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Display of Single-Chain Insulin-like Peptides on a Yeast Surface

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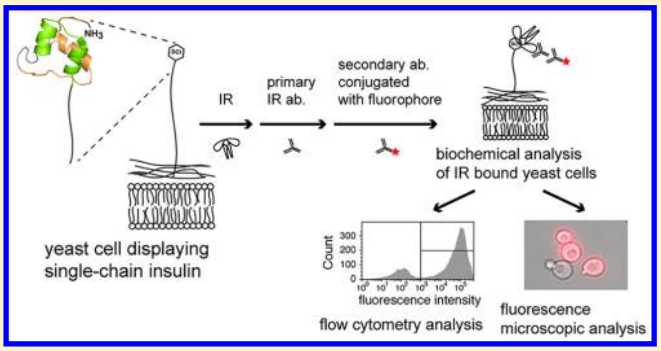
Supporting Information

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 the insulin signaling pathway and therapeutic candidates for treating metabolic diseases. Here, we report a method for genetically displaying single-chain insulin-like peptides on the surface of *Saccharomyces cerevisiae* strain DY1632. Using a previously reported single-chain insulin analogue, 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 weakened using increasing concentrations of native insulin as a soluble competitor, suggesting that the interaction is insulin-dependent. We further applied this methodology to three other single-chain insulin analogues with various lengths and confirmed their interactions with IR. In summary, we successfully displayed a number of insulin-like peptides on a 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.



Insulin-like peptides, including insulin and insulin-like growth factor 1 and 2 (IGF1 and -2, respectively), 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 (IRs) but not IGF1 receptors (IGF1Rs), while IGF1 and IGF2 strongly bind to IGF1R. Because insulin is a life-saving drug for people with type 1 diabetes, a number of novel insulin analogues 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 one or more amino acid substitutions compared to native insulin. On the other hand, insulin detemir and degludec have direct chemical modifications on B29 lysines. All of these insulin variants were rationally engineered through knowledge gained 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 make the generation of large libraries of insulin-like peptides more difficult.

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; 10^7−10^9 molecules can then be displayed for binding assays. This is an especially promising route for displaying insulin-like peptides because thousands of tons of therapeutic insulin analogues are produced using yeast each year.[9] We hypothesize that the 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 for displaying functional insulin-like peptides and confirmed their interactions with IR ectodomains. This method can therefore be used to construct a large library of insulin-like peptides to screen for therapeutic insulin analogues or probes to study IR and IGF1R.

