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Multivalent Site-Specific Phage Modification Enhances the Binding Affinity of Receptor Ligands

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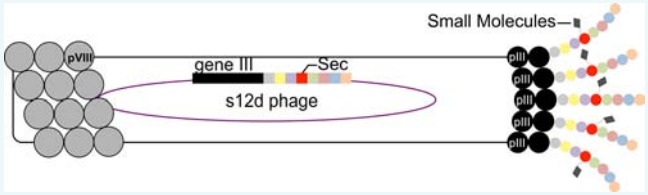
Supporting Information

ABSTRACT: High-throughput screening of combinatorial chemical libraries is a powerful approach for identifying targeted molecules. The display of combinatorial peptide libraries on the surface of bacteriophages offers a rapid, economical way to screen billions of peptides for specific binding properties and has impacted fields ranging from cancer to vaccine development. As a modification to this approach, we have previously created a system that enables site-specific insertion of selenocysteine (Sec) residues into peptides displayed pentavalently on M13 phage as pIII coat protein fusions. In this study, we show the utility of selectively derivatizing these Sec residues through the primary amine of small molecules that target a G protein-coupled receptor, the adenosine A_1 receptor, leaving the other coat proteins, including the major coat protein pVIII, unmodified. We further demonstrate that modified Sec-phage with multivalent bound agonist binds to cells and elicits downstream signaling with orders of magnitude greater potency than that of unconjugated agonist. Our results provide proof of concept of a system that can create hybrid small molecule-containing peptide libraries and open up new possibilities for phage−drug therapies.



INTRODUCTION

The last few decades have seen enormous progress in proteomic, genomic, and metabolomic understanding and treatment of disease. However, off-target effects of small molecules have hampered drug usage due to toxic side effects, resulting in the removal of patients from treatment regimens. Targeting strategies have become a prominent focal point for new drug design, with the promise of increasing drug delivery to targeted cells while reducing toxicity. Unless a drug itself is capable of targeting a desired cell type, such as a therapeutic antibody, hybrid complexes or multifunctional nanoparticles are needed to combine a therapeutic drug with a targeting moiety. Small molecules, aptamers, peptides, and antibodies comprise the most common moieties used in these applications. While each has its benefits, peptides are easy to tether, can be rapidly screened, can be chemically synthesized, and can bind with high affinity and specificity without eliciting an immune response. These qualities make them attractive for targeting.

Of the known ways for producing and screening high-affinity peptides, phage display has been one of the most effective and economical. Over the past 3 decades, phage display has been used to discover thousands of specifically targeted peptides. A common system uses bacteriophage expressing random peptides on the pIII coat protein, resulting in a diverse library that can contain $\geq 10^9$ different permutations. However, the peptide sequences are limited in the sense that they must be made from the pool of the 20 standard ribosomally encoded amino acids. Methods have been developed to expand upon this pool, including the incorporation of phosphorylated proteins[1] or modification with unnatural amino acids such as citrulline.[2,3] However, these expansions have been incremental, and any chemically large modification of the pIII protein could compromise the phage coat or more likely decrease the infectivity and expansion of the phage.[4] As an alternative, postbiosynthetic modification of the phage would circumvent these limitations and enable the incorporation of rationally selected small molecules. This would involve using a biological linker produced by the phage to enable proteins or synthetic compounds to be tethered selectively to the linker after phage replication. An example of this was described by Barrett et al. in 2007 using icosahedral phage and a streptavidin−biotin linking system in the coat proteins.[5]

We sought to create a means of producing targeted incorporation of small molecules onto phage using the

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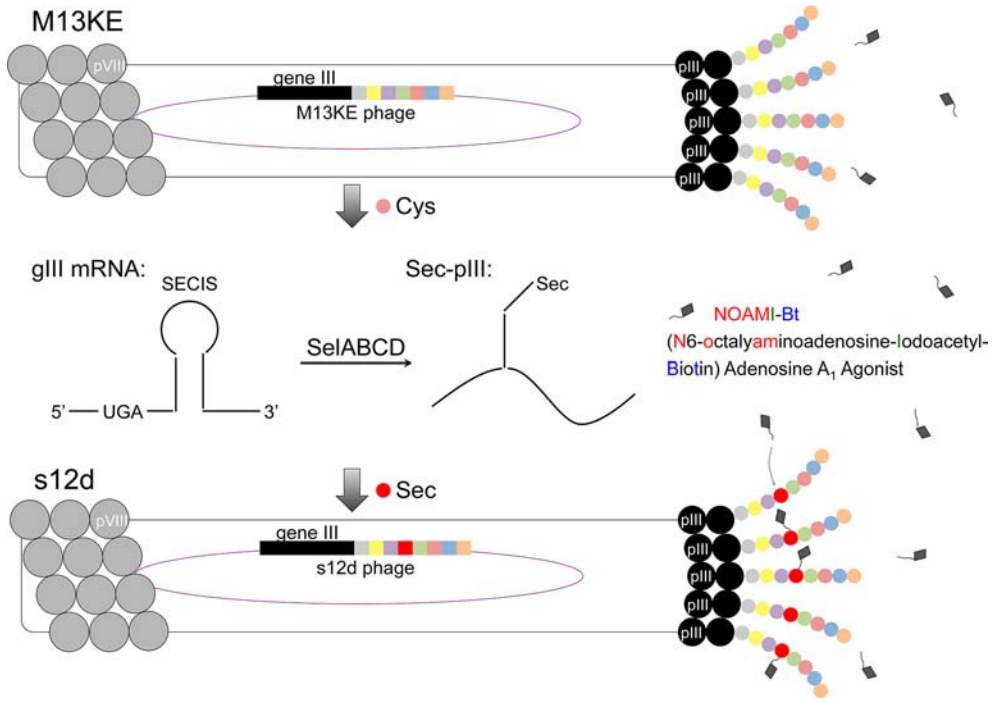

Figure 1. Schematic of the M13KE bacteriophage with incorporation of selenocysteine for site-specific modification. A Sec insertion sequence and UGA codon were inserted at the 5′ end of M13 gIII, which encodes for the pIII coat protein, providing five Sec residues for small molecule tethering under appropriate conditions.

