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Display of single-chain insulin-like peptides on yeast surface

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Abstract

Insulin and insulin-like peptides play a pivotal role in a wide variety of cellular and physiological events including energy storage, proliferation, aging and differentiation. Variants of insulin and insulin-like peptides may therefore be probes for studying insulin signaling pathway and therapeutic candidates for treating metabolic diseases. Here, we report a method to genetically display single-chain insulin-like peptides on the surface of *Saccharomyces cerevisiae* strain DY1632. Using a previously reported single-chain insulin analog, SCI-57, as a model, we demonstrate that nearly 70% of yeast binds to insulin receptor (IR) suggesting that SCI-57 is folded correctly and maintains its IR-binding property. Furthermore, the interaction between displayed SCI-57 and IR can be reduced using increasing concentrations of native insulin as a soluble competitor suggesting that the interaction is insulin-dependent. We further applied this methodology on three other single-chain insulin analogs with various length and confirm their interactions with IR. In summary, we successfully displayed a number of insulin-like peptides on yeast surface and demonstrated insulin-dependent interactions with IR. This method may, therefore, be used for construction of libraries of insulin-like peptides to select for chemical probes or therapeutic molecules.

Graphical Abstract

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Conflict of interest statement

There is no conflict of interest related to this work.

One-sentence summary:

Single-chain insulin-like peptides can be genetically displayed on yeast surfaces to bind to insulin receptors.

Introduction

Insulin-like peptides including insulin, insulin-like growth factor 1 and 2 (IGF1, 2) are involved in development, growth, metabolism, and other biological events(1). All insulin-like peptides are expressed as a single chain pro-polypeptide with B, C and A domains. While IGF1 and IGF2 remain as a single chain peptide, insulin is proteolytically cleaved into A and B chains with the intervening C-peptides removed from proinsulin(2). Insulin selectively binds to insulin receptors (IR) but not IGF1 receptors (IGF1R) while IGF1 and IGF2 strongly bind to IGF1R. Since insulin is a life-saving drug for people with type 1 diabetes, a number of novel insulin analogs have been developed to enhance the clinical benefits by shortening the onset time after injections or extending the serum half-life(3). Insulin lispro, aspart, glulisine and glargine are insulin variants with 1 or more amino acid substitutions from native insulin. On the other hand, insulin detemir and degludec have direct chemical modifications on B29 lysines. All these insulin variants were rationally engineered through knowledges from structural insights and biochemical principles, which is different from screening approaches for most small-molecule or antibody drugs(4). The three disulfide bonds in insulin-like peptides represent a significant challenge for correct folding after synthesis, which may increase the difficulty of generating large libraries of insulin-like peptides.

Yeast surface display is a powerful platform for library screening and binder isolation of bound molecules(5). A variety of methods have been reported previously, with the most common approach being fusion of a protein of interest to Aga2p, a yeast cell wall protein(6, 7). Such fusions are displayed on the surface of an engineered yeast strain expressing galactose-inducible Aga1p, which tethers Aga2p to the yeast cell wall. Recently, McMahon et al. reported a new surface display system by using a synthetic tether mimicking the low-complexity sequence of yeast cell wall proteins(8). In this case, a protein of interest is tethered to a 649 amino acid stalk sequence, which sticks to the surface of yeast. 10^7 to 10^9 molecules can then be displayed for binding assays. This is an especially promising route to display insulin-like peptides because thousands of tons of therapeutic insulin analogs are produced using yeast each year(9). We hypothesize that yeast surface display technique can be used to display insulin-like peptides with correct folding and disulfide bond patterns. In this report, we established a system to display functional insulin-like peptides and confirmed their interactions with IR ectodomains. This method can therefore be used for constructing a large library of insulin-like peptides to screen for therapeutic insulin analogs or probes to study IR and IGF1R.

Materials and Experimental Details

Yeast strain and growth condition

Saccharomyces cerevisiae DY1632 (MAT A, pep4::HIS3, prb1 1.6R, His3 200, Leu2 1, Lys2-801, Trp1, Ura3-52, Can1) was used as host stain for yeast surface display. For yeast surface display, plasmids were transformed in DY1632 by lithium acetate method. Cells were grown in synthetic complete minus tryptophan (SC-W) media supplemented with 2% glucose overnight. Next day, cells were back-diluted into the same media at OD₆₀₀ of 0.2 and incubated at 30°C. When the growth reached OD₆₀₀ of 1, cells were harvested and reinoculated in SC-W media containing 2% galactose to induce *GAL1* promoter and protein expression and incubated at 20°C for 20 hrs.

