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A high resolution interaction map of three transcriptional activation domains with an essential co-activator from photocross-linking and multiplexed mass spectrometry**

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Transcriptional activators play a central role in gene regulation, stimulating the assembly of the transcriptional machinery at a promoter in a signal responsive manner.[1] Because of the significant role that mis-regulated or malfunctioning transcriptional activators play in human disease, there has been a strong effort towards identifying molecules that inhibit the ability of an activator to function via binding interactions with the transcriptional machinery.[2–4] One difficulty associated with this strategy is that distinct transcriptional activators often share binding partners within the transcriptional machinery, making the design or discovery of molecules specific for a particular transcription factor quite challenging.[5] For example, the activators p53, c-Myc and the viral oncoprotein E1A all interact with the coactivator and chromatin modifier TRRAP, yet it is not known if these activators utilize a shared binding surface for activation or if there are several distinct binding sites within the coactivator.[6–8] Covalent cross-linking has been successfully used to identify coactivator binding partners of activators, but high resolution analysis for binding site identification has not been reported; [9–15] a real challenge to high resolution analysis is the acid-rich composition of many eukaryotic activators, a characteristic that renders them difficult to analyze by standard cross-linking and mass spectrometric strategies.[16–20] Here we describe a multiplexed mass spectrometric strategy to produce for the first time a high-resolution map of the binding sites of transcriptional activators, focusing on three prototypical activators (Gal4, Gcn4, and VP16) in complex with the key coactivator Med15(Gal11) (Figure 1). These data in combination with genetic experiments demonstrate that while Gal4 and Gcn4 target the same binding surfaces within Med15, the VP16-derived activator utilizes a distinct binding surface. This finding suggests that some degree of inhibitor specificity for a given activator or activator class may indeed be achievable. Further, this general approach can be applied to a broader range of activator-coactivator binding partners to expand the high-resolution interaction map of transcriptional activators.

The largest class of eukaryotic transcriptional activators is the acid-rich or amphipathic class of which Gal4, Gcn4 and VP16 are the most extensively studied members.[1] The descriptor ‘amphipathic’ refers to the primary amino acid sequence of their transcriptional activation domain or TAD, the domain responsible for contacting coactivators within the

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transcriptional machinery and thereby initiating transcription (Figure 2a). The coactivator Med15, a component of the Mediator complex, has been implicated by a variety of techniques to be a target of amphipathic TADs.[13,15,21–24] The amino terminal half of the protein appears particularly important for the function of Gal4, Gcn4 and VP16.[22–24] What is not known, however, is if these TADs utilize a shared binding surface or if each has a distinct binding site within the coactivator. Due to the similarity of Med15 to several metazoan coactivators and the ability of Gal4, Gcn4 and VP16 to activate transcription across species,[25,26] investigating TAD-Med15 interactions serves as an outstanding model for elucidating the mechanism of coactivator recruitment and developing a versatile approach to determine TAD-coactivator binding sites.

To localize the fragment of Med15 that interacts with activators, we designed a series of fragments that span its entire sequence and performed cross-linking reactions with isolated TADs from Gal4, Gcn4 and VP16(VP2) containing the photoactive cross-linker *p*-benzoyl-L-phenylalanine (pBpa).[27–29] In each TAD, the position chosen for pBpa incorporation was originally a phenylalanine and in each case had been shown to be substitutable with other hydrophobic amino acids without detriment to function or to affinity for coactivators.[30–32] For example, Phe869 in the Gal4 transcriptional activation domain can be replaced with cysteine without attenuation of transcription function.[32] The Med15 expression constructs for the cross-linking experiments were designed using a combination of two solubility tags to maximize yield and stability.[33] For each cross-linking reaction, a solution containing 10 μ M of activator and 1 μ M of a Med15 fragment was irradiated with 365 nm light for 5 min and the mixture was subsequently analyzed via Western blot. Consistent with previous studies highlighting the importance of the N-terminus and the middle of the protein for activator contacts, all of the TADs interacted with Med15(1–416) (Supporting Figure 1).[23,24] These findings suggested that at least one binding site was contained within this region. Additionally, fluorescence polarization binding experiments with this region revealed that the Gal4, Gcn4 and VP2 TADs interact with low micromolar apparent dissociation constants (Supporting Figure 2). Thus, Med15(1–416) was used for all subsequent mass spectrometry experiments.

