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An Assay Based on SAMDI Mass Spectrometry for Profiling Protein Interaction Domains

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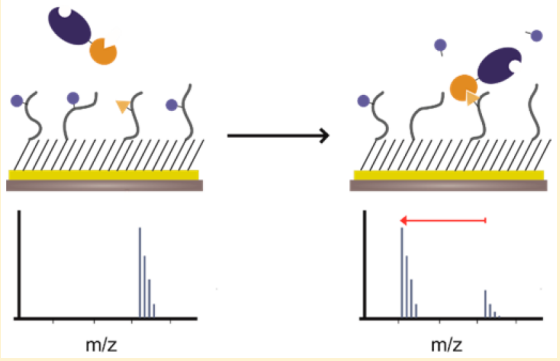
Supporting Information

ABSTRACT: This paper describes an assay that can profile the binding of a protein to ligands and can rank the affinities of a library of ligands. The method is based on the enhanced rate of an enzyme-mediated reaction that follows from colocalization of the enzyme and substrate by a protein−ligand interaction. This assay uses a self-assembled monolayer that presents a candidate peptide ligand for a receptor and a peptide substrate for an enzyme. The receptor is prepared as a fusion to the relevant enzyme so that binding of the receptor to the immobilized ligand brings the enzyme to the surface, where it can more rapidly modify its substrate. The extent of conversion of the substrate to product is therefore a measure of the average time the ligand−receptor complex is present and is quantified using the SAMDI mass spectrometry technique. The approach is used to profile the binding of chromodomain proteins to methylated lysine peptides derived from the histone 3 protein. The relative affinities for the peptide ligands found in this work agreed with results from prior studies. Additionally, this work revealed cross-talk interactions whereby phosphorylation of certain residues impaired binding of chromodomains to the peptide ligands. The method presented here, which we term protein interaction by SAMDI (PI-SAMDI), has the advantages that it is applicable to low-affinity interactions because the complexes are not observed directly, but rather leave a "covalent record" of the interaction that is measured with mass spectrometry and because it is compatible with laboratory automation for high-throughput analysis.



INTRODUCTION

Adaptor domains are protein modules that bind to peptide motifs having post-translationally modified residues.[1] When fused to enzyme domains, adaptor domains can localize the enzyme to specific locations within multiprotein complexes in the cell. When present in proteins lacking catalytic domains, they can serve scaffolding purposes. They can direct enzyme activity, through coevolution of protein interaction domains alongside related enzyme domains, and generate complex reaction networks.[2,3] Adaptor domains are very relevant in the epigenetic regulation of gene expression, where they bind to modified forms of histone proteins and are involved in chromatin maintenance and regulation. The chromodomains are a class of adaptor domains that bind to peptides with methylated lysine residues. Understanding the specificities of the various family members for discrete sites on histones has been important in elucidating the histone code as well as uncovering new druggable targets.[4,5] However, assays that can identify the preferred binding sites for the proteins are challenging, and our understanding of the specificities of the chromodomains and other adaptor domains is still incomplete. In this paper, we report a protein−ligand binding assay based on the self-assembled monolayers for MALDI-TOF (SAMDI) mass spectrometry technique, and we illustrate its use to profile the binding of five chromodomain proteins to the methylated forms of histone 3.

The chromodomains in this study are from the Cbx protein family. In humans, there are eight Cbx proteins that include a unique chromodomain as part of a larger, multidomain, protein. Of these, three are homologous to the *Drosophila* heterochromatin protein 1 (HP1) protein and five to the *Drosophila* polycomb (Pc) protein.[6,7] These chromodomains are known to bind to peptide motifs containing a trimethylated lysine residue on the histone 3 tail, with HP1 homologues binding H3K9Me3 (that is, a histone trimethylated at the lysine residue in position 9) and the Pc protein homologues favoring binding at H3K27Me3.[8,9]

Assays that measure protein−protein interactions are challenging and time-consuming, particularly in comparison to assays that measure enzyme activities. For example, isothermal calorimetry can be used for precise measurements of binding interactions but requires large quantities of protein and has a low throughput, making it unsuited for profiling large numbers of interactions.[9−12] STD NMR can also be used to measure ligand−receptor interactions in solution quantitatively and without labeling, though with a limited throughput.[13]

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Evanescent wave fluorescence biosensors can be used to detect ligand interactions as well, but require the use of fluorescent labeling strategies.[14] Fluorescence techniques based on energy transfer (FRET) and polarization offer higher throughput but require the synthesis of non-native, fluorescently labeled ligands and can be challenging to adapt to new protein−ligand interactions. Solid phase binding assays—including the use of peptide arrays based on the SPOT technology—are often used to profile binding to hundreds of possible peptide ligands but are not effective for low-affinity interactions and are often not quantitative.[15,16]

