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Artificial Ligands of Streptavidin (ALiS): Discovery, Characterization, and Application for Reversible Control of Intracellular Protein Transport

Takuya Terai,[*,†,∥] Moe Kohno,[†] Gaelle Boncompain,[#,¶] Shigeru Sugiyama,[▽] Nae Saito,[‡] Ryo Fujikake,[†] Tasuku Ueno,[†,∥] Toru Komatsu,[†,⊥] Kenjiro Hanaoka,[†] Takayoshi Okabe,[‡] Yasuteru Urano,[†,∥,§] Franck Perez,[#,¶] and Tetsuo Nagano[*,†,‡]

[†]Graduate School of Pharmaceutical Sciences, [‡]Drug Discovery Initiative (DDI), and [§]Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

[∥]CREST and [⊥]PRESTO, Japan Science and Technology Agency, 7 Gobancho, Chiyoda-ku, Tokyo 102-0076, Japan

[#]Institut Curie, Centre de Recherche, PSL Research University, [¶]CNRS, UMR144, 26, rue d'Ulm Paris 75248, France

[▽]Graduate School of Science, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Supporting Information

ABSTRACT: Artificial ligands of streptavidin (**ALiS**) with association constants of ~10^6 M^{-1} were discovered by high-throughput screening of our chemical library, and their binding characteristics, including X-ray crystal structure of the streptavidin complex, were determined. Unlike biotin and its derivatives, **ALiS** exhibits fast dissociation kinetics and excellent cell permeability. The streptavidin-**ALiS** system provides a novel, practical compound-dependent methodology for repeated reversible cycling of protein localization between intracellular organella.

Chemical biology aims to utilize chemical methods and tools to understand and control complex biological systems,[1] and one of the most widely used systems consists of biotin and its partner protein, (strept)avidin.[2] Biotin and (strept)avidin have surprisingly large binding constants (K_a = 10^{14}–10^{16} M^{-1}),[2a] which are not greatly affected by conditions such as pH, temperature, and buffer composition, although greater stability would be desirable in some cases.[2c] Consequently, biotin has been used as a chemical "glue" to connect pairs of biomolecules in various applications, including protein purification,[3] cell imaging,[4] and drug delivery.[5] However, a small dissociation rate constant (2.4×10^{-6} s^{-1})[6] is not always desirable because quick and complete regeneration of free streptavidin is required in applications such as protein purification and cell manipulation.[2b] To overcome this limitation, efforts have been made to modulate the binding by developing (strept)avidin mutants[7] or biotin derivatives.[8] However, mutant proteins are usually less stable than native (strept)avidin,[7a] and biotin derivatives tend to show only slow and partial dissociation in the absence of excess biotin as a competitor (vide infra). To our knowledge, there are no compounds with practical binding constants (K_a) of over 10^6 M^{-1} that can be detached from native streptavidin under mild conditions, though such compounds would complement and extend current streptavidin–biotin technologies.

In this study, we addressed the issue by random screening of our chemical library to find novel (i.e., unrelated to biotin) streptavidin-binding scaffolds (Figure S1). We expected that this strategy would uncover ligands that are more readily derivatizable and have better properties for biological applications than biotin analogues.[8] We named the discovered ligands **ALiS** (artificial ligands of streptavidin), characterized their binding properties, and used them to develop a novel, practical methodology for repeated reversible cycling of protein localization between intracellular organella in living cells, induced by a simple addition/washout procedure.

To identify novel ligands that are readily removable from streptavidin, we adopted a three-step approach using fluorescence polarization (FP) and surface plasmon resonance (SPR) successively (Figure 1a). First, to discover moderate binders in our large chemical library, we developed a competitive primary screening system based on FP. For this assay, we used avidin from egg instead of streptavidin due to its high structural similarity and lower cost, and we newly developed a fluorescent probe, *N*,*N*′-dimethylbiotin-6-carboxyfluorescein, (diMeB-6CF: **1**, Figures 1b and S2), based on *N*,*N*′-dimethylbiotin,[8d] which we recently reported as an avidin-interacting moiety. We optimized the assay condition to obtain high reliability (Z' = 0.97, Figure S3) and used the developed assay to screen 164,480 compounds provided by Drug Discovery Initiative, the University of Tokyo (Table S1 and Figure S4). The cutoff was set at the mean +4 × SD for within-day assays, and 963 compounds were selected as primary hits. For 773 compounds that were reproducibly picked up in repeated screening (Figure S5), we performed a second FP screening using streptavidin and a stronger-binding probe, biotin-5-aminofluorescein (B-5AF: **2**, Figures 1b and S6). This step reduced the number of candidates to 44. Removal of biotin analogues and compounds with unfavorable properties afforded 19 candidates, which were subjected to dose–response assays (Figure S7), leaving 11 compounds. Finally, SPR assay was performed with immobilized streptavidin (Figure 1c) to