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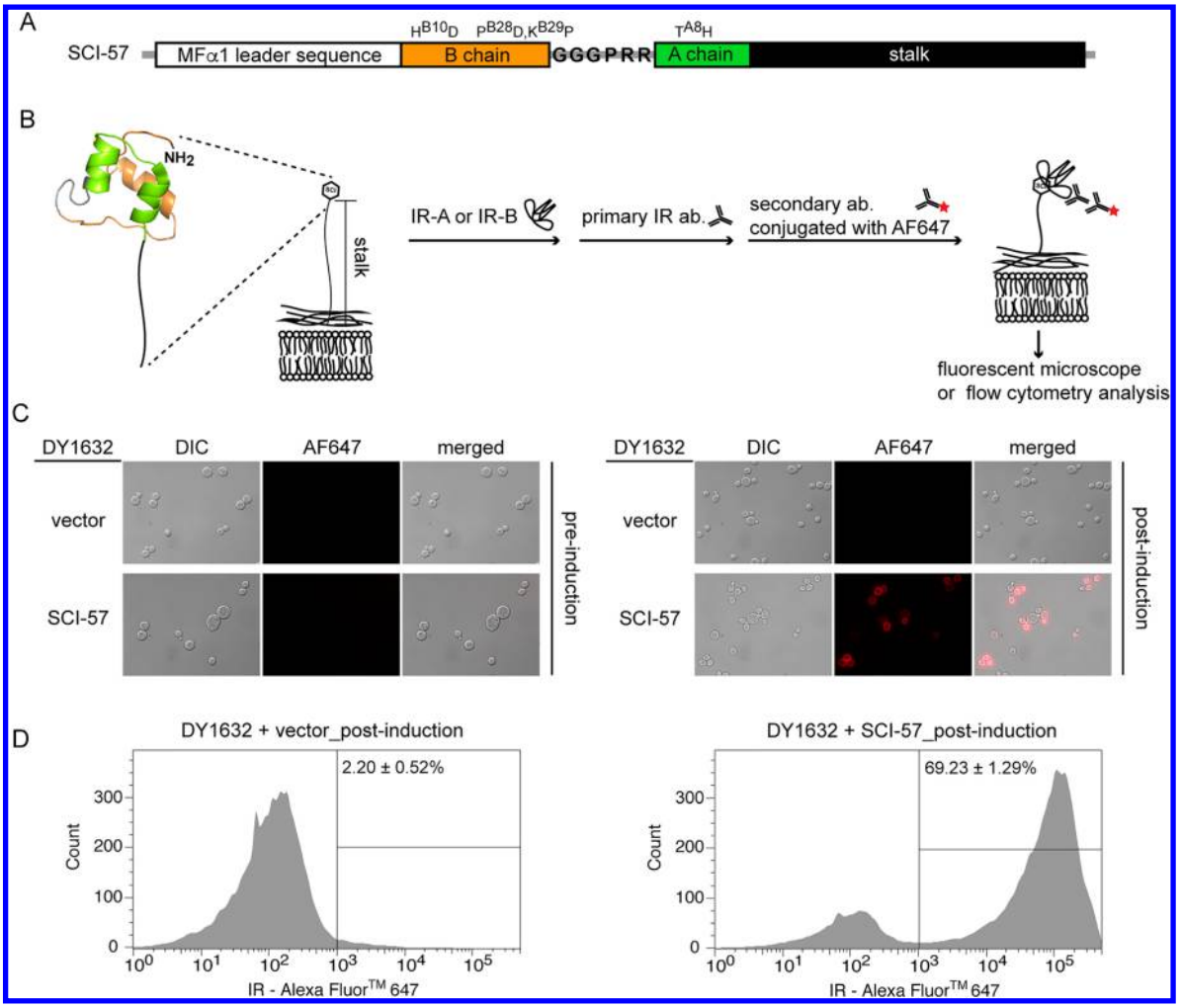

Figure 1. IR-A recognizes and binds to SCI-57 displayed on a yeast cell surface. (A) Schematic of the pCT-SCI-57 construct for yeast surface display. SCI-57 with the MFα1 leader sequence and the stalk sequence amplified from pYDS649 were cloned under the *GAL1* promoter in the pCT-con vector. (B) Schematic of SCI-57 displayed on the yeast cell surface and IR binding activity assay. The SCI-57 NMR structure (Protein Data Bank entry 1ZNI) is adopted to illustrate the yeast surface-displayed SCI-57 protein. Color codes are identical in panels A and B: B chain in orange, A chain in green, C-peptide in gray, and stalk in black. Yeast cells expressing SCI-57 or the control vector were incubated with IR, anti-IR antibodies, and the mouse secondary antibody conjugated with Alexa Flour 647 (AF647) sequentially. Immunostained cells were subjected to fluorescence microscopy or flow cytometry analysis. (C) IR-A binds to yeast cells displaying SCI-57 only after galactose induction. Cells harboring the vector or pCT-SCI-57 were collected before or 20 h after galactose induction. Cells were incubated with IR-A and subsequently stained by the immunostaining method and then observed using a Zeiss Axio Observer Z1 instrument. (D) Flow cytometry analysis of cells after an IR binding assay. Cells from panel C (1×10^7) were subjected to flow cytometry analysis using BD Celesta to quantify AF647 positive cells. The percentage of the AF647 positive cells in total events is shown in the flow cytometry box (n = 3).