Our group has developed an assay platform that uses self-assembled monolayers of alkanethiolates on gold.[17] The monolayers are used to immobilize a peptide that is subsequently treated with an enzyme that can modify the peptide. The monolayers present with a background of tri(ethylene glycol) groups to prevent the nonspecific adsorption of protein to the surface and, therefore, enable quantitative and reproducible assays.[18,19] Most significantly, the monolayers can be characterized with matrix-assisted laser desorption-ionization mass spectrometry—in a technique known as SAMDI MS—which provides the masses of the substituted alkanethiolates and therefore the mass change in the immobilized peptide that results from enzyme activity.[20−23] The method is compatible with standard array formats and liquid handling robotics, allowing a throughput in the tens of thousands of reactions per day.[24] Importantly, the matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) analysis provides a fast and quantitative readout without the need for labels. In this paper, we describe the adaptation of this technique to an assay—which we term protein interaction by SAMDI assay (PI-SAMDI)—that can profile the binding of adaptor domains to arrays of candidate peptide ligands.

DESIGN RATIONALE

Our group has pioneered the use of SAMDI for measuring enzyme activities, where mass changes corresponding to the conversion of substrates to products are directly identified to quantitate enzyme activity. However, the noncovalent binding of a protein to an immobilized ligand does not cause a change in mass in the alkanethiolate and therefore cannot be directly measured by SAMDI. Bound proteins can be observed in the SAMDI MS spectra,[25,26] but many biologically relevant interactions are of low affinity and are not stable during the rinsing of the arrays prior to analysis by mass spectrometry (a limitation common to many affinity-based isolation methods). In order to profile these interactions where the enzyme functions as a reporter of the protein−ligand interaction (Figure 1), we have designed an assay that couples an enzyme activity with the protein−ligand interaction.

In this scheme, we prepare the adaptor domain to be assayed as a fusion to an enzyme domain, and we also prepare monolayers to which two peptides have been immobilized; one is a prospective ligand for the adaptor domain and the other is a substrate for the reporter enzyme. In previous work, we showed that enzymes act on their substrates as much as 20 times more rapidly when they are localized to the surface through a protein−ligand interaction.[27,28] In this method, it is important to select a poor substrate, ensuring minimal modification of the substrate by the soluble enzyme but efficient modification of the substrate when the enzyme is brought to the surface by way of a protein−ligand interaction. The fraction of time that the protein−ligand interaction exists can then be determined by using SAMDI to measure the extent of substrate conversion; this method is advantageous in that it couples a low-affinity interaction that localizes an enzyme at the interface to a permanent, covalent modification.

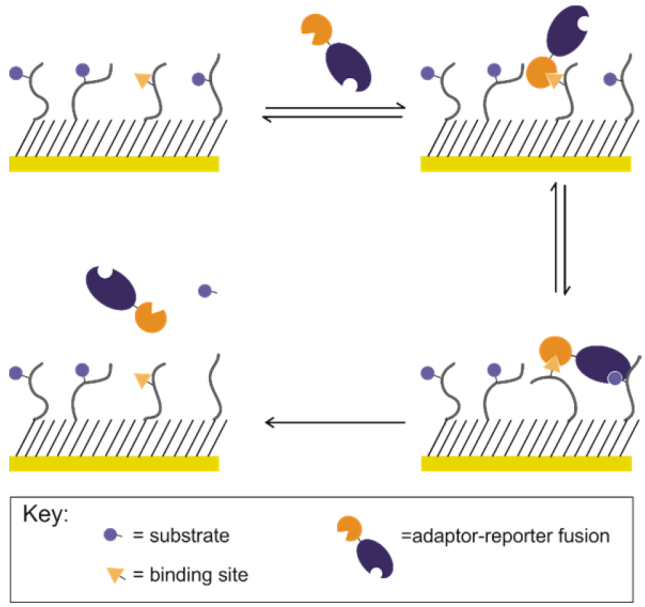

Figure 1. Binding of a fusion protein to an immobilized ligand recruits a reporter enzyme to the surface, where it can efficiently modify an immobilized substrate. This product represents a covalent record of the protein−ligand interaction and can be quantified by SAMDI mass spectrometry.

EXPERIMENTAL SECTION

Preparation of Monolayer Arrays. Array plates with 384 gold spots on steel plates were soaked in a solution of disulfide molecules for 24 h to allow formation of a self-assembled monolayer on the gold surface. The solution consisted of a mixture of EG3-alkane disulfide and a mixed disulfide of EG3-alkanethiol and a maleimide-terminated EG3-alkanethiol.[29] The solution of disulfides contains an overall concentration of 1 mM of the two monolayer compounds in an appropriate stoichiometric ratio to yield a 10% maleimide surface density.