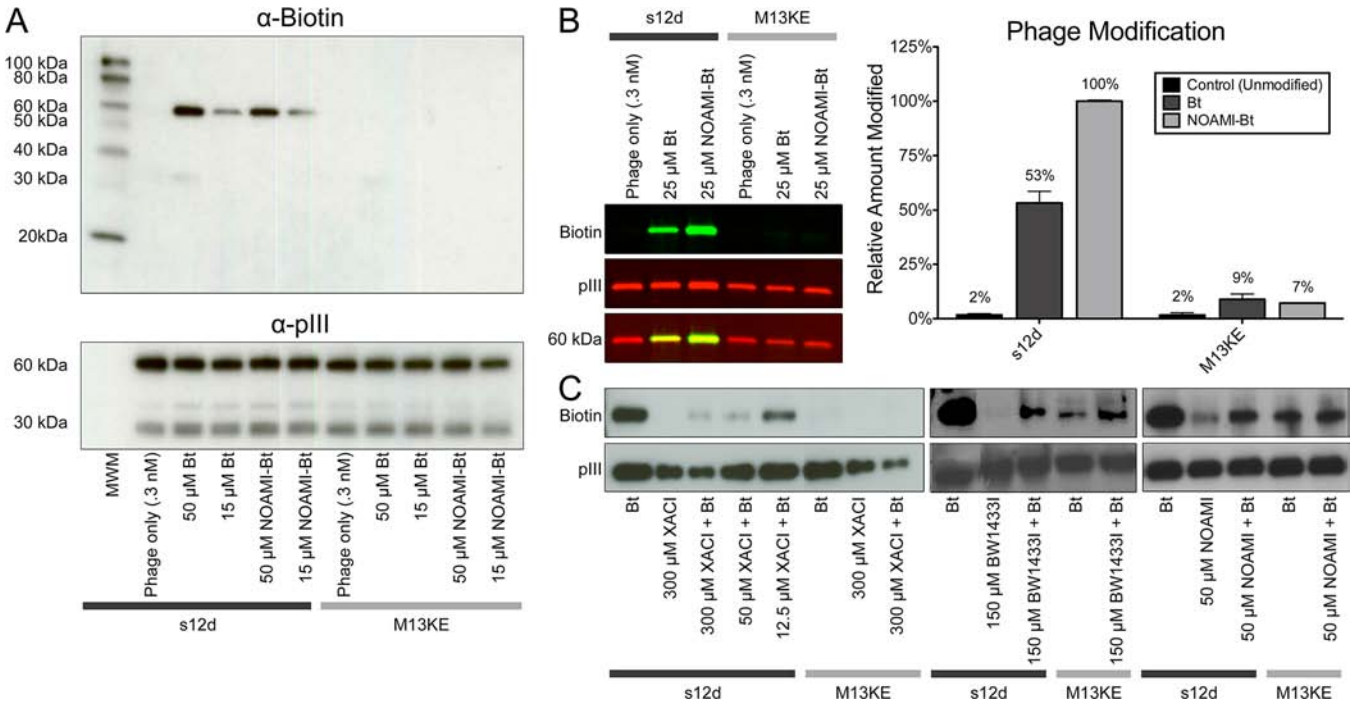

Figure 2. Site-specific modification of seleno-pIII coat protein of s12d phage. (A) Phage modified with the indicated reagents were analyzed via western blotting for the presence of biotin (top) and the pIII coat protein (bottom). Bands are seen at molecular masses near 60 kDa (the apparent molecular weight of the pIII protein) for biotin-tethered and NOAMI-Bt-tethered s12d phage but not for negative control, Sec-free M13KE phage. (B) Phage−small molecule complexes were reacted and quantified using qWestern blotting (left) to determine the relative efficiency of small molecule tethering (right). (C) Conjugation of additional small molecules to s12d or M13KE phage. Phage were incubated with the indicated molecules and subsequently incubated with Bt. As the small molecules did not contain a biotin group, the decrease in a band corresponding to the presence of biotin indicates successful tethering of small molecules.

previously identified selenocysteine (Sec) insertion sequence.[6,7] Sec, the 21st amino acid, is expressed in a ribosome-mediated protein synthesis manner, and the selenol side chain has a pK_a of ∼5.47, which is lower than that of the chemically related cysteine (Cys, pK_a ∼ 8.0), thus allowing Sec to display stronger nucleophilicity and reactivity than Cys at physiological pH.

