

Identification of Mammalian Mediator Subunits with Similarities to Yeast Mediator Subunits Srb5, Srb6, Med11, and Rox3*[§]

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From the [‡]Stowers Institute for Medical Research, Kansas City, Missouri 64110, [¶]Harvard Microchemistry and Proteomics Analysis Facility, Harvard University, Cambridge, Massachusetts 02138, [|]Division of Biology, California Institute of Technology, Pasadena, California 91125, ^{**}Department of Biochemistry and Molecular Biology, Kansas University Medical Center, Kansas City, Kansas 66160, and ^{‡‡}Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

The Mediator is a multiprotein coactivator required for activation of RNA polymerase II transcription by DNA binding transactivators. We recently identified a mammalian homologue of yeast Mediator subunit Med8 and partially purified a Med8-containing Mediator complex from rat liver nuclei (Brower, C. S., Sato, S., Tomomori-Sato, C., Kamura, T., Pause, A., Stearman, R., Klausner, R. D., Malik, S., Lane, W. S., Sorokina, I., Roeder, R. G., Conaway, J. W., and Conaway, R. C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 10353–10358). Analysis of proteins present in the most highly purified Med8-containing fractions by tandem mass spectrometry led to the identification of many known mammalian Mediator subunits, as well as four potential Mediator subunits exhibiting sequence similarity to yeast Mediator subunits Srb5, Srb6, Med11, and Rox3. Here we present direct biochemical evidence that these four proteins are *bona fide* mammalian Mediator subunits. In addition, we identify direct pairwise binding partners of these proteins among the known mammalian Mediator subunits. Taken together, our findings identify a collection of novel mammalian Mediator subunits and shed new light on the underlying architecture of the mammalian Mediator complex.

Transcription of eukaryotic protein-coding genes by RNA polymerase II and the general initiation factors is controlled by a large collection of DNA binding transcriptional activators through the action of an intermediary multiprotein coactivator referred to as Mediator. The Mediator complex was first identified in *Saccharomyces cerevisiae* and found to be composed of more than 20 proteins (designated Srb2, Srb4, Srb5, Srb6, Srb7, Srb8, Srb9, Srb10, Srb11, Med1, Med2, Pgd1, Med4, Med6, Med7, Med8, Med11, Rox3, Cse2, Nut1, Nut2, Gal11, Rgr1, and Sin4 (1)). Multiprotein mammalian Mediator complexes with structural and functional properties similar to *S.*

cerevisiae Mediator were subsequently identified in several laboratories and variously designated TRAP¹ (thyroid hormone receptor-associated proteins)/SMCC (SRB-MED-containing cofactor) (2, 3), DRIP (vitamin D receptor-interacting proteins) (4), ARC (activator-recruited cofactor) (5), CRSP (cofactor required for Sp1 activation) (6), and mouse Mediator (7). Biochemical characterization of these different mammalian Mediator complexes has revealed that they are composed of many of the same proteins. Among the consensus mammalian Mediator subunits are easily identifiable structural homologues of several *S. cerevisiae* Mediator subunits, including Srb7, Med6, Med7, Nut2, and Rgr1; in addition, recent bioinformatic evidence suggests that, among the consensus mammalian Mediator subunits, TRFP, TRAP80, TRAP240, TRAP230, Cdk8, Cyclin C, TRAP220, and TRAP36 are homologous to *S. cerevisiae* Mediator subunits Srb2, Srb4, Srb8, Srb9, Srb10, Srb11, Med1, and Med4, respectively (8).

We recently identified a mammalian homologue of *S. cerevisiae* Mediator subunit Med8 and found that it is an Elongin BC-interacting protein that can assemble with Elongins B and C and a Cul2/Rbx1 module to reconstitute a potential E3 ubiquitin ligase. As part of our effort to explore the possible relationship between Mediator and the ubiquitin pathway, we have purified a Med8-containing Mediator complex from rat liver nuclei. Analysis of proteins present in the most highly purified Med8-containing fractions by ion-trap MS/MS identified many consensus mammalian Mediator subunits, as well as additional proteins not previously recognized as subunits of mammalian Mediator. In this report, we identify four of these proteins as integral subunits of the mammalian Mediator complex.