Substrate and Ligand Synthesis. The peptide substrate for KDAC8 and the peptide ligands for the chromodomains were synthesized using standard FMOC solid phase peptide synthesis on Rink-amide resin. All peptides used were synthesized with a C-terminal cysteine for immobilization to the maleimide-terminated monolayer. For the experiments presented here, the substrate peptide has the sequence GMK(Ac)FGC. Additionally, the N-termini of all peptides were acetylated to improve stability and ionization efficiency. The peptides used as ligands are methylated versions of the sequences surrounding the H3K9 and H3K27 positions. These ligand peptides were synthesized by standard solid phase synthesis and are 10 residues long, containing the lysine of interest centered in the peptide. All peptides were purified by reverse phase HPLC after synthesis.

Surface Preparation. Peptide immobilization reactions were carried out by premixing the two peptides in 1:4 stoichiometric ratio of methylated ligand to KDAC8 substrate and a total peptide concentration of 100 μM. For each spot, 3 μL of peptide was incubated on the surface presenting 10% maleimide in Tris buffer at pH 7.5 for 1 h at 37 °C. With similar immobilization rates to the free maleimide, this yields a surface of 2% methylated ligand and 8% substrate peptide for the deacetylase.

Protein Design. Artificial fusions of KDAC8 to chromodomains Cbx1, Cbx3, Cbx4, Cbx5, and Cbx6 were constructed in the

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commercial PET-303b bacterial vector. Each construct was made by first using PCR to amplify the KDAC8 gene while introducing a polyserine linker and restriction sites for insertion of the chromodomains on the N-terminal side. This was then inserted into the commercial PET-303b vector by restriction site digest and ligation using a standard T7-ligase protocol. PCR was then used to amplify each chromodomain while introducing the appropriate restriction sites to allow for ligation into the KDAC8-containing plasmid.

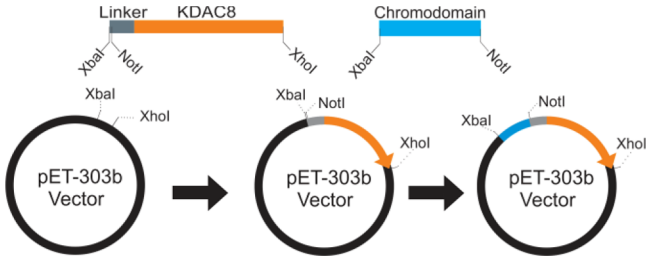

Figure 2. Fusion proteins of chromodomains (CDs) to KDAC8 were designed by sequential digestion and ligation of the KDAC8 catalytic domain and the CDs into a pET-303b vector.

Protein Expression. For expression, each vector was transformed into the BL21 (DE3) strain of *E. coli* by electroporation. Cultures in 2XTY media containing carbenicillin (1 L) were grown to an optical density of 0.6. Expression of the fusion proteins was then induced with isopropyl β-D-1-thiogalactopyranoside (1 mM) and incubated for 12–16 h at 18 °C. The cultures were then pelleted by centrifugation and lysed by sonication in buffer A (50 mM Tris, 200 mM NaCl, 5% glycerol, 5 mM BME at pH 7.5), and the overexpressed fusion proteins, which contain a C-terminal HIS-tag, were purified on a cobalt resin column. The proteins were then further purified by fast protein liquid chromatography (FPLC) using a size exclusion column and then stored at −80 °C until use in a buffer containing 80 mM Tris, 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 10% glycerol at pH 7.5.

Assays. All assays were performed on 384-spot array surfaces, in a standard 16 by 24 formatting, with each spot having a diameter of 2.5 mm. For the data presented here five adaptor domains were tested against 24 different ligand peptides, with a minimum of four replicates each. Combined, nearly 500 individual assays were performed and spectra collected in the experiments presented. When running assays, the fusion constructs were diluted to a concentration of 0.5 μM, as determined via Nanodrop, in running buffer (80 mM Tris, 150 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$ at pH 7.5). The enzyme was plated onto array plates presenting substrate and ligand peptides at 3 μL per spot, and the reactions were incubated at 37 °C for 10 to 15 min. At the end of a reaction, the surfaces were rinsed with water and ethanol, then dried. A matrix of 2,4,6-trihydroxyacetophenone in acetone was applied directly to the surface, and after drying, each spot was analyzed by MALDI-TOF mass spectrometry using an AB Sciex 4800 or 5800 series instrument. The extent of enzymatic conversion of the substrate was then determined from the mass spectra for each reaction.

