for the treatment of influenza infection

in humans. The mechanism by which the HNA/H274Y has resistance to oseltamivir is the interfering of its access to the active site owing to a pentoxyl group at position 3 in oseltamivir, whose functional group does not exist in zanamivir [18].

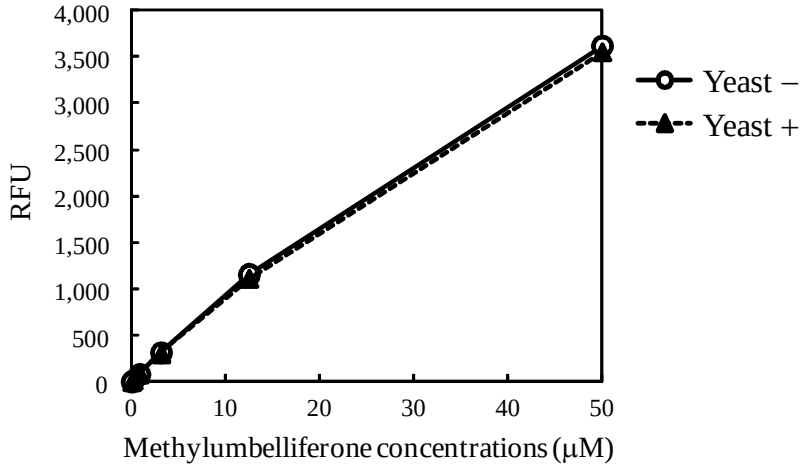

Fig. 3. Standard curve of methylumbelliferone (MU), a fluorogenic substance released after the cleavage of MUNANA by neuraminidase, with or without yeasts
Yeasts suspended in NA assay buffer at the number of 10^7 or the buffer only were transferred into 96-well microplate, and then MU solutions at the indicated concentrations were added. The fluorescent intensity of MU was measured in Fluoroskan Ascent Fluorometer with an excitation at 355 nm and an emission at 460 nm. RFU at each blank solution without MU was subtracted from each all other value.

We evaluated the NA enzymatic activity of the yeast transformants displaying HNA/WT and HNA/H274Y at different cultivation times. When HNA/WT was expressed on the yeast surface, the NA activity was significant at 24 h (79.7 ± 4.5 RFU/OD_{600}), with the peak being observed at 72 h (153.5 ± 16.5 RFU/OD_{600}). A similar trend was also observed in yeasts displaying HNA/H274Y, although the enzyme activity level was 1.5- to 2.4-fold lower than that observed in the HNA/WT-displaying yeasts at the all time points examined (Fig. 4A), and this difference in enzyme activity was statistically significant ($p < 0.05$). The control yeast without HNA on the surface showed no enzyme activity at all the cultivation times tested.

In order to confirm that the difference in the enzyme activity observed is due to the H274Y substitution in the HNA, we determined the display efficiencies of both HNAs on yeast surface by immunofluorescence staining. As seen in Fig. 4B, the display efficiencies of HNA/WT and HNA/H274Y on the yeast surface were not different. These results demonstrated that the H274Y mutation does not alter the display efficiency, but lowers the enzyme activity (Fig. 4A). In support of this conclusion, an earlier study by Collins et al. [18] reported that the Michaelis constant for the enzyme carrying the H274Y mutation was reduced when compared to that of the wild-type enzyme.

For the influence of the C-terminal FLAG tag on enzyme activity, the yeast with the FLAG tag-free NA showed 1.4-fold higher activity than that with the NA possessing FLAG tag at the C-terminus (Fig. 5).