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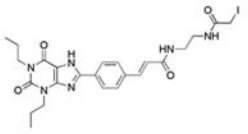
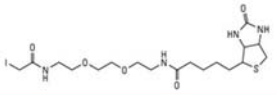
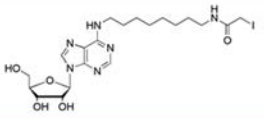
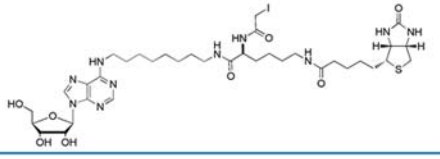
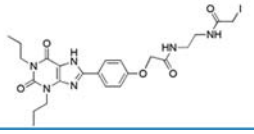
Table 1. Modified Phage Molecules[a]

Phage-Small Molecule	Tethered Molecule	Function	Chemical Structure
s12d-BW4133I	BW4133I	Non-selective adenosine receptor antagonist	
s12d-Bt	Bt	Negative control	
s12d-NOAMI	NOAMI	Selective adenosine A_1 agonist	
s12d-NOAMI-Bt	NOAMI-Bt	Selective adenosine A_1 agonist	
s12d-XACI	XACI	Non-selective adenosine receptor antagonist	

[a]A summary of the five molecules used in this article, their functions and chemical structures. The chemical structures are those of the tethered molecule.

Therefore, it is possible to tether molecules selectively to Sec and not Cys[8] in a seleno-pIII displaying phage. This method ensures that (1) no other coat proteins are affected, (2) proper orientation of the phage and small molecule occur when screening, and (3) various flanking peptide sequences can be screened. Furthermore, because Sec is coded for by UGA and requires opal suppression, a functional pIII protein (required for phage propagation) occurs only upon successful Sec insertion.[6,9]

As a proof of concept, we chose to work with several small molecule ligands that target the adenosine A_1 receptor. The A_1 receptor is one of four G protein-coupled receptors[10] found in many tissues including heart, brain, and adipose tissue. The adenosine A_1 receptors modify the function of many tissues including cardiac, neuronal, renal, and adipose.[11] N^6-substituted adenosine analogues have been synthesized that bind with high affinity and specificity to the A_1 subtype.

In the current article, we demonstrate that small molecules that target the adenosine A_1 receptor can be tethered to M13 phage via displayed Sec residues and that the resulting multivalent constructs enhance the binding of the tethered phage to adenosine A_1 receptor-expressing cells. Using competition assays, we also show that the modified phage is potent and specific for the A_1 receptor and capable of activating the receptor and its downstream signaling pathways. This technology has broad applications and introduces new ways for performing small molecule screens, which serve as platforms for developing improved peptide−drug hybrid compounds.

RESULTS

Modification of Selenopeptide-Displayed Phage with Adenosine Receptor Ligands. Phage displaying five Sec-pIII peptides, each containing a Sec residue, were produced using a modified *Escherichia coli* strain (ER2738^{selABC}) in the presence of selenite, as previously reported.[9] Sec-phage were stored anaerobically to maintain reactivity of the selenol group. The displayed Sec-pIII peptide (SARVSecHGP) is not known to have any specific targets and is thus treated as an inert sequence. A schematic of the insertion of Sec into the peptide and subsequent modification is depicted in Figure 1.

The small molecules were incorporated specifically into the pIII peptide sequence, as visualized by western blotting, demonstrating the specificity of the approach (Figure 2).

N^6-Octylaminoadenosine (NOAM), a small molecule adenosine A_1 agonist specifically designed to have selectivity for the A_1 receptor (K_D = 2.4 ± 0.3 nM) over the A_{2A} receptor (K_D = 1.9 ± 0.2 μM), was modified via its primary amine to include an iodoacetyl group for tethering to the Sec and a biotin for detection (NOAMI-Bt). Incubation of the s12d phage and NOAMI-Bt in a 1:167000 molar ratio of phage to small molecule at room temperature for 1 h resulted in the modification of pIII-Sec only with NOAMI-Bt, as only one band was present, corresponding to the pIII coat protein molecular weight (Figure 2A). As a control, an iodoacetyl-PEG_2-biotin (Bt) was reacted with the phage in the same ratio. A summary of all the phage−small molecule combinations can be found in Table 1.

A set of negative control reactions was performed with M13KE phage (no Sec) or s12d and vehicle only. M13KE phage did not react with the small molecules under these conditions. Quantitative western blotting (qWestern) revealed that the Bt reaction is 2-fold less efficient than that of NOAMI-Bt (Figure 2B). Therefore, for all subsequent experiments, a 1:333000 ratio of phage to Bt was used to have equivalent