RESULTS

Reporter Enzyme. The assay can employ a wide variety of enzymes as the reporter; here, we used the KDAC8 deacetylase. Our earlier work has demonstrated assays of KDAC8 by SAMDI and used peptide arrays to measure relative activity on hundreds of peptide substrates.[22] From that work, we selected the peptide GMK(Ac)FGC because it was a relatively poor substrate that showed little activity with soluble enzyme.

Chromodomain Fusions. We generated fusion proteins wherein each of five chromodomains (CDs) was linked to the KDAC8 catalytic domain (the CD-KDAC fusions). These five constructs represent the three HP1 homologues Cbx1, Cbx3, and Cbx5 and the two Pc homologues Cbx4 and Cbx6. We constructed these fusions in the commercial pET-303 CT-His tag vector. We first used PCR to amplify the KDAC8 catalytic site while inserting a coding region for a linker sequence in front of the catalytic sites and restriction sites that were used to digest and ligate the sequence into the vector. Through similar amplification, digestion, and ligation the chromodomains were then introduced in front of the KDAC8 catalytic domain (Figure 2). We expressed the constructs in the BL21 (DH5α) *E. coli* strain using a standard IPTG induction method and then purified the expressed protein by cobalt column affinity chromatography followed by FPLC size exclusion chromatography. We verified that the affinities of the chromodomains for their ligands are not altered by the presence of the KDAC8 fusion. We used isothermal calorimetry to measure association constants for Cbx3 with $H3K9Me_3$ and $H3K9Me_2$ and found equilibrium constants that agreed with those in the literature (see Supporting Information).

Self-Assembled Monolayers. We prepared self-assembled monolayers presenting maleimide-terminated alkanethiolates at a density of 10% against a background of tri(ethylene glycol)-terminated alkanethiolates. The latter are very effective at preventing nonspecific adsorption of protein. The maleimide group is used to immobilize cysteine-containing peptides by way of a Michael addition.[30] In previous work with a cutinase-SH2 fusion, we found that the benefit of tethering the enzyme to the interface through a protein–ligand interaction leveled off at a ligand density of approximately 1% to 1.5%, and higher densities gave only a minor increase.[28] Therefore, we immobilized two peptides—the GMK(Ac)FGC substrate for KDAC and a second peptide that is a potential ligand for the chromodomain—in a ratio of 4 to 1. This monolayer presents the binding ligand at a density of approximately 2% and the enzyme substrate at a density of 8%, ensuring maximal rate enhancement for the tethered enzyme. We note, however, that it is straightforward to evaluate and use other densities.

We performed all reactions on plates patterned with 384 gold islands arranged in the geometry of a standard microtiter plate as described previously.[22−24] These array plates are compatible with liquid-handling automation and require only 3 μL of reaction mixture for each spot. To initiate reactions, we used a Mutlidrop Combi benchtop robot to deliver a solution of the CD-KDAC in a Tris buffer to each spot and allowed the reactions to proceed for up to 30 min at 37 °C. The array plates were rinsed, treated with matrix (THAP in acetone), and analyzed by MALDI-TOF mass spectrometry to quantitate the amounts of substrate and product. We determined the extent of conversion using the integrated areas under the peaks (AUP) for the substrate and product.

$$\text{Conversion} = \frac{\text{AUP}_{\text{product}}}{\text{AUP}_{\text{product}} + \text{AUP}_{\text{Substrate}}} \quad (1)$$

We first performed the assay on monolayer arrays presenting peptides that represent the well-characterized ligands for the chromodomain proteins. These four peptides include either di- or trimethylation at residue K9 or K27 (Figure 4). In each case control spots are included that present the KDAC peptide substrate but that omit a second peptide ligand. In this way, we can measure the extent of enzyme activity that is due only to the action of a soluble (unbound) enzyme. We present the data as "fold enhancement", which corresponds to the average percent yield of the reaction in the presence of a given ligand