The general strategy for analyzing the cross-linked complexes in order to identify the TAD binding sites is illustrated in Figure 1. Particularly important was the use of a redox-sensitive biotin tag incorporated into each TAD that facilitated enrichment under mass spectrometry-compatible conditions following tryptic digest of the mixture. Second, both MALDI and ESI (the latter in both positive and negative ion modes), which have orthogonal mechanisms for ionization,[34–38] were employed. The complementary use of two ionization methods proved crucial for obtaining the full suite of TAD binding sites. For MS/MS confirmation, high energy collision induced dissociation (CID) in a tandem time-of-flight (TOF/TOF) instrument was used for MALDI-generated ions and low-energy CID in a quadrupole-Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer was used for ESI-generated ions. These MS/MS approaches also yielded complementary information.

Multiplexed mass spectrometric analysis revealed binding sites of Gal4, Gcn4, and VP2 within a few amino acid residues in Med15 (Figure 2b). In some cases, cross-linking could be localized to a single residue (e.g., Gal4-Met²¹³, Supporting Figure 8).[39] Med15(74–78) and Med15(207–217) are detected for all three activators, suggesting that these binding sites are permissive, enabling contacts with activators of different sequence compositions. In the case of the VP16-derived TAD VP2, however, we detected three additional unique cross-linked fragments Med15(79–84), Med15(222–231) and Med15(345–348).

In MS/MS analysis, low-energy CID of ESI-generated cationic cross-linked peptides (when detected) generally yielded more structural information than MALDI TOF/TOF high-energy

CID. For example, ESI MS/MS of triply protonated Gal4-Med15(74–78) yielded 12 backbone fragment ions whereas MALDI TOF/TOF analysis of the same singly protonated species yielded only five (Supporting Figure 7). However, use of MALDI was essential for detecting VP2-Med15 cross-linked species. Notably, in MALDI TOF/TOF MS/MS spectra, abundant fragment peaks resulting from gas-phase cleavage of the cross-linker to yield the free tryptic peptide from Med15 were observed. Such symmetric cross-linker cleavage, which is highly useful for verifying the two cross-linked species, has not been reported in previous sector high-energy CID of pBpa cross-linked peptides[40] and was also absent in our low-energy CID experiments (for example: Supporting Figure 11). To our knowledge, these experiments represent the first application of combined ESI low-energy CID/MALDI high-energy CID for photocross-linked peptides. Alternatives to standard low-energy CID become more pressing at higher molecular weight (~5 kDa) due to the increased number of vibrational degrees of freedom into which the activation energy is dissipated.[41]

The binding sites identified using mass spectrometry were within or in close proximity to several activator binding motifs predicted to reside within Med15 (Figure 2c). Residues 62–90 comprise helix 3 of the Med15 GACKIX module, an evolutionarily conserved activator binding motif[25, 26] and one of the identified binding sites is within this region. Residues 143–156 and 196–204 are homologous to A and B boxes found in mammalian coactivators and these sequences have been shown to contain activator binding sites;[42] two binding sites were identified proximal to these motifs in the MS/MS analysis. To assess the functional relevance of the binding sites, reporter gene assays of the TADs fused to a DNA binding domain were performed in yeast strains bearing various Med15 mutants (Figure 3). Deletion of a larger portion of Med15 (residues 1–345) attenuated the activity of all three activators, suggesting that functionally important binding sites are present in this region. Removal of a smaller portion of Med15, residues 1–186, resulted in the reduction of activity of only VP2 and did not impact the function of either Gal4 or Gcn4. This region contains the Med15(74–78) site contacted by all three activators in vitro and the Med15(161–174) binding site targeted by VP2 and Gal4. To distinguish interactions with the helix 3 binding site (residues 74–78) and Med15(161–174), the activity of VP2 was assessed in yeast strains in which either residues 61–91 or 168–172 of Med15 had been removed. As shown in Figure 3, the Med15(74–78) binding site appears to be the functionally important one as its removal reduced VP2 activity by >25%.