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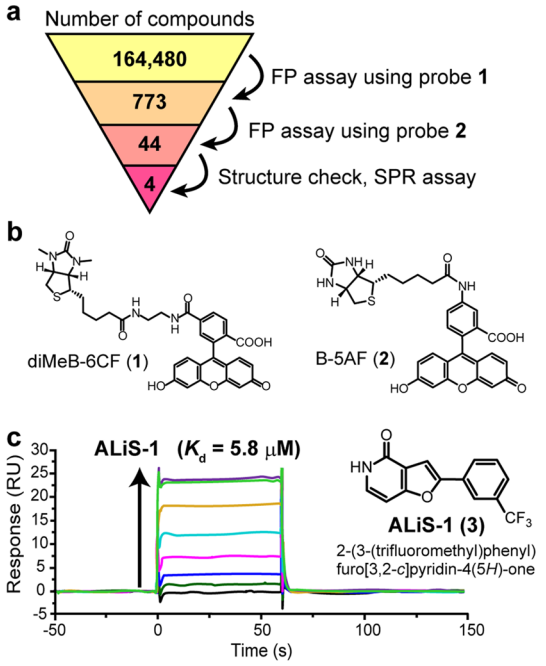

Figure 1. ALiS-1 was identified as a novel streptavidin ligand. (a) Summary of screening results. The number of compounds and the selection methods are shown schematically. (b) Chemical structures of the FP probes used for screening. (c) SPR sensorgram of **ALiS-1** (0−40 μM). The assay was performed in buffer containing 5% DMSO as a cosolvent. Inset: chemical structure of **ALiS-1** and IUPAC name.

determine the direct binding constants (K_d) and association/dissociation rates (k_{on}/k_{off}). **ALiS-1** (**3**, Figure 1c) was identified as the most interesting compound (the other three hits are shown in Figure S8) in terms of affinity and fast and complete dissociation (K_d = 5.8 μM; $k_{off} \approx 1.0\ s^{-1}$). In contrast, reported "reversible" biotin analogues such as desthiobiotin[8b] and *N*3′-ethylbiotin[8a] show slow and incomplete dissociation when washed with buffer (Figure S9). Although 4-hydroxyazobenzoic acid (HABA)[9] and its derivatives[10] show rapid dissociation kinetics, HABA has much weaker affinity (Figure S10). Thus, **ALiS-1** has unique properties that can be exploited to develop novel chemical tools.

Next, we synthesized several derivatives of **ALiS-1** (**3**) to gain insight into its binding mechanism. The derivative with CF_3 at the para-position (**4**; Table 1) had significantly lower affinity than the parent compound. Replacement of CF_3 with CH_3 (**5**) also

Table 1. Chemical Structures of Synthesized ALiS-1 Derivatives and Their Binding Constants

Comp.	R	K_d [μM][a]
3 (ALiS-1)		5.8
4		>20[b]
5		21
6 (ALiS-2)		1.2
7		>160[b]
8		12
9		—[c]
10		—[c]

[a]Determined by SPR, measured in buffer containing 5% DMSO. [b]Exact values could not be determined due to low solubility and low affinity. [c]No binding was observed under this condition (up to 160 μM).

weakened the affinity, suggesting that CF_3 enhances binding by acting as an electron-withdrawing group and/or a hydrogen-bond acceptor, although steric contribution cannot be ruled out. The increased affinity of the COOMe derivative (**6**; **ALiS-2**) is consistent with this idea. Exchange of benzene with electron-rich furan (**7**) and electron-poor pyridine (**8**) rings decreased and increased the affinity, respectively, implying that the electron-deficient benzene ring in **ALiS-1** is important. The six-membered, *cis*-amide-containing ring of **ALiS-1** was critical for binding, as **9** showed no measurable binding to streptavidin up to 160 μM. The result for **10** indicates that the relative orientation of this amide to the benzene moiety should be strictly controlled.