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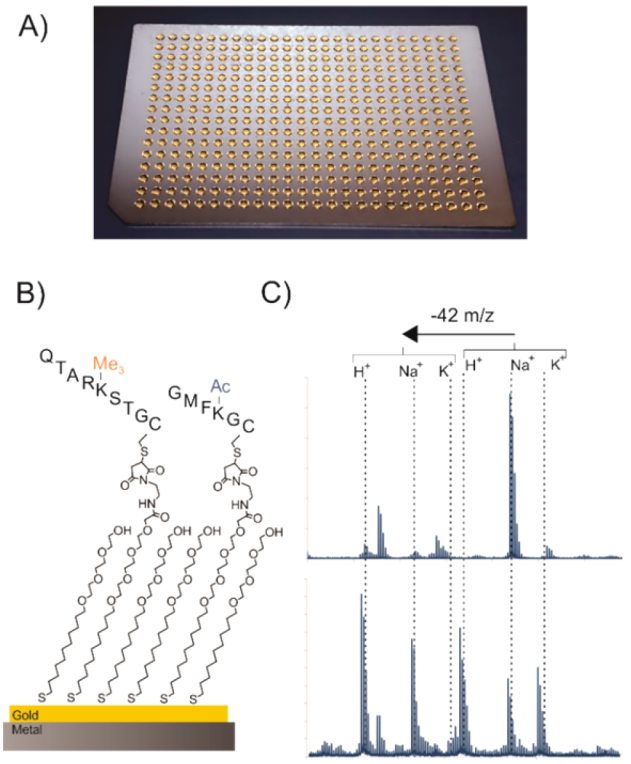

Figure 3. (A) An array of monolayers in the standard 384 spot format is treated with reactants pipetted directly onto each spot. (B) Cysteine-terminated peptides are immobilized on a maleimide-presenting monolayer. (C) MALDI-TOF mass spectrometry allows for the deacetylation reaction to be assayed; proton, sodium, and potassium ion adducts for substrate and product are observed and quantitated.

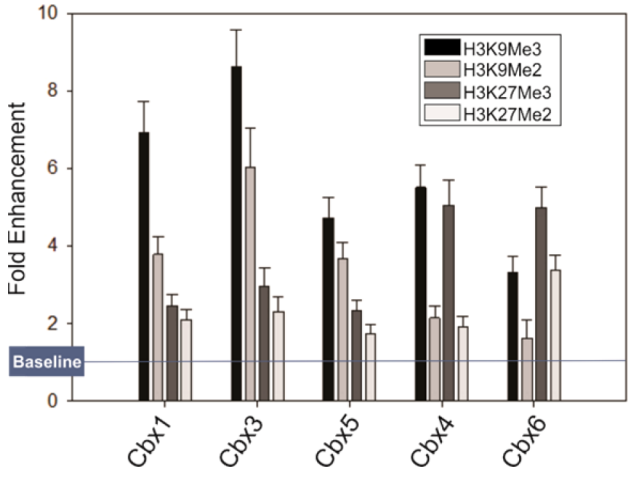

Figure 4. Increase in yield for the deacetylation of the immobilized peptide by each of the chromodomain fusions was measured for each of the four canonical binding sequences. The baseline represents the level of activity with no adaptor domain mediated enhancement. Error bars represent the standard deviation of four replicates.

peptide, divided by the average percent yield of the enzyme reaction in the absence of a ligand, i.e., the enhancement due to ligand binding (eq 2). We performed at least four replicates of each experiment on the array plate.

$$\text{Fold Enhancement} = \frac{\text{Yield with ligand}}{\text{Yield without ligand}} \quad (2)$$

For the HP1 chromodomains (Cbx1, Cbx3, Cbx5) we observe greater rate enhancement for di- and trimethylation at the H3K9 site, with the latter giving approximately 8-fold enhancements. These observations are consistent with previous reports, where it has been shown the HP1 chromodomains have the greatest affinity for methylated H3K9 and a preference for higher methylation states. For the Pc chromodomains (Cbx4 and Cbx6), a preference for higher methylation is also observed. But while Cbx6 prefers the canonical H3K27 ligand, Cbx4 has a slight preference for the H3K9 methylation site. This lack of selectivity for H3K27 has also been previously reported, where it appears that the mammalian Pc homologues do not necessarily maintain the specificity of their *Drosophila* counterparts and certain isoforms lack specificity.[31]

These results validate the ability of the PI-SAMDI assay to measure previously known protein−ligand interactions and to rank the interactions by affinity. Prior work has found no evidence that methylation at the other lysine residues in the amino-terminal tail of histone 3 gives sites that are ligands for the chromodomains. To explore the activities of these other sites, we next performed the PI-SAMDI assay on arrays that include peptides that are trimethylated on each of the 13 lysine residues derived from the histone 3 and 4 tails (Figure 5).