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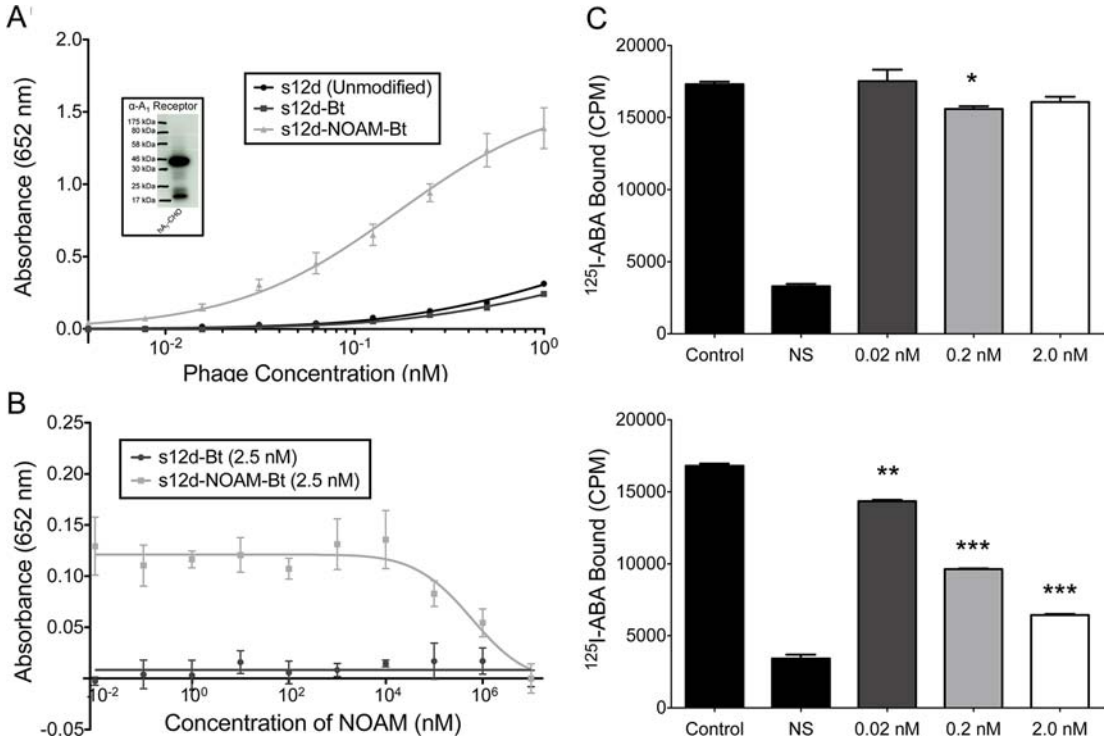

Figure 3. NOAMI-Bt-conjugated phage retain specificity for A_1. (A) Cell binding assays using human adenosine A_1 receptor-expressing CHO cells and modified s12d phage. Cells were incubated with NOAMI-Bt-modified phage and the presence of bound phage detected via ELISA. NOAMI-Bt-modified phage bind with high affinity (ED_{50} = 0.17 nM), whereas control phage (unmodified or biotin-conjugated only) demonstrated negligible binding. (Inset) Western blot of hA_1-CHO cells for the human adenosine A_1 receptor reveals a strong band at approximately 36 kDa, the reported molecular weight of the A_1 receptor. (B) Competition assays. hA_1-CHO cells were incubated with increasing concentrations of free NOAM followed by 0.5 nM NOAMI-Bt- or Bt-modified phage. As in panel A, phage binding was detected via ELISA. (C) Radioligand competition assays. ^{125}I-ABA incubated with hA_1-CHO membrane was reduced by 18, 54, and 77% with increasing concentrations of s12d-NOAMI-Bt (bottom) but not s12d-Bt (top).

tethering between the Bt and NOAMI-Bt molecules (Supporting Information Figure 1).

To demonstrate the versatility of the Sec-phage system, additional molecules were tethered to the s12d phage. Two nonselective adenosine A_1 antagonists, xanthine amine congener (XAC) and BW1433, were functionalized as iodoacetamides, forming the reactive molecules XACI and BW1433I, respectively. These modified molecules, as well as a functionalized NOAM without Bt (NOAMI), were reacted with the phage for 1 h and then chased with the Bt molecule. Detection of effective modification was observed by a lack of Bt binding in western blots. As with Bt and NOAMI-Bt, site-specific modification of the pIII coat protein was observed (Figure 2C).

NOAMI-Bt-Modified s12d Specifically Targets A_1 Receptor. To determine if NOAMI-Bt still binds to the adenosine A_1 receptor when conjugated to s12d phage, CHO-K1 cells were stably transformed to express the human adenosine A_1 receptor (hA_1-CHO). Presence of the receptor protein was validated via western blot (Figure 3A, inset). Enzyme-linked immunosorbent assays (ELISAs) were then performed to assess the modified phage's ability to bind the cells. Three different phage species were tested: unmodified s12d, Bt modified (s12d-Bt), and NOAMI-Bt modified (s12d-NOAMI-Bt). Increasing concentrations of phage were incubated with the cells for 1 h. After removing the excess phage by repeated wash steps, the cells were incubated with horseradish peroxidase (HRP) labeled anti-pVIII antibody (α-pVIII-HRP), and the phage bound to cell receptors were quantified by absorbance at 652 nm using a colorimetric substrate to HRP, 3,3′,5,5′-tetramethylbenzidine (TMB) (Pierce). The phage modified with the NOAMI-Bt molecule exhibited high binding affinity with an ED_{50} value of 0.17 nM, 14-fold lower than the ED_{50} of the free molecule, indicating a possible increased affinity due to multivalency and avidity effects. In contrast, both unmodified s12d and s12d-Bt exhibited negligible binding (Figure 3A).

Competition experiments were performed to further validate the specificity of NOAMI-Bt for the A_1 receptor. hA_1-CHO cells were preblocked for 1 h using free NOAM molecule at varying concentrations before s12d-NOAMI-Bt phage were added at 0.5 nM. After 1 h of incubation, residual bound phage were detected by absorbance as previously described (Figure 3B). As expected, the small molecule competed s12d-NOAMI-Bt away from the A_1 receptor (final ED_{50} = 577 μM).

Competition assays with radiolabeled [^{125}I]-aminobenzyladenosine (^{125}I-ABA), an adenosine A_1 receptor agonist,[12] were performed to further validate the specificity and affinity of s12d-NOAMI-Bt phage for the A_1 receptor. ^{125}I-ABA was applied for 1 h to hA_1-CHO membranes. Membranes were used because they bind agonists such as ^{125}I-ABA with high affinity through receptor-G protein complexes. Modified phage were used at increasing concentrations to compete with the bound ^{125}I-ABA. At all concentrations of the s12d-NOAMI-Bt phage tested, a significant decrease in the bound ^{125}I-ABA was detected. Specific ^{125}I-ABA binding after 1 h of incubation with 0.02, 0.2, and 2.0 nM phage was reduced by 18%, 54% and 77%, respectively. The control phage (s12d-Bt) even at the highest

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concentration competed negligibly (<10%) for ^{125}I-ABA, confirming the specificity of the modified phage for the A_1 receptor (Figure 3C). Taken together, these experiments demonstrate that specificity of the conjugated molecule for the A_1 receptor is retained.