EXPERIMENTAL PROCEDURES

Materials—Anti-c-Myc (9E10) monoclonal antibody was purchased from Roche Molecular Biochemicals. Rabbit anti-c-Myc antibody (C-3956), anti-Flag (M2) monoclonal antibody, and anti-Flag M2 agarose were obtained from Sigma. Anti-Med6 (E-20) antibody was from Santa Cruz Biotechnology. Rabbit anti-Med8 antibody was raised against a peptide corresponding to Med8 residues 247–268, and rabbit anti-p28b, anti-Surf5, anti-HSPC296, anti-LCMR1, and anti-FLJ23445 antibodies were raised against full-length recombinant proteins produced in either

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[§] The on-line version of this article (available at <http://www.jbc.org>) includes as Supplemental Material (i) a sequence comparison of yeast and mammalian Mediator subunits and (ii) peptide sequences obtained from the purified rat liver Mediator complex.

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¹ The abbreviations used are: TRAP, thyroid hormone receptor-associated protein; Med, Mediator; Srb, suppressor of RNA polymerase B; SMCC, Srb-Med-containing cofactor; DRIP, vitamin D receptor-interacting protein; ARC, activator-recruited cofactor; CRSP, cofactor required for Sp1 activation; MS/MS, tandem mass spectrometry; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; TRFP, TATA-binding protein-related factor-proximal protein.

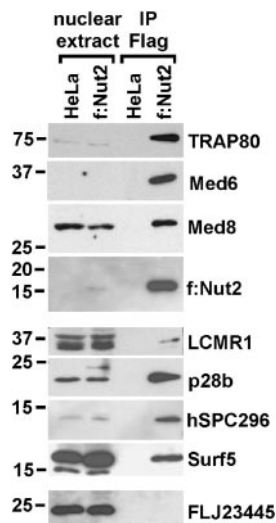


FIG. 1. The p28b, Surf5, HSPC296, and LCMR1 proteins are present in the purified TRAP/SMCC mammalian mediator complex. Immunoaffinity purification of the TRAP/SMCC Mediator complex was carried out essentially as described (11). Six ml of undialyzed nuclear extract (12) (~30 mg protein) from either parental HeLa cells (HeLa) or HeLa M10 cells (11) stably expressing Flag-tagged mammalian Mediator subunit Nut2 (*f:Nut2*) were incubated with 100 μ l of anti-Flag M2 agarose in buffer A (10 mM Hepes-NaOH, pH7.9, 10 mM KCl, 1 mM MgCl₂, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.1% Triton X-100) containing 0.3 M KCl for at least 4 h at 4 °C. Beads were washed five times with 5 ml of buffer A containing 0.3 M NaCl and once with buffer A containing 0.1 M NaCl. Bound proteins were eluted by incubating beads twice with 100 μ l of buffer A containing 0.1 M NaCl and 0.2 mg/ml Flag peptide and analyzed by Western blotting with the antibodies indicated in the figure. Western blots were developed using horseradish peroxidase-labeled secondary antibodies and either SuperSignal West Dura extended duration substrate or SuperSignal West Femto maximum sensitivity substrate (Pierce).

Escherichia coli or insect cells (Cocalico Biologicals, Inc). Rabbit anti-TRAP80 antibody was kindly provided by Dr. R. G. Roeder.