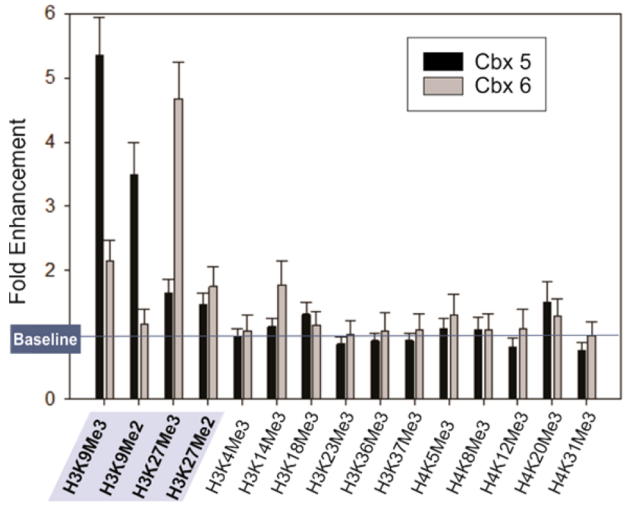

Figure 5. Binding of the HP1 chromodomain Cbx5 and the Pc chromodomain Cbx6 was evaluated using an array presenting peptides that correspond to trimethylation of each lysine residue in the amino-terminal tails of histones 3 and 4. Little to no enhancement is observed for the noncanonical sites (i.e., those other than H3K9 and H3K27).

These peptides represent methylation of residues 4, 14, 18, 23, 36, and 37 of histone 3 and residues 5, 8, 12, 16 of histone 4. We used the PI-SAMDI assay to measure binding of Cbx5 (from the HP1 family) and Cbx6 (from the Pc family) chromodomains to these peptides. As expected for these non-native positions, there is little to no observed increase in KDAC activity and, therefore, little binding of the chromodomains to these other sites.

We next used the PI-SAMDI assay to identify whether binding of chromodomains to the H3K9/K27 sites was influenced by a second post-translational modification in the peptide ligands, that is, whether there existed a "cross-talk" of distinct modifications to the peptide (Figure 6).[32] It is common for histone tails to possess multiple modifications *in vivo*, and the residues surrounding the H3K9 and H3K27 sites are also

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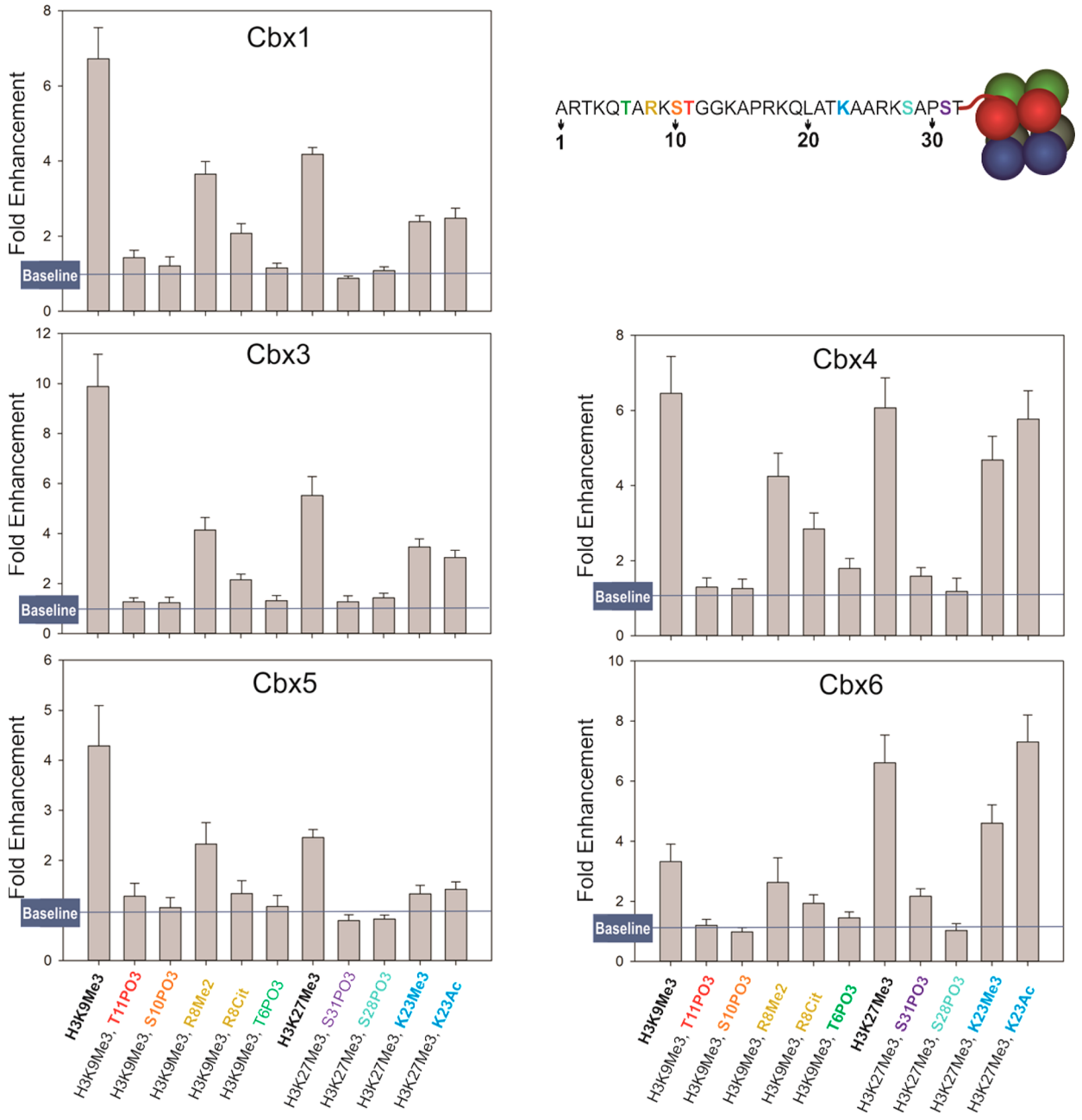