Phage-Binding Kinetics. s12d-NOAMI-Bt, s12-Bt, and unmodified s12d phage were added to hA_1-CHO cells for different time periods, and the binding kinetics of the phage to hA_1-CHO cells were determined (Figure 4).

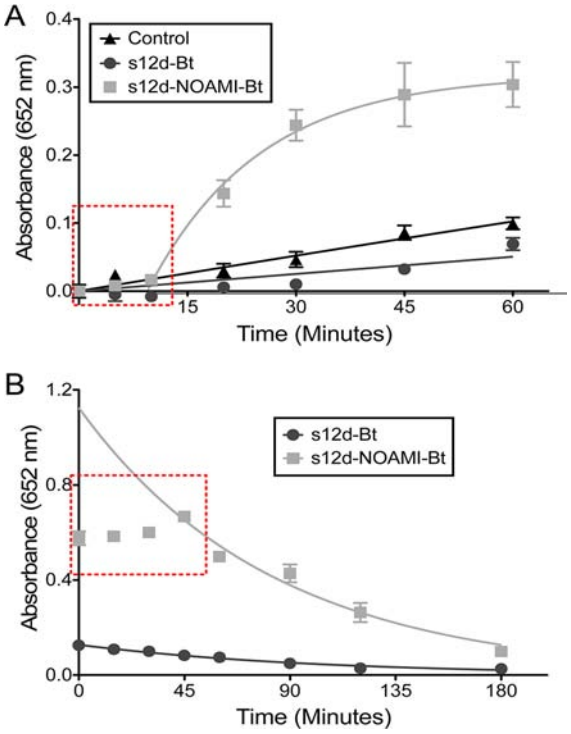

Figure 4. Binding kinetics of modified s12d phage. (A) Aliquots containing 0.5 nM of phage were incubated with hA_1-CHO cells at 4 °C for the indicated times. Due to deviation from standard binding kinetics (red box), association kinetic curve fits of s12d-NOAMI-Bt binding (squares) excluded the first 10 min were fit with an R^2 value of 0.868. Unmodified (triangles) and Bt-modified (circles) phage are fit without omission. (B) Phage were incubated for 1 h with hA_1-CHO cells and then removed. The cells were washed at various time, and remaining phage was quantified via ELISA. Data beyond 45 min were fit to a single-exponential decay curve (R^2 = 0.879) as illustrated for s12d-NOAMI-Bt.

As can be seen from the graph in Figure 4A, association kinetics past 10 min conformed to standard exponential binding kinetics with k_{on} = 1.98 × 10^6 M^{-1} s^{-1}. However, during the first 10 min, phage binding was minimal, suggesting that phage are not freely accessible to cell receptor or that binding is cooperative. Similarly, for s12d-NOAMI-Bt dissociation kinetics, a 45 min lag is seen where the data deviates from typical exponential decay. Beyond 45 min, however, the data can be fit to an exponential equation with k_{off} = 2.02 × 10^{-4} s^{-1} ($t_{1/2}$ = 57 min). Combining observed apparent k_{on} and k_{off} values assessed at long incubation times, we can calculate an apparent K_D = 0.10 nM. Fits to early and late data points are shown in Supporting Information Figure 2.

NOAM Retains Its Agonist Function When Tethered to s12d Phage. In addition to binding to the A_1 receptor, we wanted to determine if the tethered molecule retains its agonistic function. Active human adenosine A_1 receptors expressed in CHO-K1 cells have previously been reported to signal through the AKT pathway. Cheng et al. were able to use the small molecule agonist N^6-cyclohexyladenosine (CHA) to stimulate the receptor and increase P-ATK within 2 min and found peak activation around 5 min.[13] We were able to reproduce this observation using CHA in our own hA_1-CHO cells (Figure 5A).

Therefore, we used this pathway to determine the receptor activation potency in hA_1-CHO cells. The presence of a doublet in the western blot for T-AKT or P-AKT was consistent with previously published reports.[14] Various concentrations of s12d-NOAMI-Bt phage were incubated with cells, and the extent of AKT activation was compared to that of s12d-Bt and several free A_1 agonists, CHA, N^5-ethylcarboxamido-adenosine (NECA), and NOAMI-Bt. To assess extent of AKT activation, the 15 min time point was chosen to account for the 10 min delay of phage binding to cells as observed in the phage binding kinetics (Figure 4A). Remarkably, the NOAMI-Bt-modified phage were able to very potently stimulate a response in the AKT pathway (EC_{50} = 3.20 pM), whereas s12d-Bt control phage were unable to elicit a response (Figure 5A). Interestingly, conjugated NOAMI-Bt invoked a response at a concentration of ~5 orders of magnitude lower than that of free NOAMI-Bt (EC_{50} = 5.11 × 10^5 pM) (Figure 5B). When compared to free CHA (EC_{50} = 2.44 × 10^3 pM) and NECA (EC_{50} = 1.88 × 10^3 pM), s12d-NOAMI-Bt activation of AKT was 3-fold greater.