Expression of Recombinant Proteins in Insect Cells—Sf21 cells were cultured at 27 °C in SF-900 II SFM (Invitrogen) with 10% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. 1×10^8 Sf21 cells in suspension (for large-scale preparations used in preparative chromatography) or 1×10^7 Sf21 cells in monolayer cultures (for small-scale preparations used for analytical immunoprecipitations) were infected at a multiplicity of infection of ~5–10 with the recombinant baculoviruses indicated in the figures. Forty-eight hours after infection, cells were collected and lysed in ice-cold buffer containing 50 mM Hepes-NaOH (pH 7.9), 500 mM KCl, 5 mM MgCl₂, 0.2% (v/v) Triton X-100, 20% (v/v) glycerol, 0.28 μ g/ml leupeptin, 1.4 μ g/ml pepstatin A, 0.17 mg/ml PMSF, and 0.33 mg/ml benzimidazole. For large-scale preparations, lysates were centrifuged at 100,000 $\times g$ for 30 min at 4 °C, and for analytical preparations, lysates were centrifuged at 10,000 $\times g$ for 20 min at 4 °C. Where indicated in the figure legends, histidine-tagged proteins were applied to a 1-ml HiTrap chelating column (Amersham Biosciences) charged with nickel ions according to the manufacturer's instructions. The column was washed with buffer I (40 mM Hepes-KOH (pH 7.6), 100 mM KCl, 20% (v/v) glycerol, 5 mM 2-mercaptoethanol, 0.28 μ g/ml leupeptin, 1.4 μ g/ml pepstatin A, 0.17 mg/ml PMSF, and 0.33 mg/ml benzimidazole) containing 10 mM imidazole. The column was eluted with a 20-column volume gradient from buffer I containing 10 mM imidazole to buffer I containing 500 mM imidazole.

RESULTS AND DISCUSSION

We previously identified in rat liver nuclear extracts a Med8-containing Mediator complex with an apparent native molecular mass by SW4000 gel filtration of more than 1000 kDa (9). Although the Med8 complex appeared to fractionate chromatographically as a discrete species, it proved to be extremely labile and refractory to complete purification, with estimated yields as poor as 10% at each step of purification. As a consequence, in an effort to identify proteins present in the Med8 complex, we resorted to a proteomics approach involving large

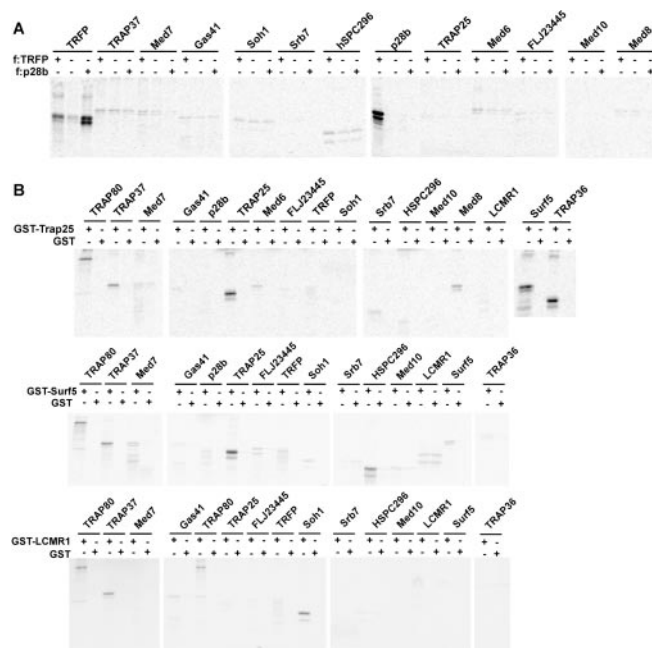


FIG. 2. Screening for binding partners of the p28b, Surf5, HSPC296, and LCMR1 proteins among the known mammalian Mediator subunits. A, reticulocyte lysates containing the indicated *in vitro* translated, ³⁵S-labeled proteins were mixed with purified, Sf21 insect cell expressed His-Flag-p28b or His-Flag-TRFP bound to anti-Flag agarose beads or to anti-Flag agarose beads alone and incubated for 2 h with rocking at 4 °C. The beads were washed three times in phosphate-buffered saline. Bound proteins were eluted with SDS sample buffer, fractionated by SDS-PAGE, and visualized by PhosphorImager analysis. B, reticulocyte lysate containing the indicated ³⁵S-labeled proteins were incubated for 3 h at room temperature with GST or the indicated GST fusion protein that had been expressed in and purified from *E. coli* and prebound to glutathione-agarose. The beads were washed three times with 40 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.2% Triton X-100. Proteins were eluted with the same buffer containing 30 mM glutathione and 10% glycerol, fractionated by SDS-PAGE, and visualized by PhosphorImager analysis. Expressed sequence tags encoding full-length human TRAP80 (BE883062), mouse Med6 (AW990333), human Med7 (AI417176), human TRAP37 (AA447877), human TRAP36 (BG77869), mouse TRFP (BF165981), mouse TRAP25 (BF657838), human Srb7 (AA449015), mouse Soh1 (BE626280), human p28b (AA460637), human Surf5 (BE618264), mouse HSPC296 (BE625435), mouse LCMR1 (AW044776), mouse Gas41 (BE308730), mouse Nut2/Med10 (BE625965), and mouse FLJ23445 (BF179247) (GenBank™ accession numbers in parentheses) were purchased from Invitrogen and subcloned into mammalian expression vector pcDNA3.1 with N-terminal Flag or C-Myc tags. pcDNA3.1 encoding full-length human MED8 with an N-terminal T7 epitope tag was described previously (9). ³⁵S-Labeled proteins used in binding studies were synthesized using the TnT T7 quick coupled transcription/translation system (rabbit reticulocyte lysate from Promega) programmed with the indicated pcDNA3.1 vectors.