Figure 6. Affinity of each chromodomain for the H3K9Me3 site was evaluated for peptides that additionally had a second post-translational modification. The HP1 isoforms Cbx1, Cbx3, and Cbx5 are shown in the left panel, and the Pc isoforms Cbx4 and Cbx6 are shown in the right panel. The error bars represent the standard deviation of four replicates. The secondary modification on each peptide is color-coded to match the sequence shown in the upper right.

frequently post-translationally modified. We synthesized nine peptides that were trimethylated at either H3K9 or H3K27 and that further had a secondary site of phosphorylation, acetylation, citrullination, or methylation; these modifications have all been reported to occur in chromosomal histones.[33−39]

We find that some of these secondary modifications can strongly influence the affinity of the chromodomain. Specifically, nearby phosphorylation acts to dramatically decrease affinity and, in some cases, causes complete loss of binding all together. For the H3K9 methylation site, nearby phosphorylation at T6, S10, or T11 dramatically reduces the affinity of the chromodomain. Citrullination at R8 strongly reduces affinity, as does methylation at R8, but to a lesser extent.

At the H3K27 methylation site, we again see strong reduction in affinity when nearby S28 or S31 residues are phosphorylated. Additionally, there is some sensitivity to methylation at K23, whereas acetylation at K23 has no observable effect on affinity of the PC chromodomains.

■ DISCUSSION

We describe a new label-free assay for measuring protein−ligand interactions. The assay relies on a coupling of the protein−ligand binding event to an enzyme-mediated modification of a peptide or small molecule on the monolayer. In this way, the enzyme can modify nearby substrates when the protein interaction domain binds to its ligand, leaving a

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"covalent record" of the transient complex. The yield of the enzymatic reaction increases with the fraction of time the complex is present, allowing this method to rank affinities of a receptor for several prospective ligands. Because this assay does not use affinity methods to isolate the complex, it is useful for measuring low-affinity interactions. Second, because it uses standard microtiter plate formats and automation, it can be performed in high throughput with small sample volumes.[24] In this way, the PI-SAMDI assay represents an important complement to the use of fluorescence-based assays, which also have substantially higher throughput than assays based on isothermal calorimetry and surface plasmon resonance. However, it can be challenging to optimize the attachment of fluorophores to proteins, and the fluorophores can often lead to artifacts due to non-native interactions with the binding partner.[40−42] We recognize the PI-SAMDI assay requires that the binding protein be modified with an enzyme fusion and that it is important to confirm that the enzyme domain does not interfere with binding.

The use of self-assembled monolayers was critical in enabling the assay reported here. The oligo(ethylene glycol)-terminated monolayers are highly effective at preventing the nonspecific adsorption of protein, which would block the ligand and enzyme substrate from interactions with the fusion protein. The monolayers also offer excellent control over density of the two peptides, which allows optimization of the sensitivity of the assay. The use of mass spectrometry to analyze the monolayers provides a sensitive detection method that benefits from the high-throughput implementation of SAMDI, which can measure tens of thousands of spots per day.[24,43] The assay also allows the flexibility to use a broad range of reporter enzymes in the assay.

