DOI: 10.1002/cbic.200700062

Disrupting Mediator with a Short Peptide Ligand

Jenifer K. Lum,[a] Zhiqian Wu,[b] and Anna K. Mapp*[a, b]

Although RNA polymerase II is the core enzyme that carries out transcription, it requires a large number of accessory proteins and protein complexes to regulate its activity. One of the most important of these complexes is Mediator, a group of 20–25 protein components that is highly conserved from *Saccharomyces cerevisiae* through to humans.[1–6] The yeast Mediator is subdivided into three portions: a head region that contacts RNA polymerase II, a middle region, and a tail (Figure 1).[7]

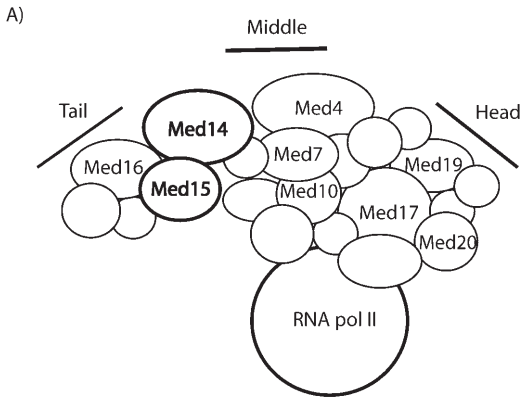

Figure 1. A) Schematic of the organization of the Mediator complex, with Med14(Rgr1) and Med15(Gal11) highlighted. B) Ligand 32 was isolated from a binding screen against Med15(186–619) and has significant similarity to a sequence within Med14(Rgr1); scr32 is the scrambled form of ligand 32.

While the functions of most of the Mediator subunits are unknown, a preponderance of evidence suggests that Mediator integrates information from DNA-bound transcriptional activators and repressors to RNA polymerase II, informing it as to the extent and level of up-regulation required.[8] It has been difficult to precisely assign the function and role of the Mediator complex and its subunits both because most are important for different subsets of genes and because all participate in multiple protein–protein interactions. Deleterious and/or pleiotropic effects are often observed upon either deletion or mutation of these proteins. Molecules that inhibit particular protein–protein interactions within this complex would thus be invaluable for parsing the role(s) of these proteins in transcriptional regulation. Here, we report the first inhibitor of a protein–protein interaction within the Mediator complex, an eight-residue peptide that prevents the association of Med15(Gal11) and Med14-(Rgr1) at low micromolar concentrations. This peptide thus serves as a key starting point for the development of a new class of artificial transcriptional regulators.

Although the locations of individual proteins within each Mediator subcomplex are not known with high precision, several lines of evidence suggest that Med14(Rgr1) (middle module) and Med15(Gal11) (tail module) reside at the interface between the tail and middle portions.[1,9] Genetic studies indicate that Med14(Rgr1) and Med15(Gal11) affect overlapping but distinct sets of genes.[10] Med14(Rgr1) (also known as ARC/DRIP150, TRAP170, or CRSP150 in humans) is an essential protein and has been identified in multiple genetic screens in *S. cerevisiae* as both a positive and negative regulator of transcription.[9,10] Mutations in Med14(Rgr1) diminish Ty, GAL, and MATα gene expression.[9] In addition, Med14(Rgr1) is required for the repression of the MAL structural genes and RME1-mediated repression of meiosis in haploid yeast cells under starvation conditions.[11,12] However, little is known about its specific function or interaction partners.

Med15(Gal11) is commonly identified as a target of transcriptional activators, interacting in vitro with more than 10 activators; studies in *S. cerevisiae* support the functional relevance of these interactions.[13–22] Although a direct homologue of Med15(Gal11) has not been identified in metazoans, the amino terminal domain with which many activators interact is proposed to be a KIX domain, found in a number of metazoan coactivators (CBP/p300 and ARC105, for example), although this domain can be removed without a significant impact on function.[1,23] Unlike Med14(Rgr1), Med15(Gal11) is not an essential protein, as deletion is not lethal but rather induces a slow-growth phenotype.[24] Although a direct interaction has not been demonstrated, Med14(Rgr1) appears necessary for the association of Med15(Gal11) with Mediator. Mediator complex that contains truncated versions of Med14(Rgr1) does not contain Med15(Gal11), for example.[9] These studies thus suggest that ligands that interact with either Med15(Gal11) or Med14-(Rgr1) could directly or indirectly inhibit the association of these proteins.