scale enrichment of the complex by multistep conventional chromatography and HPLC followed by exhaustive sequencing of Med8-associated proteins by HPLC/MS/MS.

Among the proteins present in the most highly enriched Med8-containing fractions and identified by mass spectrometry were the known mammalian Mediator subunits TRAP230, TRAP220, TRAP80, Cdk8, Cyclin C, TRAP37, Med6, Med7, TRFP, TRAP25, Nut2, and Soh1 (Table I in the Supplemental Material). In addition to these known mammalian Mediator subunits were a collection of additional proteins including the p28b, Surf5, HSPC296, AK007855, and LCMR1 (lung cancer metastasis-related protein 1) proteins, which were not previously recognized as consensus mammalian Mediator subunits.

The p28b protein was previously identified by protein sequencing as a constituent of a mammalian Mediator preparation from mouse B-cells (7), and its apparent *Drosophila mela-*

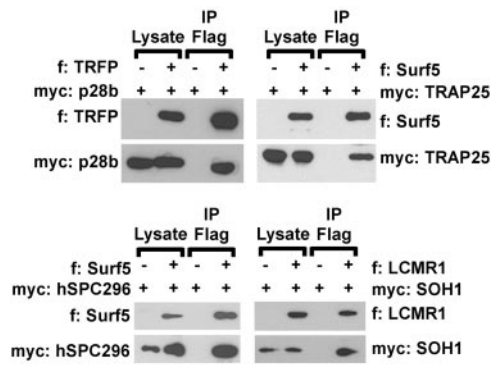


FIG. 3. Interaction of p28b, Surf5, HSPC296, and LCMR1 with known Mediator subunits in transfected mammalian cells. Two 10-cm dishes of 293T cells grown to ~50% confluency were cotransfected with 3 μ g of plasmids encoding the proteins indicated in the figure. 24–36 h after transfection, cells were lysed by incubation in 50 mM Hepes-NaOH (pH 7.9), 250 mM KCl, 0.2% (v/v) Triton X-100, and 20% (v/v) glycerol. Proteins in cell lysates or anti-Flag immunoprecipitates were fractionated by SDS-polyacrylamide gel electrophoresis and visualized by Western blotting using anti-Flag or anti-c-Myc antibodies and horseradish peroxidase-labeled secondary antibodies. Western blots were developed using the SuperSignal West Dura extended duration substrate (Pierce). *f:TRFP*, FLAG-TRFP; *f:Surf5*, FLAG-Surf5; *f:LCMR1*, FLAG-LCMR1; *myc*, c-Myc epitope; *IP*, immunoprecipitation.

nogaster homologue was recently identified as a component of a *D. melanogaster* Mediator complex (10). However, the p28b protein had not previously been identified as a subunit of other mammalian Mediator complexes, including the TRAP/SMCC, DRIP, ARC, and CRSP complexes (2–6). The proteins encoded by the Surf5, HSPC296, and AK007855 open reading frames had not previously been identified as components of mammalian Mediator complexes, but their apparent *D. melanogaster* homologues were recently identified as the Med24, Med21, and Med23 components of a *D. melanogaster* Mediator complex (10). The LCMR1 protein is defined as a metastasis-related protein (GenBank™ accession number AAN16075). However, no information about the function of LCMR1 has been reported, and the LCMR1 protein had not previously been identified as a constituent of either mammalian or *D. melanogaster* Mediator complexes.