DISCUSSION

Phage display is a powerful tool enabling the discovery of peptide ligands that bind to targets of interest. The efficiency of this process is strongly correlated to the complexity of the displayed library, which can be increased by using semisynthetic phage display. In the current study, we demonstrate that incorporating Sec residues in the displayed peptide on the pIII phage coat protein can substantially improve this technique because it allows specific multivalent modification of the phage with small molecules. We demonstrate that ligands of a G protein-coupled receptor, the adenosine A_1 receptor, can be tethered to the pIII peptide and maintain its ability to bind and signal.

Throughout this article, we had worked with the assumption that five small molecules were tethered to each phage. Although 100% efficiency is not plausible, we do believe that we had >90% tethering efficiency, based on our findings in Figures 2C and S1. After the s12d phage were reacted with XACI, BW1433I, or NOAMI (none of which have a biotin group), excess Bt was added to react with any remaining Sec groups. The lack of strong biotin bands in the western blots, < 10% of the s12d-Bt control, implies a high small molecule tethering efficiency. Probabilistically, a 90% tethering efficiency would result in 59% of the phage having all five Sec residues tethered (0.9^5) and 92% having at least four small molecules tethered ($0.9^5 + 5 \times 0.9^4 \times 0.1^1$). Only 0.001% of phage (0.1^5) would not have a small molecule bound.

The modification of the pentavalently displayed pIII-Sec with NOAMI-Bt results in a striking increase in the affinity of the tethered NOAMI-Bt molecule compared to the free form. Although the kinetics of phage binding to adenosine receptors on CHO-A1 cells suggest that the phage are not as accessible to receptors as small molecules, a shift of ~5 orders of magnitude in the EC_{50} of NOAMI-Bt compared to free small molecule for

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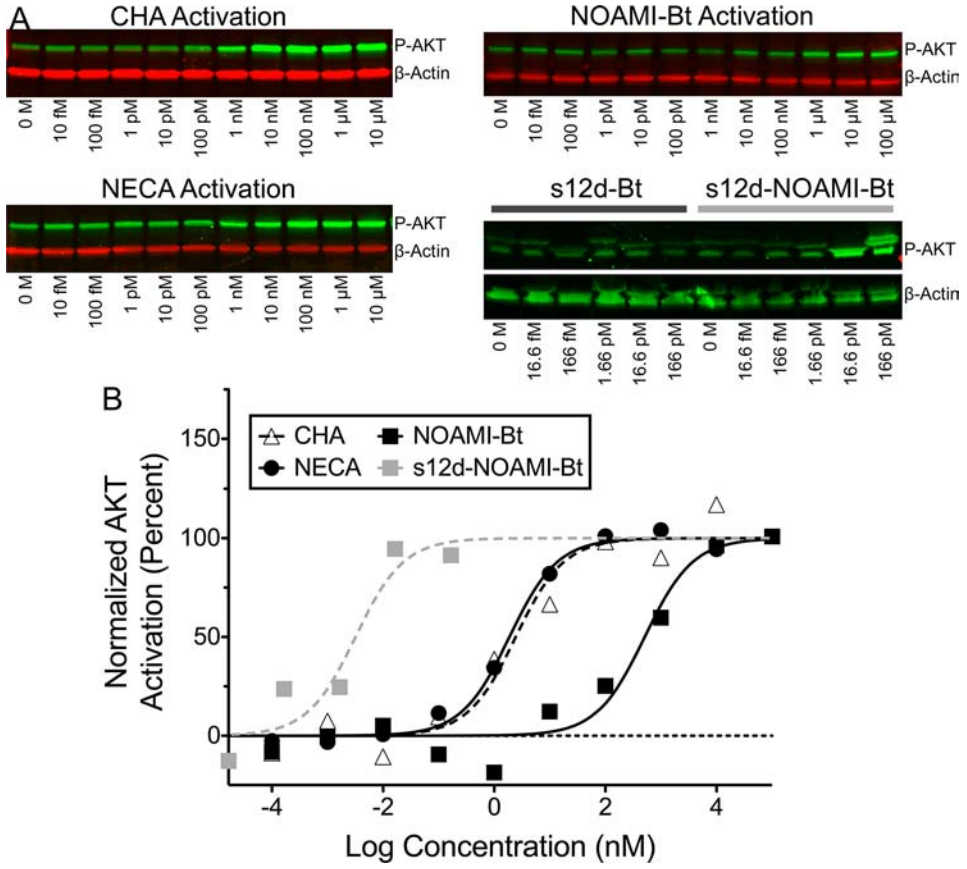

Figure 5. NOAMI-Bt is functional when tethered to s12d phage. (A) AKT pathway activation by small molecules and modified phage. hA_1-CHO cells were incubated with the indicated concentrations of CHA, NECA, and NOAMI-Bt molecules or modified phage for 15 min. Activation of AKT was analyzed by qWestern blotting for phospho-AKT and normalized to β-actin. (B) Activation plot fits based on the qWestern blots and normalized to baseline for AKT in response to increasing concentrations of s12d-NOAMI-Bt (gray squares, EC_{50} = 3.20 pM), free NOAMI-Bt (black squares, EC_{50} = 510 nM), CHA (triangles, EC_{50} = 2.44 nM), or NECA (circles, EC_{50} = 1.88 nM).

activation of AKT is observed when the molecule is tethered to s12d. This is a remarkable difference that we attribute largely to an avidity effect in binding due to the pentavalent display of the NOAMI-Bt-modified pIII-Sec phage coat protein. It has been reported that the covalent linking of two or more ligands within physical proximity to target molecules results in affinity enhancement that is primarily manifested as a result of a slowed dissociation.[15] Therefore, the tethering of five NOAMI-Bt molecules to the surface of the phage was expected to exhibit a very high apparent binding affinity. If all or most of the closely spaced tethered ligands are capable of binding receptors simultaneously, then it is unlikely that all of the tethered ligands will dissociate simultaneously, resulting in very slow dissociation kinetics. Also, the local concentration of tethered agonists in the region of the A_1 receptor is effectively high due to the closely packed selenocysteines displayed on the tip of the phage; one tethered molecule bound to a receptor keeps four other tethered molecules in very close proximity to other receptors.