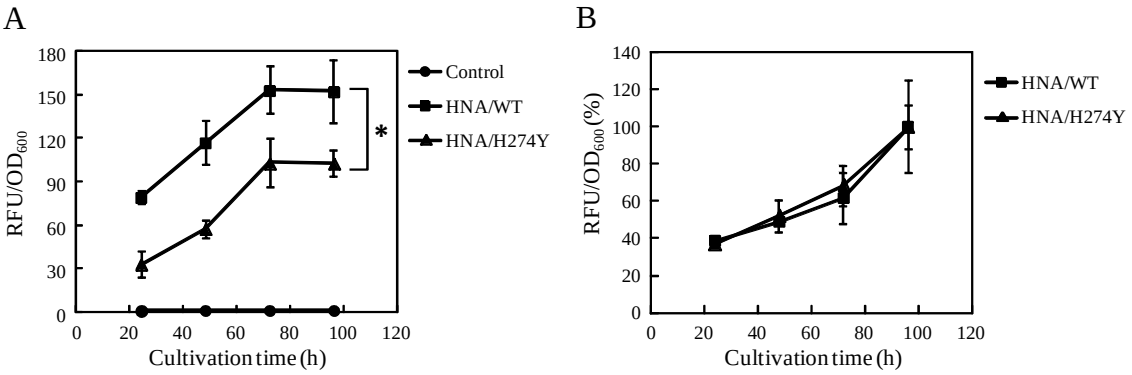

Fig. 4. Time course of the enzyme activity (A) and display efficiency (B) for HNA/WT and HNA/H274Y

The enzyme activity is expressed in relative fluorescence units (RFU) divided by OD_{600}. The display efficiency was determined by immunofluorescence staining as described above, and then the intensity was measured by Fluoroskan Ascent Fluorometer (Labsystems) with an excitation at 485 nm and an emission at 537 nm. The data were expressed as percent of RFU in which the value of HNA/WT-displaying yeast at 96 h was 100%. The data represents the average of 3 independent experiments, and the error bars depict the SEM. *$p < 0.05$, determined by two-way ANOVA

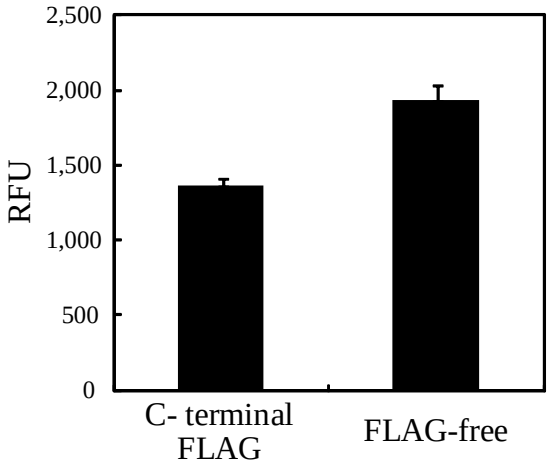

Fig. 5. Enzyme activity of yeast-displayed HNA with or without FLAG-tag at the C-terminus

A plasmid encoding a FLAG tag-free NA was constructed and introduced Saccharomyces cerevisiae BY4742, and then the NA enzyme activity was compared with the yeast displaying NA with FLAG tag at the C-terminus. NA assay was performed as indicated in the Materials and Methods. The data represent means ± SD.

Inhibition of the displayed HNAs on yeast by NA inhibitors

Both HNA/WT and HNA/H274Y showed significant enzymatic activity and we examined the effect of 2 types of selective NA inhibitors on this activity. Oseltamivir carboxylate (the active ingredient of Tamiflu) and zanamivir (the active ingredient of Relenza) were used as inhibitors. When we conducted a test for concentration-response curve on the HNA/WT on yeast, the IC_{50} was 9.1 and 8.8 nM for oseltamivir carboxylate and zanamivir, respectively (Fig. 6). On the other hand, while zanamivir showed the near or higher inhibition activity on the HNA/H274Y whose IC_{50} was 4.1 nM, oseltamivir carboxylate was only able to inhibit the activity by 28% even at 160 nM, the highest concentration in this test. This inhibition properties and IC_{50} values of oseltamivir carboxylate and zanamivir on the HNA/WT and the HNA/H274Y were consistent with the previous reports in which NA derived from influenza virus grown in hen eggs or MDCK cells was used for carrying out inhibition study [18,19]. Thus, we

conclude that the HNAs displayed on the yeast surface have the same property as the native and mutated NAs present on the virus surface.