On the basis of bioinformatic evidence, Bourbon and co-workers (8) recently predicted that the p28b and Surf5 proteins are higher eukaryotic homologues of the *S. cerevisiae* Mediator subunits Srb5 and Srb6. Results of our PSI-BLAST searches and multiple sequence alignments revealed structural similarities between LCMR1 and HSPC296 and the yeast Mediator subunits Rox3 and Med11, respectively, suggesting that LCMR1 and HSPC296 could be mammalian homologues of yeast Rox3 and Med11 (Fig. 1 in the Supplemental Material).

Because of their apparent structural similarities to *S. cerevisiae* Mediator subunits, we sought to determine whether the p28b, Surf5, HSPC296, and LCMR1 proteins are *bona fide* subunits of the mammalian Mediator complex. To begin to address this possibility, we took advantage of a HeLa cell line that stably expresses Mediator subunit Nut2 with an N-terminal Flag tag (f:Nut2). This HeLa cell line has been used extensively as a source for immunoaffinity purification of the transcriptionally active TRAP/SMCC Mediator complex (11). Rabbit polyclonal antibodies were raised against the p28b, Surf5, HSPC296, and LCMR1 proteins and used in Western blotting experiments to assay for the presence of these proteins in immunoaffinity-purified preparations of the HeLa cell TRAP/SMCC complex. As shown in Fig. 1, all four proteins were readily detected by Western blotting in immunoaffinity-purified preparations of the TRAP/SMCC complex, along with

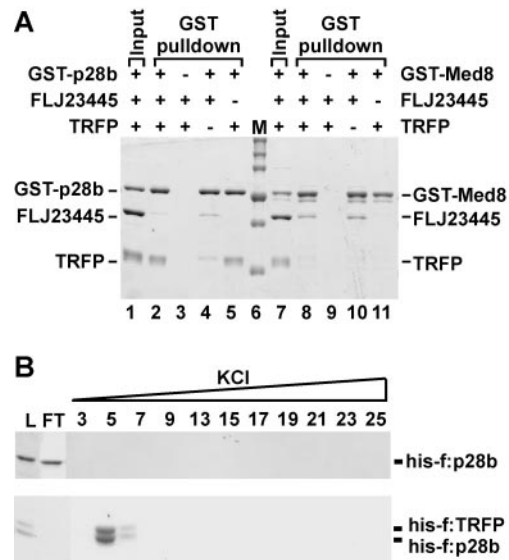


FIG. 4. Interaction of p28b with TRFP. *A*, specific interaction between bacterially expressed p28b and TRFP. ~10 μ g of GST-p28b, His-TRX-FLJ23445, and His-TRFP were mixed in the combinations indicated in the figure and incubated for 3 h at room temperature in a buffer containing 40 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.2% (v/v) Triton X-100. Protein mixtures were then incubated for 1 h with 15 μ l of glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences), washed three times with 1 ml of 40 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.2% Triton X-100, and eluted with 25 μ l of 30 mM glutathione, 40 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, and 0.15 M NaCl. Eluted proteins were fractionated by SDS-polyacrylamide electrophoresis and visualized by staining with Coomassie Blue. *B*, copurification of insect cell-expressed p28b and TRFP. His-Flag-p28b (upper panel) or His-Flag-p28b and TRFP (lower panel), which had been expressed in insect cells and purified by nickel chromatography as described under “Experimental Procedures” were mixed and incubated at 4 °C for 60 min. The mixture was then diluted with buffer A (40 mM Tris-HCl (pH 7.9), 10% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.28 μ g/ml leupeptin, 1.4 μ g/ml pepstatin A, 0.17 mg/ml PMSF, and 0.33 mg/ml benzamide) to a conductivity equivalent to that of buffer A containing 100 mM KCl. Proteins were applied to a 0.6 ml TSK DEAE-NPR HPLC column (Tosoh-BioSep) equilibrated in buffer A containing 100 mM KCl. The column was eluted with a 6-ml linear gradient from 100 to 500 mM KCl in buffer A, and 200 μ l fractions were collected. Aliquots of the indicated column fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and proteins were visualized by Coomassie Blue staining. The identities of the protein bands were confirmed by Western blotting (data not shown).