In an earlier study we carried out a screen against the middle portion of Med15(Gal11) (residues 186–619) by using two 8-residue peptide libraries of general composition Ala-$(Xaa)_4$-(Pro/Leu)-Ser-Glu and identified 37 ligands for this protein (see ref. [28] for a complete list). To identify ligands that might target binding surfaces that Med15(Gal11) uses for interactions with other Mediator proteins, we carried out a BLAST search against these short peptides to identify sequences that had overlap with potential binding partners of Med15(Gal11). For this purpose we used the 2.1.3 BLAST protocol specifically developed for short sequences and focused upon hits for which there was some genetic and/or biochemical evidence of an interaction.[25] Peptide 32 (Ala-Tyr-Phe-Glu-Val-Pro-Ser-Glu) exhibited significant ($E = 75$) overlap with residues 277–283

[a] *J. K. Lum, Prof. A. K. Mapp*
Department of Medicinal Chemistry, University of Michigan
930 N. University Avenue, Ann Arbor, MI 48109 (USA)
Fax: (+1) 734-615-8553
E-mail: lumj@umich.edu

[b] *Dr. Z. Wu, Prof. A. K. Mapp*
Department of Chemistry, University of Michigan
930 N. University Avenue, Ann Arbor, MI 48109 (USA)

(Tyr-Phe-Thr-Val-Pro-Asn-Glu) in Med14(Rgr1) (Figure 1B). These residues reside in the N-terminal portion of Rgr1, which is essential for viability and has the highest sequence similarity to homologues in higher eukaryotes.[1] This result was suggestive both of a direct interaction between Med15(Gal11) and Med14(Rgr1), and of a mechanism by which association of the two proteins could be inhibited.

To investigate this hypothesis, we first identified conditions under which the association of Med14(Rgr1) and Med15(Gal11) could be observed. Although several fragments of Med15(Gal11) have been bacterially over-expressed and studied, constructs for Med14(Rgr1) (123 kDa) bacterial expression have not been reported. We thus elected to examine the interaction of the entire protein with Med15(Gal11), using a protein A-tagged variant for isolation and detection. Homologous recombination in *S. cerevisiae* was used to tag Med14(Rgr1) for isolation and detection purposes by using standard protocols.[26,27] Protein A incorporation was confirmed by PCR, and expression of the tagged protein was verified by analysis of yeast extracts by SDS-PAGE followed by Western blotting with the PAP antibody to detect the protein A-tagged Med14(Rgr1). To probe the association of Med14(Rgr1) with Med15(Gal11), recombinant Med15(Gal11) (residues 186–619) was expressed as a glutathione *S*-transferase (GST) fusion in Rosetta2(DE3) pLysS *E. coli* and directly bound to glutathione agarose beads. Yeast extracts containing protein A-tagged Med14(Rgr1) in extract buffer (200 mM Tris pH 8.0, 150 mM ammonium sulfate, 10% glycerol, 1 mM EDTA, 2 mM dithiothreitol (DTT) and protease inhibitors) were added to the beads, which were then rotated at 4 °C for 2 h. The beads were thoroughly washed and heated in SDS buffer, and equal volumes of the supernatant were analyzed by SDS-PAGE and Western blotting (Figure 2A, lane 3). A single protein band could be seen at approximately 120 kDa, the molecular weight of protein A-tagged Med14(Rgr1); this indicated that Med14(Rgr1) was retained by the solid-support-bound Med15(Gal11) under these conditions. Med14(Rgr1) was not retained by the glutathione beads in control experiments in which Med15(Gal11) was not present (Figure 2A, lane 2).

With conditions for observing the association of Med14(Rgr1) and Med15(Gal11) in hand, we then tested whether or not peptide 32 could inhibit the formation of this complex. For this purpose, peptide 32 was synthesized by using standard solid-phase synthesis techniques, and the crude material thus obtained was purified to homogeneity by reversed-phase HPLC. The identity of the isolated peptide was verified by electrospray mass spectrometry. A portion of peptide 32 was labeled at the amino terminus with fluorescein, purified by reversed-phase HPLC, and used in a fluorescence-polarization experiment to measure the affinity of peptide 32 for Med15(Gal11). Peptide 32 interacts with Med15(Gal11) with a K_D of 2.4 μM, similar to that of other Med15(Gal11) peptide ligands isolated from binding screens.[28] In addition to peptide 32, a scrambled version of this sequence (scr32: Tyr-Glu-Val-Phe-Ala-Ser-Pro-Glu) was synthesized for use as a negative control.