MATERIALS AND EXPERIMENTAL DETAILS

Yeast Strain and Growth Condition. *Saccharomyces cerevisiae* DY1632 (MAT A, pep4::HIS3, prb1Δ1.6R, His3Δ200, Leu2Δ1, Lys2−801, Trp1, Ura3−52, Can1) was used as the host stain for yeast surface display. For yeast surface display, plasmids were transformed in DY1632 by the lithium acetate method. Cells were grown in synthetic complete minus tryptophan (SC-W) medium supplemented with 2% glucose overnight. The next day, cells were back-diluted into the same medium at an OD_{600} of 0.2 and incubated at 30 °C. When the OD_{600} reached 1, cells were harvested and reinoculated in SC-W medium containing 2% galactose to induce *GAL1* promoter and protein expression and incubated at 20 °C for 20 h.

Plasmid Construction. The native human insulin DNA sequence with a short C-peptide (amino acid sequence RRLQKR) was cloned between NheI and BamHI in pCT-con2 and named pCT-INS. The MF α1 leader sequence and the 649-amino acid stalk region were amplified by polymerase chain reaction (PCR) using pYDS649 as a template. The MF α1 leader sequence was substituted with the Aga2 gene in pCT-INS, and the stalk DNA sequence were cloned at the 3′ end of the 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 sequences 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 the MF α1 leader sequence and the stalk sequence in frame.

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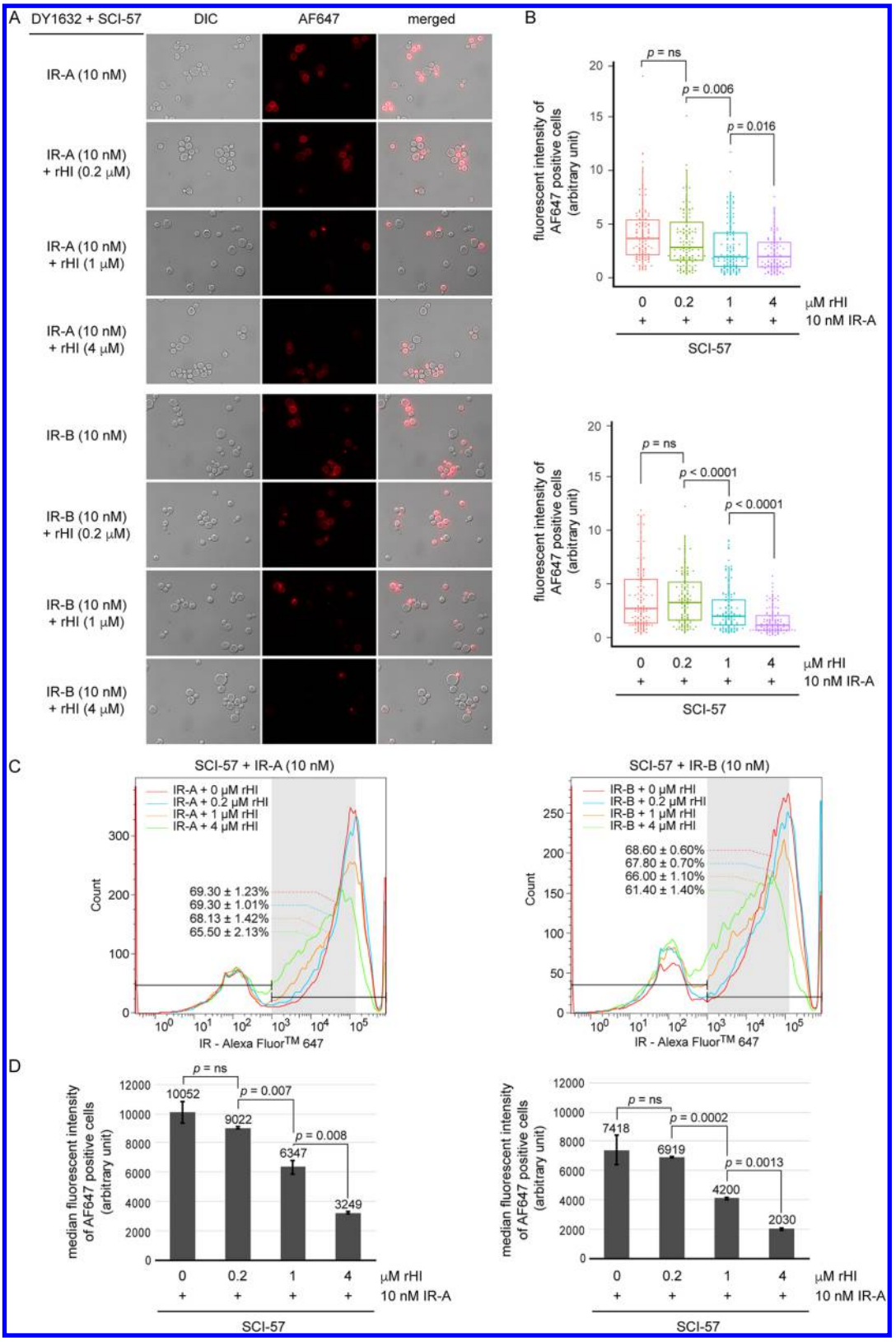