f:Nut2 and known Mediator subunits TRAP80, Med6, and Med8. A protein encoded by the FLJ23445 open reading frame, which was also identified by mass spectrometry in the most highly enriched Med8-containing fractions, was not detected by Western blotting in purified preparations of the TRAP/SMCC complex (Fig. 1). Thus, although the p28b, Surf5, HSPC296, and LCMR1 proteins were not previously identified in the TRAP/SMCC complex by protein sequencing, they were all detected by Western blotting in purified preparations of the complex, suggesting that they are subunits of the complex.

To obtain additional evidence supporting assignment of the p28b, Surf5, HSPC296, and LCMR1 proteins as mammalian Mediator subunits, we sought to identify pairwise binding partners of these proteins among the known mammalian Mediator subunits. To this end, we used a convenient and scalable screen to assess the ability of these proteins to interact with known mammalian Mediator subunits prepared by *in vitro* translation in rabbit reticulocyte lysates. pcDNA3.1 expression vectors encoding the known mammalian Mediator subunits indicated in Fig. 2 were constructed and used to program rabbit reticulocyte lysates for translation of 35 S-labeled Mediator proteins. Binding of 35 S-labeled Mediator proteins to purified recombinant Flag-p28b, Flag-TRFP, GST-Surf5, GST-TRAP25, and GST-

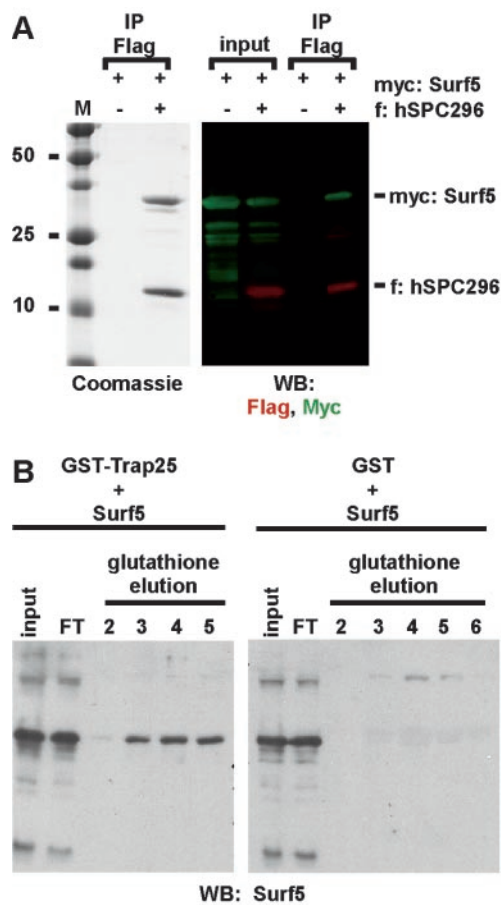


FIG. 5. Interaction of HSPC296 with Surf5 and TRAP25. *A*, extracts from Sf21 cells infected with baculoviruses encoding the hSPC296 protein with an N-terminal Flag tag and the Surf5 protein with an N-terminal Myc tag and a 16-amino acid C-terminal extension replacing the last two Surf5 amino acids with DPLEDLVPSSNSRAAA (*input*) were immunoprecipitated using anti-FLAG agarose. Bound proteins were eluted from anti-FLAG beads with 150 μ g/ml FLAG peptide, subjected to SDS-polyacrylamide gel electrophoresis, and detected by Coomassie staining or by Western blotting (*IP Flag*). *Myc:Surf5* was detected with rabbit anti-c-Myc antibody and IR DyeTM 800-labeled goat anti-rabbit IgG secondary antibody (*green*). *f:hSPC296* was detected with mouse anti-FLAG monoclonal antibody and Alexa Fluor 680-labeled anti-mouse IgG (α light chain-specific) secondary antibody (*red*). Light chain-specific anti-mouse antibody was obtained from Bethyl Laboratories and labeled with Alexa Fluor 680 (Molecular Probes) according to the manufacturer's instructions. Fluorescently labeled secondary antibodies were detected using a Li-Cor Odyssey infrared imaging system. *B*, His-Surf5 and either GST or GST-TRAP25 were coexpressed in *E. coli* and purified on a glutathione-Sepharose FastFlow column. Aliquots of the column load (*input*), flow-through (*FT*), and fractions eluted with glutathione were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were detected by Western blotting (*WB*) with anti-Surf5 antiserum using horseradish peroxidase-labeled secondary antibody. Bands were visualized using the SuperSignal West Femto maximum sensitivity substrate (Pierce).