Plasmid construction

Native human insulin DNA sequence with short C-peptide (amino acid sequence RRLQKR) was cloned between NheI and BamHI in pCT-con2 and named pCT-INS. MF α1 leader sequence and the 649 amino acid long stalk region were amplified by PCR using pYDS649 as template. MF α1 leader sequence was substituted Aga2 gene in pCT-INS and the stalk DNA sequence were cloned at the 3' of insulin gene in-frame. The resulting plasmid was named pCT-SCI-RRLQKR. Other SCI constructs used in this study were generated by replacing a short C-peptide in pCT-SCI-RRLQKR by site-directed mutagenesis PCR. Point mutations in pCT-SCI-57 were introduced by QuickChange site-directed mutagenesis PCR. To generate a negative control plasmid, DNA sequence encoding insulin were removed by NheI and BamHI digestion from pCT-SCI-RRLQKR. The digested DNA fragment then was self-ligated after Klenow fill-in which makes MF α1 leader sequence and the stalk sequence in-frame.

IR binding assay and immunostaining

To examine the interaction between the yeast surface displayed SCI and the IR isoforms, 1×10^7 cells displaying SCI were harvested and washed with the IR binding assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.1% BSA) and resuspended in 1 ml of the same buffer. Recombinant human IR isoforms was added at the final concentration of 10 nM to the cell suspension and incubated at room temperature with rotation for 40 min. After washing with PBST (PBS plus 0.2% Tween 20) two times, cells were subject to immunostaining to visualize the IR bound to SCI on yeast cell surface. Cell pellets from the IR binding assay were resuspended in 100 μl of PBS/3% BSA containing anti-INSR antibody (1:100 dilution) and incubated at room temperature for 30 min. After washing with PBS/3% BSA two times, cells then were incubated with Alexa Fluor™ 647 conjugated mouse secondary antibody in PBS/3% BSA (1:100 dilution) for 30 min in the dark. After washing, cells were resuspended in PBS/0.1% BSA and either observed under a fluorescence microscope or subject to flow cytometry analysis. For the competition assay, recombinant human insulin was mixed to the cell suspension prior to IR proteins. To reduce disulfide bond in cell surface displayed SCI-57, yeast cells were pre-incubated in 25 mM Tris-Cl, pH 8.0 containing 5 mM DTT for 30 min at room temperature.

Flow cytometry analysis

After the IR binding assay and immunostaining, cells were suspended in PBS/0.1% BSA at 2×10^7 cells/ml and the Alexa Fluor™ 647 positive cells in each sample were quantified using BD Celesta.

Analysis of fluorescence intensity of the Alexa Fluor™ 647 positive cells

Fluorescence intensity of cells in images obtained from fluorescence microscopy were analyzed using ImageJ. Integrated intensity of selected fluorescent cell was measured and the corrected total cell fluorescence (CTCF) was calculated using the following formula: CTCF (Corrected Total Cell Fluorescence) = Integrated Density - (Area of selected cell X Mean fluorescence of background readings). CTCF of individual cell was plotted as dot-boxplot.

Recombinant proteins and antibodies

Recombinant human insulin receptor isoforms were purchased from the R&D system (IR-A; cat. # 1544-1R, IR-B; cat. # 8974-1R). Anti-INSR alpha antibody (cat. # AHR0231) and anti-mouse IgG (H+L) conjugated with Alexa Fluor 647™ (cat. # A21235) used in immunostaining were purchased from Invitrogen. Recombinant human insulin was purchased from Life Technologies (cat.# A113821J).

Results and Discussion

We sought to use the McMahon strategy to display a single chain insulin, SCI-57 which was reported to maintain native insulin like affinity to IR(10). DNA sequence encoding yeast mating factor (MF) α 1 leader sequence and the 649 amino acid long stalk sequence were amplified from pYDS649 and inserted 5' and 3' of the SCI-57 gene in pCT plasmid, respectively (Figure 1A). The resulting plasmid pCT-SCI-57 was transformed in *Saccharomyces cerevisiae* strain DY1632. To verify that the displayed SCI-57 on yeast surface was folded correctly, we performed a binding assay using IR ectodomains. Cells with empty vector or pCT-SCI-57 were grown in synthetic complete medium without tryptophan (SC-W) supplemented with 2% galactose to induce the expression of SCI-57. After 20 hrs incubation at 20°C, 1×10^7 cells were collected and incubated with 10 nM of recombinant IR ectodomain followed by immunostaining to visualize IR ectodomain bound to the displayed SCI-57 as shown in Figure 1B. Cells were then examined by fluorescence microscopy and flow cytometry. Yeast cells harboring either pCT-SCI-57 or empty vector were not positive for IR binding prior to the galactose induction. After 20 hrs cultivation in galactose, only cells expressing SCI-57 were observed to be stained with Alexa Fluor™ 647 (AF647) as shown in Figure 1C. Quantitative flow cytometry analysis revealed that about 70 % of cells expressing SCI-57 were AF647 positive with high fluorescence intensity during IR binding reaction (Figure 1D), whereas only 2% cells with empty vector were AF647 positive. Lastly, to ascertain that IR binds to correctly folded SCI-57, we incubated the SCI-57 displaying yeast cells with a reducing agent, dithiothreitol (DTT) prior to the IR binding activity assay to disrupt disulfide bonds in the protein. As the cell surface displayed protein in our system is covalently linked to the polysaccharide of yeast cell wall, DTT treatment should not release the surface displayed SCI-57 but reduce the disulfide bonds in