Compared to the unconjugated small molecule radioligands, we did notice deviations in both association and dissociation kinetics of tethered phage. There are a number of factors that may contribute to this phenomenon. Since phage are large molecules, it may take time for them to diffuse and orient to enable productive binding to receptors. Also, their multivalency likely contributes to changing effective affinity, that is, as phage bind, the local concentration increases; thus, the overall effective concentration increases, driving the process forward. Multivalency could also account for the delayed release of phage; if a single phage is bound to multiple receptors, then that would keep it bound to the surface even when one receptor−agonist bond breaks. Phage dissociation may appear to accelerate as a consequence of receptors being taken up into cells and possibly uncoupled from G proteins that favor high-affinity binding. Another possibility is that a change in receptor conformation is needed to switch from a low-affinity to a high-affinity state for binding and the reverse for dissociation. This may result from the formation of R−G complexes.

Potential applications of this new technology are numerous. Most apparent is the development of an expanded phage display library that incorporates both randomized peptides and small molecules. Starting with a small molecule of interest and tethering it to the phage would allow for screening of enhancing peptides that could change the way the small molecule performs. This may cause the small molecules to become more specific, selective, and potent or to target them to a specific cell type. In essence, it would be a peptide screen but with a single, specific small molecule in the center of the peptide.

Another possibility would be to change the way small molecule screens are performed. First, each small molecule would need to be paired with a unique barcode peptide sequence. For example, peptide XXXX(Sec)XXX would be paired with molecule x, peptide YYYY(sec)YYY would be

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paired with molecule y, and so on. Once modified, the phage could be pooled and subsequently used in a screen. This would allow for all tethered molecules to be screened together as a single pool of phage and performed without the need for a very large number of wells, making the screen much more efficient and economical. The high-affinity molecules could later be identified via their tethered barcode peptides/phage.

CONCLUSIONS

To expand the functionality of phage display, we have developed Sec-displaying phage that are capable of being tethered to small molecules. The specific reactivity of the Sec selenol group allows us to tether these molecules specifically to the expressed pIII peptide and nowhere else on the phage coat. As proof of concept, we tethered several adenosine agonists to selenocysteine phage (s12d) and validated the site specificity by western blotting. We further demonstrated that the small molecules maintained their ability to bind to receptors and signal. We accomplished this by showing that s12d-NOAMI-Bt phage had increased binding to hA_1-CHO cells over that of biotin-modified or unmodified phage. Furthermore, we observed significantly tighter binding of modified phage compared with that of the untethered small molecule, suggesting higher avidity due to the pentavalency of the displayed selenol-peptide. This technology permits wide latitude in the nature of the small molecule being tethered, the peptide flanking the small molecule, or both, creating new possibilities for small molecule screens.

MATERIALS AND METHODS

Antibodies and Drugs. Antibodies were purchased from Abcam [Adenosine A_1 Receptor (EPR6179)], Cell Signaling Technology (CST) [AKT (C67E7), P-AKT (Ser473 9271), β-Actin (8H10D10), Streptavidin-HRP (3999)], GE Healthcare [M13-HRP (27-9421-01)], and Li-Cor [Rabbit-800CW (926-32213), Mouse-800CW (926-32212), Mouse-680LT (926-68022), Streptavidin-800CW (926-32230)]. CHA and NECA were purchased from Abcam and prepared in dimethyl sulfoxide (DMSO).

Preparation of hA_1-CHO Cells. An expression plasmid for the human A_1 receptor in the CLDN10B mammalian expression vector was prepared as described.[16] The adenosine receptor was introduced into CHO-K1 cells by means of Lipofectin (Life Technologies), and colonies were selected by growth in 1 mg/mL G418 (Life Technologies) and maintained in 0.5 mg/mL G418. The agonist radioligand ^{125}I-ABA was found to bind to a total of 3.5 pmol/mg recombinant human A_1 receptors on hA1-CHO cell membranes with a K_D of 1.02 nM.[16]

Preparation of Modified s12d Phage. Selenocysteine phage was amplified using ER2738^{SelABC} *E. coli* in the presence of selenite and quantified as described previously.[9] The phage was resuspended in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5 and 150 mM sodium chloride (NaCl)) with 50% glycerol and stored at −20 °C under anaerobic conditions (95% N_2, 5% H_2, and O_2 < 1 ppm) to prevent oxidation of selenocysteine to unreactive species.

Tethering of all small molecules was performed in either small (40 μL) or large (250 μL) reaction volumes in glycine buffered saline (GBS), pH 2.5. Small reactions used a phage concentration of 0.3 pM, and the large reactions, 30 nM, with small molecules at final concentrations for the desired ratio. The reactions were performed for 1 h at room temperature in the dark, with the large reactions performed using a stir bar for constant mixing. For molecules without a biotin incorporated (XACI, BW1433I, and NOAMI), a second reaction step where Bt was added at a final concentration of 50 μM for 30 min was performed to react with any unmodified phage. All reactions were subsequently quenched using 40 mM DTT (Thermo Sci). Overnight dialysis was performed in TBS to remove the excess untethered small molecules before use.