Yeast extracts containing protein A-tagged Med14(Rgr1) were combined with 10 μg of recombinant GST-Med15(Gal11) (186–619) and glutathione agarose beads. Also included in the mixture was peptide 32 at concentrations ranging from 1 μM to 5 mM, and each reaction mixture was incubated with rotation for 2 h at 4 °C. The beads were washed several times with extract buffer, and the bound protein complex was separated by SDS-PAGE and detected by Western blot analysis. As shown in Figure 2B, at a peptide 32 concentration of 10 μM, significant inhibition of Med15(Gal11)–Med14(Rgr1) association was observed with complete inhibition at 100 μM. In contrast, the scrambled version of peptide 32 (scr32) did not inhibit the association of these two proteins over the same concentration range (Figure 2C). Future in vitro cross-linking experiments in combination with mutagenesis approaches in vivo will be useful in distinguishing the mechanism of peptide 32-mediated inhibition. The observed inhibition could be due to direct disruption of a Med15(Gal11)–Med14(Rgr1) interaction or an indirect mechanism either through an allosteric change in Med15(Gal11) upon binding or by inhibition of other Med15(Gal11)–Mediator protein interactions.

In summary, the eight-residue peptide Ala-Tyr-Phe-Glu-Val-Pro-Ser-Glu disrupts the association of the Mediator components Med14(Rgr1) and Med15(Gal11), transcription proteins important for Mediator recruitment and integration of activator

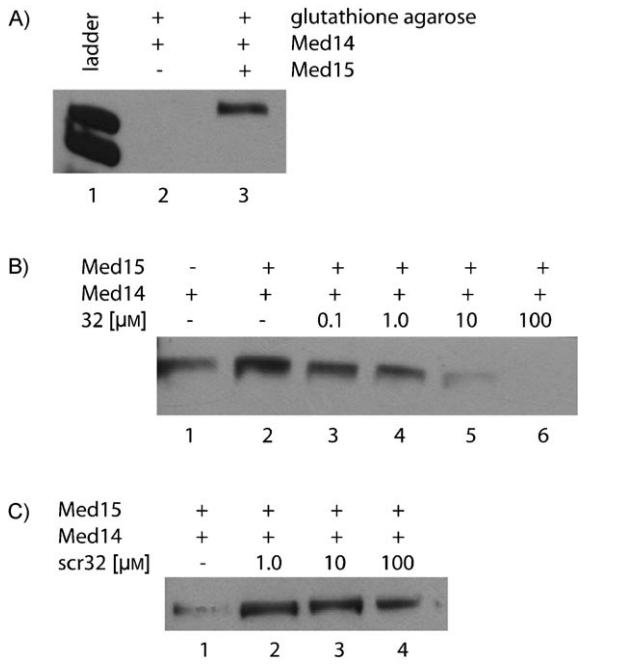

Figure 2. Med14 coprecipitates with Med15(Gal11), and this association is specifically inhibited by peptide 32. A) When yeast lysates containing protein A-tagged Med14(Rgr1) were combined with glutathione agarose beads, no detectable Med14(Rgr1) was precipitated (lane 2). In contrast, when GST-tagged Med15(Gal11) was added to the mixture, Med14(Rgr1) colocalized with Med15(Gal11) on the solid support (lane 3). B) Increasing concentrations of peptide 32 added to a mixture of GST-tagged Med15(Gal11) and yeast lysates containing protein A-tagged Med14(Rgr1) inhibit Med14(Rgr1)–Med15(Gal11) association. Lane 1: yeast lysate; lane 2: Med14(Rgr1) coprecipitated with Med15(Gal11) by glutathione agarose beads; lanes 3–6: as lane 2, but with increasing concentrations of peptide 32. C) The scrambled version of peptide 32 (scr32) does not inhibit the association of Med15(Gal11) and Med14(Rgr1) at concentrations up to 100 μM under the same conditions as used for (B).

signals for a range of genes, at micromolar concentrations. Importantly, there is some level of specificity associated with this inhibition. The experiments were carried out with yeast lysates, such that there was a high number of potential binding partners within the assay mixture. In addition, when the sequence of the peptide was scrambled, no discernable inhibition occurred under identical conditions. As the first reported inhibitor of a Mediator protein–protein interaction, peptide 32 will be a useful mechanistic tool on a number of fronts. Perhaps most promising, peptide 32 can be used as the starting point for identifying nonpeptidic inhibitors through competitive binding assays, and such molecules could have enhanced utility in cell-based experiments. Finally, other mediator components can be submitted to similar peptide/small-molecule screens to identify not only potential protein partners and inhibitors of contacts between Mediator components, but, depending on the target, novel artificial transcriptional activation domains. Future peptide/small-molecule inhibitors of Mediator interactions will be useful for dissecting the roles of individual subunits and mediator modules in conveying signals from transcriptional regulators to RNA polymerase II and in preinitiation complex (PIC) assembly.