Although the reversible binding ability and the order of relative affinity of these compounds were highly reproducible, we stress that the measured K_d values depend on experimental conditions. For example, when the DMSO concentration was decreased from 5% to 0.1% in SPR, the calculated K_d value of **ALiS-2** changed from 1.2 μM to 180 nM (Figure S11). For **ALiS-2**, isothermal calorimetry (ITC), performed to demonstrate that the compound can bind to streptavidin in solution, provided a lower K_d value of 200 nM (Figure S12). Although K_d values in a biological environment are difficult to determine, these data strongly suggest that **ALiS-2** (and maybe also **ALiS-1**) has a binding affinity of high-nanomolar order in cells. The ITC data also indicate that the stoichiometry of binding is 1:4, and the ligands fill all the pockets of streptavidin tetramer without apparent allosteric effects. ESI-MS experiments[11] on streptavidin−**ALiS-1** complex (Figure S13) also support this stoichiometry.

Next, we performed cocrystallization of **ALiS** with streptavidin and determined the 3D structure of the complexes (Figures 2 and

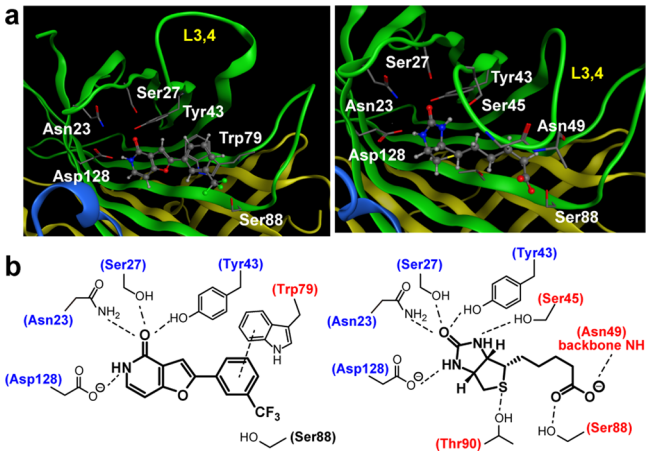

Figure 2. Binding mechanism of **ALiS-1**. (a) Magnified view of the 3,4-loop and the ligand-binding pocket of **ALiS-1** with streptavidin (left). The same view was created for biotin−streptavidin complex from PDB 3RY2[12a] and is shown for comparison (right). (b) Schematic representation of interactions between amino acid residues and ligands (**ALiS-1** and biotin). Residues that interact with both compounds are shown in blue, and those involved in compound-specific interaction are shown in red.

S14−S16 and Table S2). First, we confirmed that **ALiS-1** binds to the biotin-binding pocket of streptavidin. However, a significant conformational change of the 3,4-loop (L3,4: residues 45−52) was observed upon binding of compounds (Figure 2a). This "flexible" loop is reported to be "open" in the absence of ligands, and changes to "closed" form when biotin is bound

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(Figure S17).[12] This conformational change may prevent fast release of biotin from the complex.[2c,13] In the case of **ALiS**, the loop remains "open" in the complex; thus, the ligand is highly exposed to solvent, compared with biotin (Figure S18). Though further study is necessary, this may account for the fast dissociation kinetics of **ALiS-1**. Interactions between ligands and protein are illustrated in Figure 2b. As expected, the *cis*-amide moiety of the compound forms four hydrogen bonds with the protein and functions as an anchor, just like the ureido moiety of biotin. In addition, the benzene ring has a $\pi-\pi$ interaction with Trp 79. Its meta position is oriented toward the outer surface of the protein (Figure S18), and the CF_3 or COOMe group partially interacts with the hydroxy group of Ser 88 (Figures 2a and S16). This position is likely to be available to accommodate a linker for attachment of biological molecules, as is the case with the carboxy group of the biotin side chain.