Immunoblotting for Detection of s12d Modification and Cho Cell Activity. Phage was denatured via heating in SDS sample buffer containing 3× loading dye and DTT (NEB). Equivalent amounts of protein were resolved on 20% Tris glycine gels (Novex) and transferred onto poly(vinylidene difluoride) (PVDF) membranes (Millipore) for immunoblot analysis.

All CHO cell blots used cells grown to ∼90% confluence in 6-well plates, treated with the indicated testing conditions, and lysed in 150 μL of lysis buffer (PBS plus 1% Triton X-100, 1× protease/phosphatase inhibitor (CST)). Loading dye (3×) plus DTT (NEB) was added before heat denaturation, and samples were run on 4−15% TGX gels (Bio-Rad). Proteins were transferred to PVDF membranes and incubated with the appropriate antibodies. Quantitative western blots were performed using fluorescent secondary antibodies (800 and 680 nm, Li-Cor) and a Li-Cor Odyssey imager. Quantification was performed using the manufacturer's software.

Enzyme Linked Immunosorbant Assays (ELISAs). hA_1-CHO cells were grown to ∼90% confluence in 96-well plates. Cells were washed with phosphate buffered saline plus calcium and magnesium (PBS+) containing 1% BSA and 0.1% Tween-20 three times before the addition of phage in 20 μL PBS+. After 1 h incubation at 4 °C (to minimize phage internalization), unbound phage was removed, and the cells were washed three times. The cells were then fixed in 4% paraformaldehyde for 10 min. The cells were washed three more times before incubation with anti-M13-HRP (1:3000 dilution in PBS + 2% BSA, GE Healthcare) for 1 h. The cells were then washed five times, and Ultra-TMB (Thermo Sci) was added for 10 min before the absorbance was measured at 652 nm.

Kinetic assays were performed in a 96-well plate using the reverse order of phage addition, that is, all phage incubations were designed to finish at the same time with their initial addition point being variable. Three wells were washed per condition, and the phage, 0.5 nM, was added for the longest time point. After a certain period of incubation at 4 °C, the next set of wells was washed, the second longest time point phage was added, and the same procedure was repeated until the shortest time point phage was added followed by the appropriate incubation time. The plate was then processed as detailed above. All curve fits were performed using GraphPad Prism software.

Competition Binding Assays. hA_1-CHO cells grown in 96-well plates were washed with PBS+ three times before adding varying concentrations of NOAMI-Bt in 20 μL of PBS. After 1 h incubation at 4 °C, unbound NOAMI-Bt was removed, and the cells were washed again three times in PBS+. Twenty microliters of phage was then added at a concentration of 0.5 nM and incubated with cells for 1 h at 4 °C. The cells were then fixed and processed the same as that described for the ELISAs.

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For the radiolabeled competition assays, hA_1-CHO cells were washed with PBS and harvested in buffer A (10 mM HEPES, 20 mM EDTA, pH 7.4), supplemented with protease inhibitors (20 pg/mL benzamidine, 100 PM phenylmethylsulfonyl fluoride, and 2 pg/mL of each aprotinin, pepstatin, and leupeptin). The cells were homogenized in a Polytron (Brinkmann) for 20 s and centrifuged at 30 000*g*, and the pellet was washed twice with buffer HE (10 mM HEPES, 1 mM EDTA, pH 7.4, containing protease inhibitors). The pellet was resuspended in buffer HE, supplemented with 10% sucrose, and frozen in aliquots at −80 °C.

Binding assays for human A_1 adenosine receptors were performed with the agonist [^{125}I]-aminobenzyladenosine (^{125}I-ABA). Experiments were performed in triplicate using 96-well GF/C filter plates (Millipore). To each well were added 10 μg of hA_1-CHO membrane protein, 0.5 U/mL adenosine deaminase, Hepes buffer + 5 mM MgCl, and various concentrations of phage or competing ligands followed by 0.1−0.2 nM of carrier free ^{125}I-ABA, resulting in a total volume of 100 μL. After a 1 h incubation at room temperature, the liquid in the wells was drawn through the filters under vacuum, and the filters were washed by vacuum filtration three times with 200 μL of ice-cold buffer. The filters were removed with a filter punch, and the ^{125}I-ABA bound to receptors in the membranes was retained on the filters and counted in a gamma counter. Nonspecific binding was measured in the presence of 10 μM *N*-ethylcarboxamidoadenosine.

AKT Activation Assays. hA_1-CHO cells were grown in 96-well plates until they were ∼90% confluent. The media was removed, the cells were washed once with PBS+, and then fresh medium with varying concentrations of small molecule or phage was added. After a 15 min incubation at 37 °C, the medium was removed, and the cells were washed three times with PBS+. Cells were lysed in 150 μL of lysis buffer, and immunoblotting was performed as indicated above.

Materials. N^6-Octylamineadenosine (NOAM) and N^6-aminobenzyladenosine (ABA) were gifts from Dr. Susan M. Daluge of GlaxoSmithKline PLC. ABA was radio-iodinated and purified as described previously.[16] The K_i of NAOM for binding to the human A_1 receptor was determined by competition for radioligand binding as described previously.[10,16]

■ ASSOCIATED CONTENT

Supporting Information

Small molecule tethering efficiency and alternative plot fits for Figure 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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