the SCI-57 and unfold them. We observed that the total number of AF647 positive cells and their fluorescence intensity significantly decreased in the sample upon 5 mM DTT treatment (Figure S1A). The median fluorescence intensity (MFI) of the DTT treated cells was determined to be about 25% of the non-treated sample by flow cytometry analysis (Figure S1B). These results suggest that SCI-57 on yeast surface is correctly folded and binds to IR ectodomain. The merely 2% AF647 positive cells with empty vectors suggest that low background non-specific binding occurs in this yeast surface display system.

To further confirm the specificity of IR binding, we sought to use recombinant human insulin (rHI) as a soluble competitor. In this competition assay, cells displaying SCI-57 were incubated with 10 nM IR ectodomain isoforms A or B (IR-A or IR-B) in the presence of increasing amount of rHI. Cells were then analyzed by fluorescence microscopy and flow cytometry. Fluorescence microscopy images show that the number of IR-bound cells as well as their fluorescence intensity decreased as rHI concentration increased in the competition assay (Figure 2A, Figure S3-4). The integrated intensity of the stained cells (by ImageJ) collected from >100 cells of each sample was plotted as dot-boxplot as shown in Figure 2B. The fluorescence intensity of AF647 positive cells with 4 μ M rHI dropped to 54% in cells incubated with IR-A and 43% in cells incubated with IR-B compared to that of cells without rHI treatments. Using flow cytometry for quantification, AF647 positive cells gradually decreased from 69% to 65% in cells incubated with IR-A and from 69% to 61% in cells incubated with IR-B, as rHI increased in the competition assay (Figure 2C). Furthermore, the peak in flow cytometry histogram shifted toward low fluorescence intensity as rHI concentration increased. The MFI reduced to 30% when cells incubated with IR in the presence of 4 μ M of rHI (Figure 2D).

As SCI-57 is a single chain insulin which was engineered to maintain native insulin like IR binding potency, removal of the mutated residues in SCI-57 should decrease its IR binding activity. To test this, we generated a construct in which native insulin A and B chain is connected with GGGPRR C-peptide (SCI-GGGPRR) and expressed in DY1632. Removal of mutated residues, indeed, resulted in reduction of IR binding activity (Figure S2). The MFI of AF647 positive cells measured to be 20% in yeast cells displaying SCI-GGGPRR when compared to that of SCI-57. Along with the rHI competition result, this result strongly support that the both IR isoforms specifically binds to SCI-57 displayed on the yeast cell surface.

To further demonstrate the utility of this method, we displayed new single chain insulin-like peptide constructs with different intervening C-peptide on yeast surface. The single chain insulin “SCI-010”, consisting of the insulin A and B chain with a connecting C-peptide derived from IGF1 (Figure 3A), was previously reported to have similar binding affinity toward IR isoforms compared to native insulin(11, 12). As a comparison, we also constructed another single chain insulin “SCI-020” in which the A and B chain of native insulin is connected by the C-peptide from IGF2 (Figure 3A). “SCI-RRLQKR” containing the 6 amino acids RRLQKR as a C-peptide was originally designed to display two-chain insulin based on the report by Thim(13). However, this construct was expressed as single-chain in our system and we evaluated its IR binding ability compared to other SCIs. These SCI variants were expressed in DY1632 along with SCI-57 and their binding affinity for