Experimental Section

Isolation of protein A-tagged Med14(Rgr1): To C-terminally protein A-tagged Med14(Rgr1) under its natural promoter, primers containing 40 base pairs homologous to the C terminus of Med14(Rgr1) and the 3′ untranslated region of Med14(Rgr1) were designed to PCR and then clone the protein A tag and Kan marker from pFA6a-KanMX6 with protein A.[26,27] The forward primer was 5′-CATAATATCCTCAAAGTGGACTCGAACTCAAGTTCATCTCGGATCCC-CGGGTTAATTAA-3′. The reverse primer was 5′-CTCCTAAGGGATAGT-AGCGCCGGTGACATTTTATTCGCTGAATTCGAGCTCGTTTAAAC-3′. PCR reactions contained dNTPs (200 μM), the forward and reverse primer (2 μM), and pFA6a-KanMX6 (100 ng; 50 μL total volume). The amplification profile was one cycle for 3 min at 95 °C followed by 30 cycles of 15 s at 95 °C, 1 min at 50 °C, and 2 min at 72 °C. The final extension step was 7 min at 72 °C. The correct PCR fragment was verified by gel electrophoresis. Several PCR reaction mixtures were combined and transformed into *S. cerevisiae* (BJ2168, genotype: *MATalpha,ura3-52,leu2,gal2,prb1-1122,1pep4-3,prc1-407*) by using the lithium acetate method. Integration of the protein A tag at the C terminus of Med14(Rgr1) was verified by Western blot and PCR analysis of the transformed yeast colonies that survived on SC G418 (500 $\mu g\,mL^{-1}$) plates. Yeast containing the integrated protein A tag at the C terminus of Med14(Rgr1) were used to inoculate an overnight culture (50 mL), which was subsequently diluted 500-fold to inoculate YPD media (1 L). The culture was grown to an OD of 3 before the cells were centrifuged at 5000 rpm for 5 min. The pellets were stored at −80 °C until needed. (http://www.fhcrc.org/science/labs/hahn/methods/methods_index.html) In order to lyse, the cells the pellet was washed with sterilized ddH_2O (50 mL) then extract buffer (200 mM Tris-HCl pH 8.0, 150 mM ammonium sulfate, 10% glycerol, 1 mM EDTA, 2 mM DTT, Roche protease inhibitor tablet; 25 mL) both prechilled to 4 °C. The cells were resuspended in extract buffer and lysed by using glass beads at 4 °C with a 1 min vortex, 1 min cooling cycle, repeated 5 times. The glass bead–cell lysate mixture was centrifuged for 3 min at 14000 rpm and 4 °C, and the supernatant was centrifuged again for 30 min at 14000 rpm and 4 °C. The supernatant was concentrated in a spin filter, and aliquots (1 mL) were used in pull-down experiments.

GST-Med15(Gal11)(186–619) was over-expressed in Rosetta2(DE3) pLysS *E. coli* cells and isolated as previously described.[28]

Competitive inhibition experiments: For the peptide competition experiments, GST–Med15 (186–619) (50 μL, 20 μM) was added with yeast extracts (1 mL) and a range of concentrations of peptide 32 or scrambled peptide 32 (scr32) to glutathione beads (50 μL) and mixed end on end at 4 °C for 2 h. The beads were washed with extract buffer (3×), and the bound protein was eluted by boiling in NUPAGE LDS 4× loading buffer. The supernatant (15 μL) was loaded and separated by SDS-PAGE and detected by Western blot by using chemiluminescence.

Acknowledgements

A.K.M. acknowledges support for this work from the NIH (GM65330), the Alfred P. Sloan Foundation, the Burroughs Wellcome Fund and the March of Dimes Foundation. J.K.L. was supported by the Michigan C.B.I. Training Program (NIH GM09597-07). We thank A. Z. Ansari for helpful discussions and P. Stromhaug for technical assistance.