Figure 2. Recombinant human insulin (rHI) outcompetes SCI-57 on a yeast cell surface for IR binding. (A) The population and fluorescence intensity of the AF647 positive cells in the IR binding assay decrease as the rHI concentration increases. Cells displaying SCI-57 were premixed with the denoted amount of rHI before being incubated with IR-A or IR-B. After being immunostained, cells were subjected to fluorescence microscopic analysis. (B) Quantification of the fluorescence intensity of AF647 positive cells. The 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 the rHI concentration increases in the binding reaction but a marginal change in the AF647 positive cell population. The percentage of the AF647 positive cells in total events is shown in the flow cytometry box (n = 3). (D) Bar graph that represents the MFI of the AF647 positive cells in the flow cytometry analysis (n = 3).

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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 IR binding assay buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, and 0.1% bovine serum albumin (BSA)] and resuspended in 1 mL of the same buffer. Recombinant human IR isoforms was added at a final concentration of 10 nM to the cell suspension and incubated at room temperature with rotation for 40 min. After being washed twice with PBST (PBS with 0.2% Tween 20), cells were subjected to immunostaining to visualize the IR bound to SCI on a yeast cell surface. Cell pellets from the IR binding assay were resuspended in 100 μL of a PBS/3% BSA mixture containing the anti-INSR antibody (1:100 dilution) and incubated at room temperature for 30 min. After being washed twice with a PBS/3% BSA mixture, cells then were incubated with the Alexa Fluor 647-conjugated mouse secondary antibody in a PBS/3% BSA mixture (1:100 dilution) for 30 min in the dark. After being washed, cells were resuspended in a PBS/0.1% BSA mixture and either observed under a fluorescence microscope or subjected to flow cytometry analysis. For the competition assay, recombinant human insulin was mixed with the cell suspension prior to IR proteins. To reduce the disulfide bond in cell surface-displayed SCI-57, yeast cells were preincubated in 25 mM Tris-HCl (pH 8.0) containing 5 mM dithiothreitol (DTT) for 30 min at room termperature.

Flow Cytometry Analysis. After the IR binding assay and immunostaining, cells were suspended in a PBS/0.1% BSA mixture at a density of 2×10^7 cells/mL and the Alexa Fluor 647 positive cells in each sample were quantified using BD Celesta.

Analysis of the Fluorescence Intensity of the Alexa Fluor 647 Positive Cells. The fluorescence intensities of cells in images obtained via fluorescence microscopy were analyzed using ImageJ. The integrated intensity of selected fluorescent cells was measured, and the corrected total cell fluorescence (CTCF) was calculated using the formula CTCF (corrected total cell fluorescence) = integrated density − (area of selected cell × mean fluorescence of background readings). The CTCF of an individual cell was plotted as a dot-boxplot.

Recombinant Proteins and Antibodies. Recombinant human insulin receptor isoforms were purchased from R&D Systems (IR-A, catalog no. 1544-IR; IR-B, catalog no. 8974-IR). The anti-INSR alpha antibody (catalog no. AHR0231) and anti-mouse IgG (H+L) conjugated with Alexa Fluor 647 (catalog no. A21235) used in immunostaining were purchased from Invitrogen. Recombinant human insulin was purchased from Life Technologies (catalog no. A11382IJ).