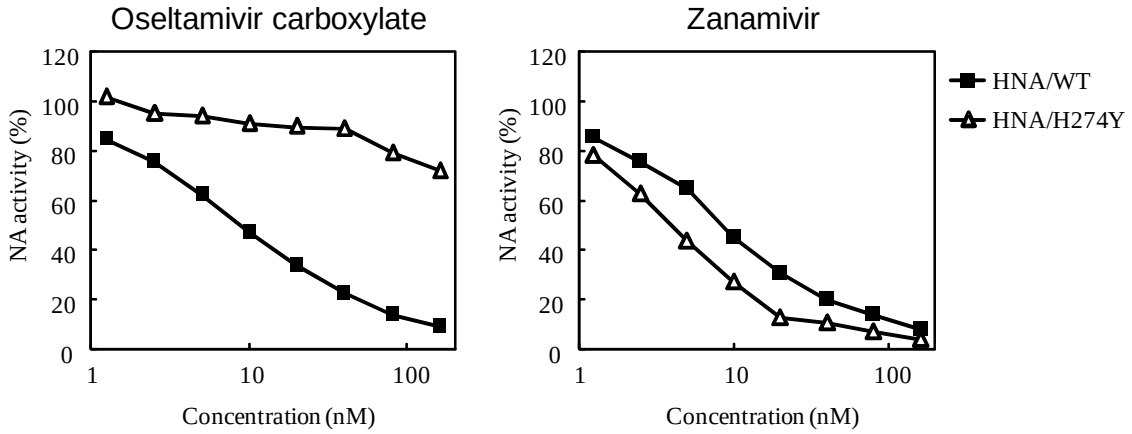

Fig. 6. Concentration-response curve on HNAs displayed on yeast with increasing dose of inhibitors, oseltamivir carboxylate (left) and zanamivir (right)

HNA/WT or HNA/H274Y displayed on the yeast cell surface was subjected to an inhibition assay by the addition of the indicated concentrations of the inhibitors. The enzyme activity without any inhibitors was expressed 100%, and IC_{50} values were calculated based on the inhibition curves.

Thermal stability

When enzymes (including NAs) are used to screen for inhibitors, the thermal stability of the displayed proteins during the enzyme reaction is important in evaluating the inhibitors. For example, certain newly acquired mutations in enzymes of interest have been reported to give rise to drug-resistance and lowered stability simultaneously, which impairs an effective screening. Specifically, Mickimm-Breshkin et al. [20] reported such mutant NAs that simultaneously displayed drug-resistance and reduced stability. Therefore, we examined the effect of heat treatment on the enzyme activity of the HNAs displayed on the yeast surface. The HNA displayed on the yeast surface retained their activity even after prolonged heating (Fig. 7). In contrast, recombinant NA produced in insect cells is less stable and is inactivated upon heat treatment [19]. These

findings suggest that the thermal stability of the NA displayed on the yeast cell surface is superior to that of the soluble NA produced in insect cells.

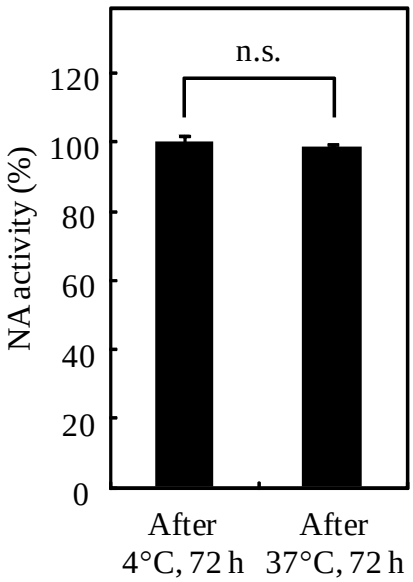

Fig. 7. Thermal stability of displayed HNA/WT

Yeast displaying HNA/WT was suspended in NA assay buffer at the concentration of 2 x 10^8 cells/mL and incubated at 37°C for 72 h, and the enzyme activity was assayed. The data represents the average of 3 independent experiments, and the error bars depict the SEM. n.s. indicates not significant.