each IR isoform was investigated. SCI-57-expressing yeast exhibited robust binding to both IR-A and IR-B (Figure 3B-C). SCI-010 showed very little binding to either IR isoform, which is different from previous studies. This discrepancy may be due to the fact that IR ectodomains instead of soluble receptors were used in our experiments(11). SCI-020 showed strong binding to IR-A but less binding to IR-B (Figure 3B-C). Both IR isoforms bind to the yeast displayed SCI-RRLQKR with low affinity. Since the C-peptide length is the same for both SCI-57 and SCI-RRLQKR, this low affinity may be due to more rigid conformation in C-peptide compared to the glycine-rich linker in SCI-57. For each variant, representative images are shown in Figure 3B (full data in Figure S5-6) and quantification of the fluorescence intensity of each stained cell in the images was measured, calculated by ImageJ and plotted as a dot-boxplot in Figure 3C. In general, IR-A exhibited stronger binding to the SCI variants compared to IR-B as the mean fluorescent intensity of cells incubated with IR-A was higher than that with IR-B. This is consistent with the fact that both IGF1 and IGF2 have been shown to bind more strongly to IR-A than IR-B(14). Finally, we performed flow cytometry-based quantification for each of these interactions (Figure 3D-E). These data, presented as a histogram in Figure 3D, largely replicated the image quantification presented in Figure 3C. We noticed that SCI-010 exhibits a broad peak reflecting wide range of IR binding affinity to the protein unlike other SCI variants. This may indicate variations in the displayed level of SCI-010 on yeast that may arise from the defective processing. The proper folding of this SCI may require additional in vitro folding process to obtain native insulin like IR binding potency as shown in earlier studies. We next compared the MFIs obtained from flow cytometry. Consistent to the fluorescence microscopic analysis result, the MFI of all SCI variants was about 1.5-fold higher when cells were incubated with IR-A than IR-B, indicating the preferred binding to IR-A of the tested SCI variants. In summary, we established a yeast display method to construct functional insulin-like peptides on yeast cell surface. This method can be used for library construction that may lead to identification of IR isoform-selective insulin analogs as probes or therapeutic single-chain insulin candidates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

IR-A recognizes and binds to SCI-57 displayed on yeast cell surface. (A) Schematic of pCT-SCI-57 construct for yeast surface display. SCI-57 with MFα1 leader sequence and the stalk sequence amplified from pYDS649 were cloned under the *GAL1* promoter in pCT-con vector. (B) Schematic of SCI-57 displayed on yeast cell surface and IR binding activity assay. SCI-57 NMR structure (PBD 1ZNI) is adopted to illustrate the yeast surface displayed SCI-57 protein. Color codes are identical in Figure 1A and 1B; B chain in orange, A chain in green, C-peptide in grey and the stalk in black. Yeast cells expressing SCI-57 or control vector were incubated with IR, anti-IR antibodies and mouse secondary conjugated with AlexaFluor 647 (AF647) sequentially. Immunostained cells were subject to fluorescence microscopy or flow cytometry analysis. (C) IR-A binds to yeast cells displaying SCI-57 only after galactose induction. Cells harboring vector and pCT-SCI-57 were collected before or 20 hrs after galactose induction. Cells were incubated with IR-A and subsequently stained by immunostaining method and then observed using a Zeiss Axio Observer Z1. (D) Flow cytometry analysis of cells after IR binding assay. 1×10^7 cells from (C) were subject to flow

cytometry analysis using BD Celesta to quantify AF647 positive cells. Percentage of the AF647 positive cells in total events is shown in the flow cytometry box (n=3).

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Figure 2.

Recombinant human insulin (rHI) outcompetes SCI-57 on yeast cell surface for IR binding. (A) Population and fluorescence intensity of the AF647 positive cells in IR binding assay diminishes as rHI concentration increase in the assay. Cells displaying SCI-57 were premixed with the denoted amount of rHI before incubating with IR-A or IR-B. Followed by immunostaining, cells were subject to fluorescence microscopic analysis. (B) Quantification of fluorescence intensity of the AF647 positive cells. Fluorescence intensity of the stained yeast cells was quantified using ImageJ. Intensity value data were plotted as a dot-boxplot. (C) Flow cytometry analysis shows that fluorescence intensity decreases as rHI increase in the binding reaction yet a marginal change in the AF647 positive cells population. Percentage of the AF647 positive cells in total events is shown in the flow cytometry box (n=3). (D) Bar graph represents the MFI of the AF647 positive cells in the flow cytometry analysis (n=3).

Figure 3.

IR isoforms show varying affinity to single-chain insulins with different C-peptide sequence. (A) Schematic of SCI variants for yeast surface display. SCI-57; insulin A and B chain with point mutations and GGGPRR C-peptide, SCI-010; native insulin A and B chain with IGF1 C-peptide, SCI-020; native insulin A and B chain with IGF2 C-peptide, SCI-RRLQKR; native insulin A and B chain with RRLQKR C-peptide. (B) IR binds to yeast cells displaying SCI variants with different affinity. Cells displaying each SCI incubated with IR-A or IR-B. Followed by immunostaining, cells were subject to fluorescence microscopic analysis. (C) Quantification of fluorescence intensity of the AF647 positive cells. Fluorescence intensity of the stained yeast cells was quantified using ImageJ. Intensity value data were plotted as a dot-boxplot. (D) Flow cytometry analysis shows a different distribution of AF647 positive cells within SCI variants. Percentage of the AF647 positive

cells in total events is shown in the flow cytometry box (n=3). (E) Bar graph represents the MFI of the AF647 positive cells in the flow cytometry analysis (n=3).

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