Keywords: inhibitors · Mediator complex · peptides · transcription · transcriptional activator

[1] L. C. Myers, R. D. Kornberg, *Annu. Rev. Biochem.* **2000**, *69*, 729.
[2] H. M. Bourbon, R. G. Roeder, R. D. Kornberg, et al., *Mol. Cell* **2004**, *14*, 553.
[3] M. Boube, L. Joulia, D. L. Cribbs, H. M. Bourbon, *Cell* **2002**, *110*, 143.
[4] A. J. Koleske, R. A. Young, *Trends Biochem. Sci.* **1995**, *20*, 113.
[5] A. J. Koleske, R. A. Young, *Nature* **1994**, *368*, 466.
[6] Y. J. Kim, S. Björklund, Y. Li, M. H. Sayre, R. D. Kornberg, *Cell* **1994**, *77*, 599.
[7] F. J. Asturias, Y. W. Jiang, L. C. Myers, C. M. Gustafsson, R. D. Kornberg, *Science* **1999**, *283*, 985.
[8] M. Ptashne, A. Gann, *Genes & Signals*, Cold Spring Harbor Laboratory Press, New York, **2001**.
[9] Y. Li, S. Björklund, Y. W. Jiang, Y. J. Kim, W. S. Lane, D. J. Stillman, R. D. Kornberg, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 10864.
[10] M. Carlson, *Annu. Rev. Cell Dev. Biol.* **1997**, *13*, 1.
[11] X. Wang, C. A. Michels, *Genetics* **2004**, *168*, 747.
[12] A. Blumental-Perry, W. Li, G. Simchen, A. P. Mitchell, *Mol. Biol. Cell* **2002**, *13*, 1709.
[13] Y. C. Lee, J. M. Park, S. Min, S. J. Han, Y. J. Kim, *Mol. Cell. Biol.* **1999**, *19*, 2967.
[14] J. M. Park, H. S. Kim, S. J. Han, M. S. Hwang, Y. C. Lee, Y. J. Kim, *Mol. Cell. Biol.* **2000**, *20*, 8709.
[15] J. Fishburn, N. Mohibullah, S. Hahn, *Mol. Cell* **2005**, *18*, 369.
[16] W. M. Reeves, S. Hahn, *Mol. Cell. Biol.* **2005**, *25*, 9092.
[17] F. Zhang, L. Sumibcay, A. G. Hinnebusch, M. J. Swanson, *Mol. Cell. Biol.* **2004**, *24*, 6871.
[18] S. Lallet, H. Garreau, C. Garmendia-Torres, D. Szestakowska, E. Boy-Marcotte, S. Quevillon-Cheruel, M. Jacquet, *Mol. Microbiol.* **2006**, *62*, 438.
[19] C. J. Jeong, S. H. Yang, Y. Xie, L. Zhang, S. A. Johnston, T. Kodadek, *Biochemistry* **2001**, *40*, 9421.
[20] Z. Lu, A. Z. Ansari, X. Y. Lu, A. Ogirala, M. Ptashne, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 8591.
[21] H. Sakurai, Y. Hiraoka, T. Fukasawa, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 8382.
[22] T. Mizuno, S. Harashima, *Mol. Genet. Genomics* **2003**, *269*, 68.
[23] M. Novatchkova, F. Eisenhaber, *Curr. Biol.* **2004**, *14*, R54.
[24] Y. Suzuki, Y. Nogi, A. Abe, T. Fukasawa, *Mol. Cell. Biol.* **1988**, *8*, 4991.

[25] S. F. Altschul, T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, *Nucleic Acids Res.* **1997**, *25*, 3389.
[26] M. S. Longtine, A. McKenzie III, D. J. Demarini, N. G. Shah, A. Wach, A. Brachat, P. Philippsen, J. R. Pringle, *Yeast* **1998**, *14*, 953.
[27] O. Puig, B. Rutz, B. G. Luukkonen, S. Kandels-Lewis, E. Bragado-Nilsson, B. Seraphin, *Yeast* **1998**, *14*, 1139.
[28] Z. Wu, G. Belanger, B. B. Brennan, J. K. Lum, A. R. Minter, S. P. Rowe, A. Plachetka, C. Y. Majmudar, A. K. Mapp, *J. Am. Chem. Soc.* **2003**, *125*, 12390.

Received: February 8, 2007
Published online on June 21, 2007