To date, biotin derivatives with moderate affinity have mainly been used for protein purification: derivatives linked to the protein of interest bind to immobilized streptavidin on the column and can be eluted with biotin.[8a−c] However, we thought the unique properties of **ALiS** (fast k_{on} and k_{off}, appropriate lipophilicity for transfer across biological membranes, and small molecular weight) could be utilized to develop a practical methodology for reversible manipulation of protein localization in living cells.

Proper function of proteins depends on correct cellular localization, especially for enzymes working in membrane-bound organelles. In this context, we recently developed the RUSH system[14] in which the streptavidin/biotin pair is used to manipulate protein transport, and hence localization, in cells. This is achieved by immobilizing a streptavidin-binding peptide[15] (SBP)-containing "reporter" protein through its binding to a streptavidin "hook" localized in a specific organelle and releasing it with free biotin. With this system, synchronized transport of an SBP-conjugated reporter protein was achieved in living cells. However, because of the slow k_{off} of biotin, the release is not reversible and the released protein cannot be recycled to its original position (Figure S19 and Video 1).

We considered that **ALiS** might be used to achieve reversible protein transport between endoplasmic reticulum (ER) and Golgi apparatus. To test this idea, we used a C-terminal ER-retention signal peptide, KDEL, conjugated to streptavidin as the hooking moiety, and a Golgi enzyme, α-mannosidase II (ManII), fused with SBP-EGFP as the reporter (Figure 3a).[14] We prepared HeLa cells expressing these two proteins and added **ALiS-1** (40 μM) to the medium. As anticipated, **ALiS-1** entered the cells and induced transport of the reporter from ER to Golgi (Figure S20). Release was fast and complete within 30 min (Figure 3b,c and Video 2). Most importantly, simple exchange of culture medium after **ALiS-1** treatment induced rapid retrograde transport of the reporter back to the ER. The retrieval was not caused by protein synthesis (Figure S21), indicating that the streptavidin hook was regenerated in cells. Subsequent addition of **ALiS-1** again induced forward transport of the same reporter (Figure 3b,c and Video 2), demonstrating the repeatability of the whole process. **ALiS-2** worked in a similar manner, with slightly better kinetics (Figures 3c and S22 and Video 3). In contrast, biotin (Figure S19 and Video 1) or desthiobiotin (Figure S23) induced only unidirectional, irreversible transport. To our knowledge, **ALiS** is the only tool currently available to achieve reversible release and retention in RUSH assay. Moreover, onset of protein transport was earlier with **ALiS** than with biotin, probably due to its high membrane permeability (Figure 3c).