LCMR1 fusion proteins was assayed in pull-down experiments using anti-Flag-agarose or glutathione-agarose beads, and bound proteins were visualized by autoradiography. The results of these experiments revealed significant and reproducible binding (i) of p28b to Mediator subunit TRFP, (ii) of TRAP25 to itself and to Mediator subunits TRAP36 and Surf5, (iii) of Surf5 to both Mediator subunit TRAP25 and HSPC296, and (iv) of LCMR1 to Mediator subunit Soh1 (Fig. 3). Additionally, GST-TRAP25, GST-Surf5, and GST-LCMR1 all bound to TRAP80 and TRAP37 in these experiments (Fig. 2).

To confirm and extend these findings, we sought to reconstitute the protein-protein interactions observed in *in vitro* trans-

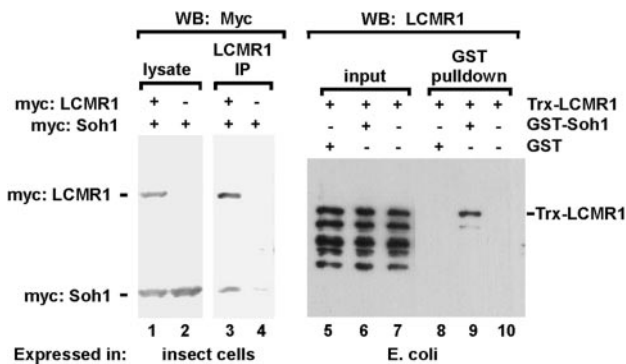


FIG. 6. Interaction of LCMR1 with Soh1. *Lanes 1–4*, 200 μ l of lysates from Sf21 cells infected with baculoviruses expressing the indicated proteins (*input*) were incubated for 30 min at 4 °C with 1 μ l of rabbit anti-LCMR1 antiserum in 50 mM Hepes-NaOH (pH 7.9), 500 mM KCl, 5 mM MgCl₂, 0.2% (v/v) Triton X-100, 20% (v/v) glycerol, 0.28 μ g/ml leupeptin, 1.4 μ g/ml pepstatin A, 0.17 mg/ml PMSF, and 0.33 mg/ml benzamide. The mixture was diluted with 0.5 ml of the same buffer and incubated overnight at 4 °C with anti-rabbit IgG-agarose (Sigma). Beads were washed three times with 1 ml of 40 mM Tris-HCl (pH 7.4), 137 mM NaCl, 3 mM KCl, and 0.02% (v/v) Tween 20. Bound proteins were eluted with SDS sample buffer, analyzed by Western blotting using rabbit anti-c-Myc primary antibody and IR DyeTM 800-labeled goat anti-rabbit IgG secondary antibody, and visualized using a Li-Cor Odyssey infrared imaging system. *Lanes 5–6*, ~10 μ g of Trx-His-LCMR1 (Trx-LCMR1) and ~15 μ g of GST or ~10 μ g of GST-Soh1 were incubated at room temperature in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol. After 2 h, the protein mixture was added to 25 μ l of glutathione agarose beads pre-equilibrated with the same buffer and incubated an additional 1 h at room. Beads were then transferred to Micro Bio-Spin chromatography columns (Bio-Rad) and washed with a total of 4 ml of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, and 0.2% Triton X-100 and then with 1 ml of phosphate-buffered saline. Bound proteins were eluted with 40 μ l of 30 mM glutathione, 40 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, and 0.15 M NaCl.

lation experiments in transfected 293T cells or with recombinant proteins expressed in Sf21 insect cells or in *E. coli*. Consistent with the results of our initial screening experiments, p28b could be coimmunoprecipitated from 293T cell lysates with mammalian Mediator subunit TRFP, Surf5 could be coimmunoprecipitated with both HSPC296 and Mediator subunit TRAP25, and LCMR1 could be coimmunoprecipitated with Mediator subunit Soh1 (Fig. 3).