Discussion

In this chapter, we successfully produced the head domain of the NA from influenza H5N1 virus on the yeast cell surface. The recombinant enzyme showed sufficient enzyme activity and high thermal stability. Importantly, the available small inhibitors were able to reasonably inhibit the activity of these enzymes displayed on the yeast surface. The successful heterologous production of the head domain of NA from avian influenza virus H5N1 was first reported using the methylotropic yeast *Pichia pastris* [9]. However, the recombinant NA was produced as a secreted, soluble protein which requires several steps for purification. On the other hand, it should be noted that in the present study, the yeast displaying the HNA on the surface only requires to be centrifuged and does not require any further purification prior to examination of the enzyme activity. This makes the yeast surface display system very effective, especially in studying the application of enzymes (mutated and non-mutated) and screening for novel enzyme inhibitors.

NA is a mushroom-shaped tetramer of identical subunits, with the head of the mushroom suspended from the virus membrane. Each of the subunits that form the head of the mushroom is made up of a six-bladed propeller-like structure [21]. Although it is unclear whether the NAs displayed on yeast surface have a proper folding or tetramer structure, the proper folding of NAs on the yeast surface will be partly supported by the fact that the wild type- or H274Y-HNA showed reasonable enzyme activities and inhibition properties by NA inhibitors. The same is true on a proper tetramerization of NA, but we think that it is not critical for application to drug screening because NA inhibitors attack the active site of NA monomer not tetramer.

Because Schmidt et al. [19] indicated that the NA bound to the virus surface was highly stable during incubation at 37°C compared with the soluble NA produced in insect cells, we considered that the reduced flexibility of NA by being fixed on the virus surface might be one of the factors contributing to thermal stabilization. Yeast surface display system is also expected to have the same stabilized effect as virus, but whether the intrinsically instable NA with E119G mutation reported by

Mickimm-Breshkin et al. [20] is stabilized as well should be investigated for further research.

In general, exogenous proteins produced in yeast are known to get high mannose type glycosylation, potentially deteriorating innate properties of target proteins. One of the examples is the soluble NA produced in *Pichia pastris,* which showed a larger molecular weight due to hyperglycosylation [9].On the other hand, when HNA was intracellularly expressed in *Saccharomyces cerevisiae*, we detected the main band around 44 kDa probably showing the HNA in western blotting (Fig. 8). In addition, the yeast without HNA on the surface showed a band at 102 kDa indicating α-agglutinin only, and the yeast displaying HNA showed a band that is 44 kDa higher than the α-agglutinin. From these findings, we think that the HNA produced in *Saccharomyces cerevisiae* is likely not to have hyperglycosylation, unlike the HNA produced in *Pichia pastris.*

In addition to application in enzyme assays, the yeast displaying HNA could be utilized for yeast-based vaccines that could be administrated subcutaneously as a whole yeast [22]. Although yeast which produces target antigens intracellularly has been investigated, yeast with desired antigens on the cell surface might be more effective because of accessibility to immune cells. The in vivo efficacy of soluble neuraminidase as vaccines has been demonstrated in mice challenged with recombinant N2 neuraminidase [7], and therefore, a prophylactic effect of the HNA-displaying yeast constructed here can be expected [23].

In conclusion, this system of using yeast to display HNA on the surface can be applied for screening chemical libraries in order to obtain inhibitors of NAs carrying various novel mutations. As it is less time consuming, this technique can also be applied for quick preparation of recombinant protein antigens from frequently mutating viruses.

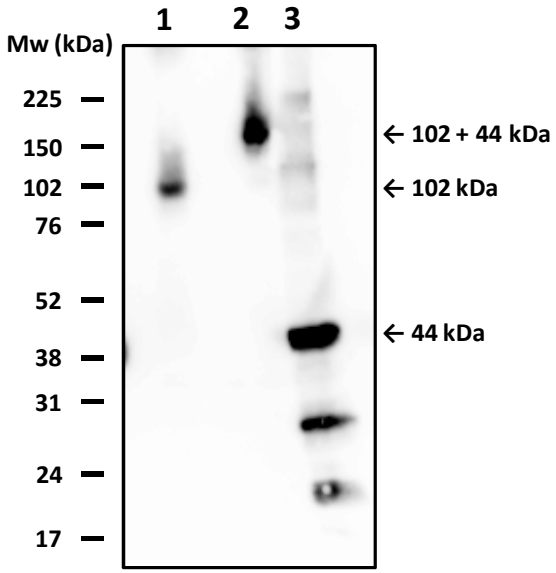

Fig. 8. Western blotting of protein extracts from yeast with or without displayed HNA and yeast producing HNA within the cell