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 for IR.[10] A DNA sequence encoding the yeast mating factor (MF) α1 leader sequence and the 649-amino acid stalk sequence were amplified from pYDS649 and inserted 5′ and 3′ of the SCI-57 gene in the pCT plasmid, respectively (Figure 1A). The resulting pCT-SCI-57 was transformed in *S. cerevisiae* strain DY1632. To verify that the displayed SCI-57 on the yeast surface was folded correctly, we performed a binding assay using IR ectodomains. Cells with an 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 incubation at 20 °C for 20 h, 1×10^7 cells were collected and incubated with 10 nM recombinant IR ectodomain followed by immunostaining to visualize the 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 the empty vector were not positive for IR binding prior to galactose induction. After cultivation in galactose for 20 h, 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 ∼70% of cells expressing SCI-57 were AF647 positive with a high fluorescence intensity during the IR binding reaction (Figure 1D), whereas only 2% of cells with an 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, 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 the yeast cell wall, DTT treatment should not release the surface-displayed SCI-57 but reduce the disulfide bonds in the SCI-57 and upfold them. We observed that the total number of AF647 positive cells and their fluorescence intensity significantly decreased in the sample upon treatment with 5 mM DTT (Figure S1A). The median fluorescence intensity (MFI) of the DTT-treated cells was determined to be ∼25% of that of the nontreated sample by flow cytometry analysis (Figure S1B). These results suggest that SCI-57 on a yeast surface is correctly folded and binds to the IR ectodomain. The merely 2% of AF647 positive cells with the empty vector suggest that low-background nonspecific 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 isoform A or B (IR-A or IR-B, respectively) in the presence of an 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 the rHI concentration increased in the competition assay (Figure 2A and Figures S3 and S4). 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 μM rHI decreased to 54% in cells incubated with IR-A and to 43% in cells incubated with IR-B compared to that of cells without rHI treatment. Using flow cytometry for quantification, the population of 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 the level of rHI increased in the competition assay (Figure 2C). Furthermore, the peak in the flow cytometry histogram shifted toward a low fluorescence intensity as the rHI concentration increased. The MFI decreased to 30% when cells were incubated with IR in the presence of 4 μM rHI (Figure 2D).

As SCI-57 is a single-chain insulin that was enginnered to maintain a 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 the GGGPRR C-