Additional experiments in which residues 198–211 and 224–232 in Med15 were removed resulted in a significant reduction of activity for Gal4, Gcn4 and VP2, indicating the importance of this region for the function of all of the activators. Consistent with the cross-linking data, deletion of residues 345–348 in Med15 only resulted in the reduction of the activity of VP2. Taken together, these data suggest a model in which VP2 can utilize at least three binding sites within Med15 for function in vivo whereas Gal4 and Gcn4 utilize the Med15 B-box proximal binding site for function. Further, it suggests that the GACKIX domain is dispensable for Gal4 and Gcn4 function but is utilized by VP2.[43]

In conclusion, using photocross-linking coupled with multiplexed mass spectrometry we show that amphiphatic transcriptional activators of distinct sequence composition nonetheless interact in vitro with several of the same binding sites within the coactivator Med15. Complementary use of ESI and MALDI was essential to maximize detection of the full suite of Med15 binding sites. Further, the combination of low and high energy CID provided complementary structural information for verification of assigned cross-linked species. Importantly, the high resolution interaction map obtained from the in vitro work enabled targeted mutagenesis in vivo to discriminate functionally important binding interactions among the activators. The in vivo experiments revealed that the VP16-derived TAD has a distinct binding pattern relative to Gal4 and Gcn4. In particular, only the VP16-

derived TAD has a functionally important binding interaction with the GACKIX domain of the coactivator; this highlights the necessity of cellular validation of in vitro binding results, particularly in the case of moderate affinity interactions such as these. Together these data have important implications for future screens to identify therapeutically useful inhibitors of activator-coactivator interactions, revealing that specificity to attenuate the function of a particular activator or activator class can likely be achieved by targeting an appropriate binding site.

Supplementary Material

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Figure 1. Schematic for mapping transcriptional activation domain (TAD) binding sites within a key coactivator. Each TAD (red helix) contains a photoactivatable amino acid (green star) as well as biotin (blue sphere) linked by a disulfide bond. Following incubation with the coactivator Med15 and irradiation to initiate cross-linking, the mixture is trypsinized and the cross-linked species affinity purified (AP) using a neutravidin column. Elution is accomplished under reducing conditions. The resulting fragments are analyzed by high-resolution multiplexed mass spectrometry.

Figure 2.

Cross-linking with Med15 and the TADs of Gal4 and Gcn4 and a sequence, VP2, from VP16. a) TAD sequences. Starred Phe indicates the position replaced by p-benzoyl-L-phenylalanine. Underlined Cys indicates biotin attachment. b) Results from multiplexed mass spectrometric analysis of cross-linking products. See Supporting Information for relevant MS/MS spectra. c) Schematic of the amino terminus of Med15, with predicted TAD binding motifs indicated in light grey and in vitro cross-linked sites indicated in dark grey.

Figure 3.

In vivo analysis of TAD binding sites. Results from β -galactosidase assays performed in yeast strains bearing plasmids expressing either wild-type Med15 (Med15 WT) or truncated forms of Med15 in addition to plasmids expressing the TAD under examination fused to the minimal DNA binding domain of Gal4. Note that because each TAD functions through interaction with several coactivators, transcriptional activity is attenuated rather than abolished upon removal of a key binding interaction. Each activity is the mean of at least three experiments (error = SDOM).