We used chromodomains in our work because this family of proteins plays an important role in epigenetic control of gene expression and because the adaptor domains have recently emerged as a new target class for drug development.[4,5,44,45] Prior work has established that the human homologues of the *Drosophila* HP1 protein (here Cbx1, Cbx3, and Cbx5) share a specificity and strongly prefer binding the H3K9 position and higher methylation states; our data using the PI-SAMDI assay are in agreement with these trends. The specific affinities of these proteins have been examined with various methods, with the *Drosophilia* HP1 chromodomain generally reported to have a K_d in the range of 1−15 μM for the H3K9 trimethylated site. While mammalian HP1 homologues have reported K_d values as high as 30 μM.[10,15,46]

The human homologues of the *Drosophila* Pc proteins (here Cbx4 and Cbx6), in contrast, are promiscuous and bind both H3K9 and H3K27, unlike the *Drosophila* Pc protein, which has selectivity for H3K9. For the *Drosophila* Pc homologues, we find that Cbx4 has a modest preference for H3K9, which has also been previously observed by fluorescence polarization. Where dissociation constants have been measured for mammalian Cbx6, the protein prefers the H3K27 position but with a weaker affinity as compared to other Pc homologues with a K_d well over 100 μM.[15,31] The binding was measurable by our PI-SAMDI assay, and we find that Cbx6 binds to both sites with a modest preference for H3K27 over H3K9. Generally, though, the trends that we observed in relative preferences agree with the picture developed from the various biochemical and biological experiments available in the literature on this family of proteins.

The compatibility of PI-SAMDI with peptide arrays makes it straightforward to evaluate binding interactions to libraries of prospective ligands. In this work, we prepared arrays wherein the peptides had a trimethylated lysine either at H3K9 or H3K27 and further had one additional post-translational modification, with the goal of surveying potential "cross-talk" interactions in chromodomain binding. We found that all five chromodomains had no measurable affinity for H3K9 in peptides that were phosphorylated at serine 10. Previous work by Peters and co-workers reported *in vivo* data that showed that inhibition of S10 phosphorylation prevented dissociation of HP1 from nucleosomes during mitosis.[47,48] They also used a radioactive labeling experiment to demonstrate a direct interaction of recombinant Cbx5 with H3-derived peptide that was inhibited with the S10 phosphorylated form. Our work provides direct evidence of the phospho-dependent binding of all three HP1 human homologues to H3-derived peptides. We also observed a reduction in binding affinity when H3S28 is phosphorylated. Evidence of this relationship *in vivo* comes from experiments showing genes normally repressed by the Pc complex are expressed when H3S28 is phosphorylated, suggesting a phosphorylation-dependent removal of the Pc complex.[49] More recently, biochemical experiments have suggested that this regulation by nearby phosphorylation may be a widespread phenomenon for methyl-lysine reader domains, with *in situ* experiments showing that the affinity of several nonchromodomain methyl-lysine readers for their corresponding binding site on the H3 tail are also reduced in the presence of nearby phosphorylation.[50] Many of the biological studies are limited by the need to develop post-translational-modification-specific antibodies and often do not resolve the binding interactions of individual homologues. The PI-SAMDI assay provides a method to directly profile the binding of recombinant homologues to a large number of prospective ligands, both naturally occurring and non-native.

We describe the PI-SAMDI assay as appropriate for profiling or screening large numbers of protein−ligand combinations and, from the results presented here, capable of rank-ordering affinities of ligands that span 3 orders of magnitude. We emphasize, however, that the assay does not provide equilibrium binding constants for the interactions. Translating the enhancement factors to equilibrium constants is challenging, in part because analytical descriptions of enzyme kinetics at interfaces are challenging[51,52] and in part because of non-linearities that are intrinsic to the PI-SAMDI assay. For example, when the fusion proteins are bound to the monolayer at low density, each of the tethered KDAC8 domains can sweep a defined area of the monolayer and convert the substrates to products. But as the density of the bound fusion protein increases, there will be an overlap of the regions that neighboring enzymes can access, leading to a decrease in the enhancement factor. This complexity makes it difficult to develop an analytical expression for the binding affinity. In part for these reasons, we suggest the PI-SAMDI assay is most suited to profiling large numbers of prospective interactions, identifying interactions of interest, and providing a rank-order of affinity for these interactions. It is also promising as an assay for screening large libraries of small molecules to identify inhibitors or promotors of interactions. Indeed, the SAMDI method has proven to be well-suited to high-throughput screening with very good Z-factors in the 0.7−0.85 range.[43]

We believe this assay makes an important contribution to methods that profile protein interaction domains. Our strategy

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couples binding to an interfacial enzyme reaction that gives a "covalent record" of transient binding interactions, allowing it to be applied to rapidly assess low-affinity interactions that would otherwise be difficult to see by affinity-based methods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b03805.

Additional data (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors note that they have filed a provisional patent application pertaining to this work.

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