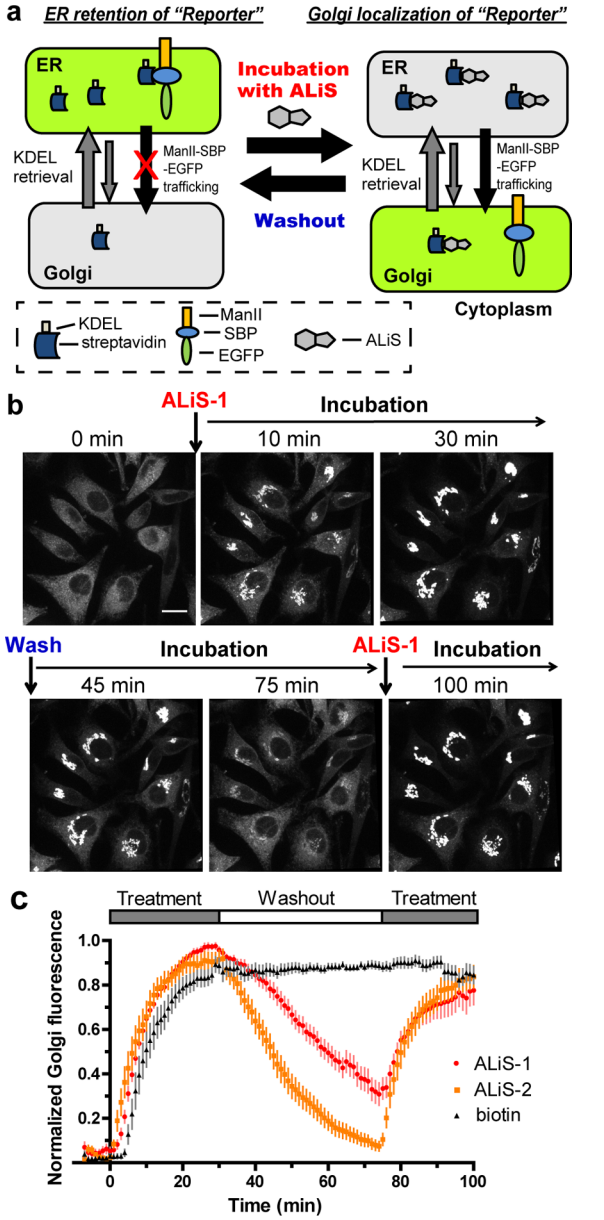

Figure 3. Reversible control of protein transport using **ALiS**. (a) Schematic representation of a reversible RUSH system between ER and Golgi, induced by addition and washout of **ALiS**. (b) Confocal fluorescence images of HeLa cells stably coexpressing streptavidin-KDEL and ManII-SBP-EGFP. **ALiS-1** (40 μM) was added to the medium at 0 min. Culture medium was changed at 30 min, and the cells were incubated for 45 min in the absence of the drug. Next, **ALiS-1** (40 μM) was added again. Scale bar: 20 μm. (c) Time-dependent analysis of Golgi localization of ManII-SBP-EGFP. Data of **ALiS-1** (red circle), **ALiS-2** (5 μM, orange square), and biotin (40 μM, black triangle) represent mean ± SEM (n = 8−12 fields, >5 cells per field) of two independent experiments. Intensities were normalized to the maximum value of the series.

The retrograde transport highlighted in this reversible RUSH assay is probably linked to the biology of KDEL receptor signaling.[16] When released from ER by **ALiS**, the SBP-containing reporter protein is transferred to the Golgi complex. When **ALiS** is washed out from the cells, the streptavidin-containing hook fused to KDEL, which recycles between ER and Golgi, can interact again with Golgi-localized reporter protein. The KDEL receptor is then able to carry the protein complex back to the ER

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(Figure 3a). Immunostaining and co-IP experiments (Figure S24 and S25) similarly indicated that the reporter protein moved to Golgi and then reverted to ER when **ALiS** was washed out, whereas no reversibility was observed with biotin. *In vitro* SPR experiments (Figure S26) also supported the occurrence of these processes. Finally, although we cannot rule out the presence of off-target proteins in cells, **ALiS** showed no cytotoxicity in the concentration range used for imaging (Figure S27) and induced no morphological change or other distinctive behavior of HeLa cells, which implies that these compounds do not interfere with critical cell signaling.

In conclusion, our large-scale screening system identified novel streptavidin ligands (**ALiS**) with K_d values of approximately 1 μM. In conjunction with the SBP tag, their fast k_{off} (>0.1 s^{-1}) makes them unique tools as competitive ligands, as illustrated herein by their application to reversibly control protein transport between ER and Golgi. This approach, for example, would be applicable to transiently activate post-translational protein modification, induce reversible signaling, or reversibly alter protein localization in cells. It will also enable kinetic studies to identify compounds/genes that specifically control forward/backward transport of proteins of interest. A straightforward extension of this work would be vesicle-independent reversible control of protein translocation from cytosol to a particular organelle. A few such systems using FKBP[17] and tobacco 14-3-3[18] have been reported recently, but we use a simpler ligand (i.e., a readily derivatizable synthetic compound) and a shorter peptide (SBP) than previous systems. Importantly, our system is orthogonal to other methods. We are currently optimizing **ALiS** in terms of solubility, affinity, and binding to streptavidin mutants[7] to pave the way for further applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05672.

Experimental procedures, characterization of synthesized compounds, and supplementary data (PDF)

Unsuccessful trial of reversible trafficking with biotin (AVI)

Reversible trafficking with **ALiS-1** (AVI)

Reversible trafficking with **ALiS-2** (AVI)

■ AUTHOR INFORMATION

Corresponding Authors

*t-terai@mol.f.u-tokyo.ac.jp

*tlong@mol.f.u-tokyo.ac.jp

Notes

The authors declare no competing financial interest.

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