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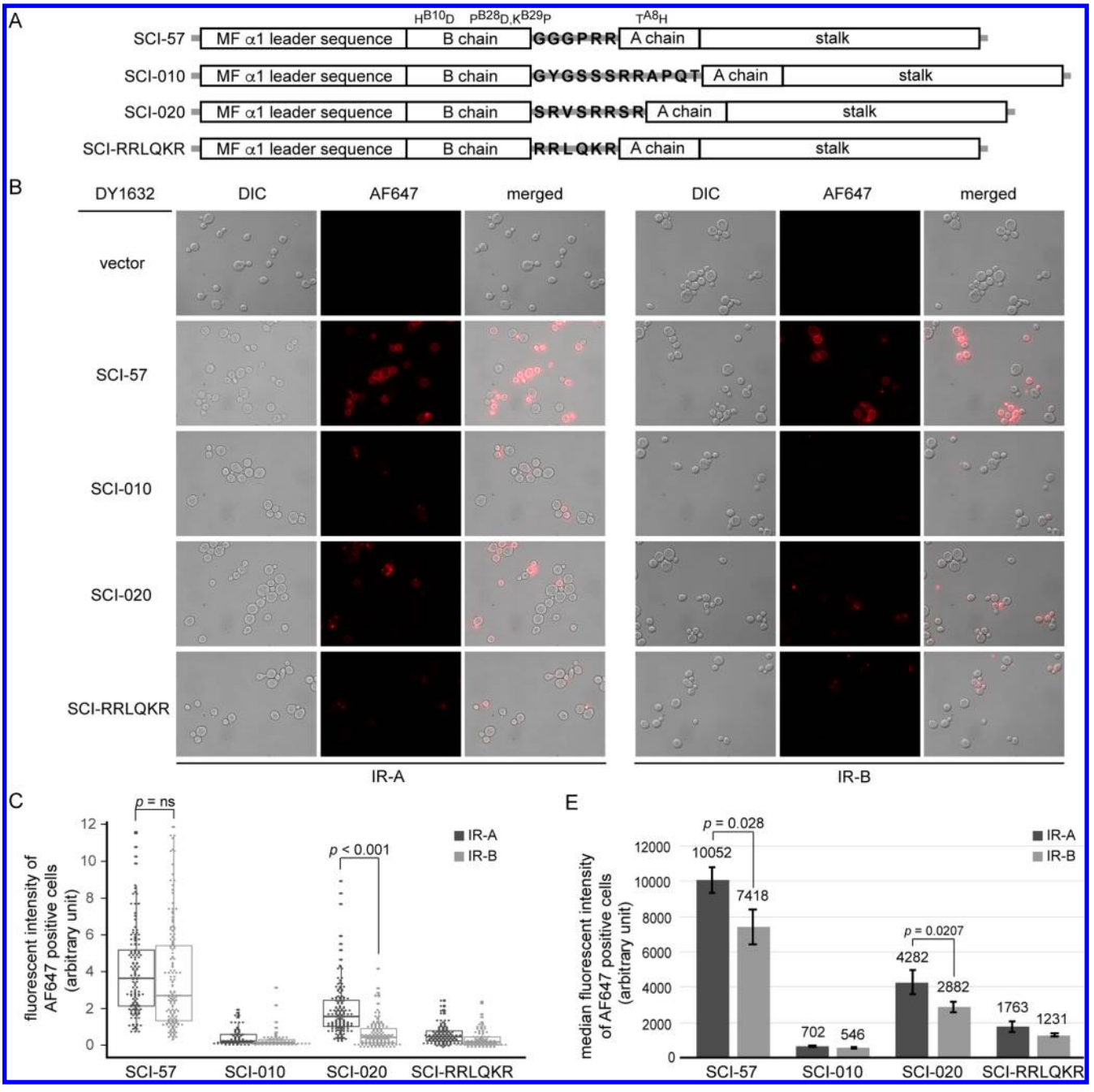

Figure 3. continued

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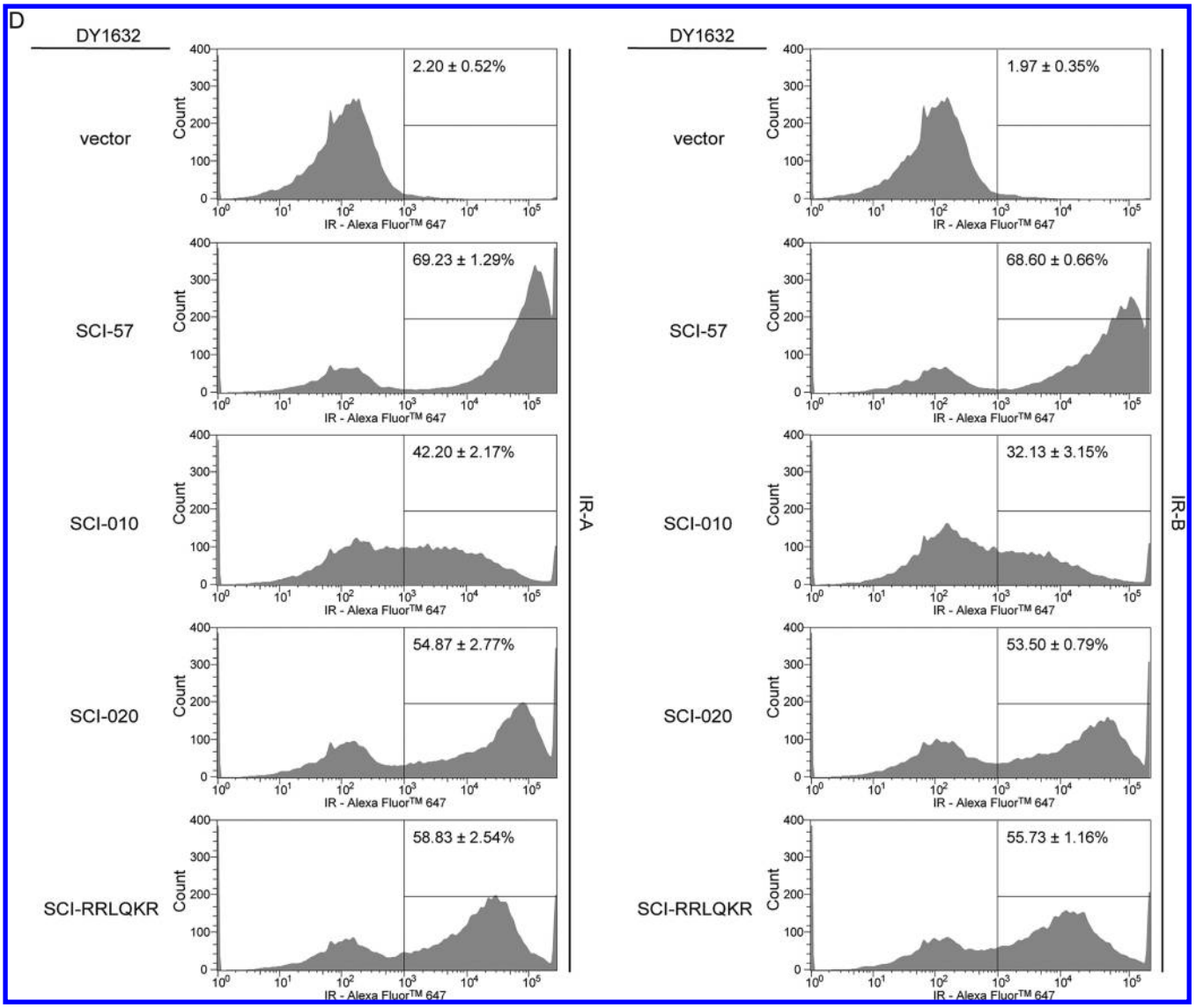