The yeasts with or without HNA on the cell surface (lane1 and 2, respectively) and the yeast producing HNA internally (lane3) were crushed by beads-shocker in PBS solution, and then, the supernatants were separated by 5-20% SDS-PAGE. Immunoblotting was conducted using anti-FLAG M2 mouse IgG antibody conjugated with horseradish peroxidase.

Summary

Neuraminidase (NA) is a surface glycoprotein produced by the influenza virus. Specific NA mutations that confer resistance to anti-viral drugs have been reported. The aim of this study was to demonstrate quick preparation of the mutated NAs using the yeast surface display and its applicability for screening inhibitors. Plasmids encoding the head domain of wild-type and drug-resistant NAs were constructed and introduced into yeast, and these were successfully displayed on the yeast surface, with biochemical properties similar to the native virus NAs. This system using mutated NAs-displaying yeast provides an efficient and convenient tool for screening novel inhibitors against the drug-resistant influenza virus.

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Conclusions

The present studies have been carried out to develop novel bioactive peptide screening systems for GPCRs agonists and influenza neuraminidase inhibitors toward further extension of peptide versatility as medicines.

In chapter I, we established a novel functional screening system of yeast-secreted peptides acting on GLP1R. The system directly detected the functional activity of yeast secreted-GLP1R agonists based on the activation of GLP1R produced on mammalian cells, without any purification and condensation of yeast-produced peptides. In addition, application of the system enabled identification of agonist-secreting yeast in the model screening.

In chapter II, we identified novel modified GLP1R agonists through the screening of randomly mutagenized GLP1 library by using an integrated yeast-mammalian assay system. To conduct this, we constructed novel high sensitive reporter assay system using secreted luciferase that is significantly induced in response to activated CREB transcription factor upon GLP1R activation and is detected without any cell lysis.

In chapter III, we successfully produced the head domain of the neuraminidase from influenza H5N1 virus on the yeast cell surface. The displayed recombinant enzyme showed sufficient enzyme activity and high thermal stability. Importantly, the available small inhibitors were able to reasonably inhibit the activity of these enzymes displayed on the yeast surface.

The existing screening systems of peptides such as phage display need chemical synthesis to evaluate the functional activity of the selected peptides. On the other hand, our novel peptide screening system consisted of the sufficient amount of yeast-secreted peptide and the high sensitive activity detection using GPCRs-producing mammalian cells or active enzyme-displaying yeast, requiring no chemical synthesis of peptides. This screening system established here should contribute to more efficient drug discovery and development to deliver novel drugs for patients all over the world.

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Laboratory of Biomacromolecular Chemistry
Division of Applied Life Sciences
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Publications

Chapter I

Shigemori T, Kuroda K, Ueda M. Novel functional screening system for yeast-secreted peptides acting on G-protein coupled receptors. submitted.

Chapter II

Shigemori T, Kuroda K, Ueda M. Screening of randomly mutagenized GLP1 library by using an integrated yeast-mammalian assay system. submitted.

Chapter III

Shigemori T, Nagayama M, Yamada J, Miura N, Yongkiettrakul S, Kuroda K, Katsuragi T, Ueda M. Construction of a convenient system for easily screening inhibitors of mutated influenza virus neuraminidases. FEBS Open Bio. **3**, 484-489 (2013)

Other publications

Hara K, Shigemori T, Kuroda K, Ueda M. Membrane-displayed somatostatin activates somatostatin receptor subtype-2 heterologously produced in *Saccharomyces cerevisiae*. AMB Express. **2**, 63-70 (2012)

Morioka S, Shigemori T, Hara K, Morisaka H, Kuroda K, Ueda M. Effect of sterol composition on the activity of the yeast G-protein-coupled receptor Ste2. Appl. Microbiol. Biotechnol. **97**, 4013-4020 (2013)