As shown in Fig. 4A, recombinant TRFP purified from *E. coli*, but not bacterially expressed FLJ23445, bound specifically to purified GST-p28b. In control experiments, TRFP did not bind to GST-Med8. Consistent with these observations, a TRFP-p28b heterodimer could be reconstituted with His-TRFP and His-p28b that had been purified from baculovirus-infected insect cells by nickel chromatography. The TRFP-p28b heterodimer was purified to near homogeneity by TSK DEAE-NPR HPLC. After mixing recombinant TRFP and p28b, both proteins bound the TSK DEAE-NPR column and were eluted with ~130 mM KCl. In control experiments p28b flowed through the TSK DEAE-NPR column when TRFP was not present (Fig. 4B). Similarly, an HSPC296-Surf5 heterodimer could be purified to near homogeneity by anti-Flag immunoaffinity chromatography of Flag-tagged HSPC296 and C-Myc-tagged Surf5 that had been coexpressed in Sf21 cells (Fig. 5A).

To determine whether Surf5 can bind directly to TRAP25, either GST-TRAP25 and Surf5 or GST and Surf5 were coexpressed in *E. coli*, and cell lysates were subjected to glutathione-agarose chromatography. Because GST and Surf5 coelectrophorese on SDS-polyacrylamide gels, the presence or absence of Surf5 in column eluates was detected by Western blotting with anti-Surf5 antibodies. As shown in Fig. 5B, bacterially expressed Surf5 bound specifically to bacterially expressed GST-TRAP25 but not to GST.

Finally, binding of LCMR1 to Soh1 was detected following pairwise coexpression of these proteins in Sf21 cells. As shown in Fig. 6, lanes 1–4, LCMR1 and Soh1 could be coimmunoprecipitated efficiently with anti-LCMR1 antibodies from lysates of cells expressing both LCMR1 and Soh1, whereas only a small amount of Soh1 was detected in immunoprecipitates from cells expressing Soh1 alone. To determine whether LCMR1 can bind directly to Soh1, thioredoxin-LCMR1, which had been expressed in and purified from *E. coli*, was mixed with either GST-Soh1 or GST and subjected to glutathione-agarose chromatography. Full-length Trx-LCMR1 and GST-Soh1 coelectrophorese on SDS-polyacrylamide gels (data not shown). Therefore, Trx-LCMR1 was detected by Western blotting with anti-LCMR1 antibodies. As shown in Fig. 6, lanes 8–10, Trx-LCMR1 bound GST-Soh1 but not GST. Further supporting the specificity of this interaction, a large fraction of Trx-LCMR1 in binding reactions had been proteolyzed during its expression and/or purification (lanes 5–7); however, the full-length protein bound most efficiently to GST-Soh1.

In summary, in this report we present direct biochemical evidence that the mammalian p28b, Surf5, HSPC296, and LCMR1 proteins are *bona fide* subunits of the mammalian Mediator complex. Taken together with the bioinformatic evidence of Bourbon and co-workers (8), which suggests that the p28b and Surf5 proteins may be homologues of *S. cerevisiae* Mediator subunits Srb5 and Srb6, our finding that the HSPC296 and LCMR1 proteins share sequence similarity with *S. cerevisiae* Mediator subunits Med11 and Rox3 suggests that yeast and higher eukaryotic Mediator complexes may be more

highly conserved throughout evolution than previously thought. Ultimately, however, experiments that directly explore the structural and functional relationships between apparent homologues of yeast and higher eukaryotic Mediator subunits will be required to determine the extent of evolutionary conservation between yeast and higher eukaryotic Mediator complexes.

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