Figure 3. IR isoforms show varying affinities for single-chain insulins with different C-peptide sequences. (A) Schematic of SCI variants for yeast surface display: SCI-57, insulin A and B chain with point mutations and the GGGPRR C-peptide; SCI-010, native insulin A and B chain with the IGF1 C-peptide; SCI-020, native insulin A and B chain with the IGF2 C-peptide; SCI-RRLQKR, native insulin A and B chain with the RRLQKR C-peptide. (B) IR binds to yeast cells displaying SCI variants with different affinities. Cells displaying each SCI incubated with IR-A or IR-B. After being immunostained, cells were subjected to fluorescence microscopic analysis. (C) Quantification of the fluorescence intensity of the AF647 positive cells. The 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. The percentage of the AF647 positive cells in total events is shown in the flow cytometry box (n = 3). (E) Bar graph that represents the MFI of the AF647 positive cells in the flow cytometry analysis (n = 3).

peptide (SCI-GGGPRR) and expressed in DY1632. Removal of mutated residues, indeed, resulted in a decrease in IR binding activity (Figure S2). The MFI of AF647 positive cells was 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 supports the finding that both IR isoforms specifically bind 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 a different intervening C-peptide on a 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 a similar binding affinity for 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 six 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 those of 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 the results of previous studies. This

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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. Because the length of the the C-peptide is the same for both SCI-57 and SCI-RRLQKR, this low affinity may be due to the more rigid conformation in the C-peptide compared to that in the glycine-rich linker in SCI-57. For each variant, representative images are shown in Figure 3B (full data in Figures S5 and S6), and 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, the binding of IR-A to the SCI variants was stronger than that of IR-B as the mean fluorescence 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 to 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 a 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 an additional in vitro folding process to produce a native insulin-like IR binding potency as shown in earlier studies. We next compared the MFIs obtained from flow cytometry. Consistent with the fluorescence microscopic analysis result, the MFI of all SCI varaints was ~1.5-fold higher when cells were incubated with IR-A than with 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 a yeast cell surface. This method can be used for library construction that may lead to identification of IR isoform-selective insulin analogues as probes or therapeutic single-chain insulin candidates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b01094.

Figures S1−S6 (PDF)

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Notes

The authors declare no competing financial interest.

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