The multiprotein Mediator complex is a coactivator required for activation of RNA polymerase II transcription by DNA bound transcription factors. We previously identified and partially purified a mammalian 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 by tandem mass spectrometry of proteins present in the most highly purified rat Mediator fractions led to the identification of a collection of new mammalian Mediator subunits, as well as several potential Mediator subunits including a previously uncharacterized protein encoded by the FLJ10193 open reading frame. In this study, we present direct biochemical evidence that the FLJ10193 protein, which we designate Med25, is a bona fide subunit of the mammalian Mediator complex. In addition, we present evidence that Med25 shares structural and functional properties with Saccharomyces cerevisiae Mediator subunit Cse2 and may be a mammalian Cse2 ortholog. Taken together, our findings identify a novel mammalian Mediator subunit and shed new light on the architecture of the mammalian Mediator complex.

The Mediator (Med)1 is a multiprotein coactivator that promotes RNA polymerase II transcriptional activation through direct interactions with transcription factors bound at enhancers and upstream promoter elements and with polymerase and the general initiation factors at the core promoter. The Mediator was first identified in Saccharomyces cerevisiae and found to be comprised of more than twenty proteins including Srb2, Srb4, Srb5, Srb6, Srb7, Srb8, Srb9, Srb10, Srb11, Med1, Med2, Med4, Med6, Med7, Med8, Med11, Pgd1, Rox3, Cse2, Nut1, Nut2, Gal11, Sin4, and Rgr1 (1).

A mammalian Mediator complex similar to yeast Mediator was subsequently identified in several laboratories and designated mouse Mediator (2), TRAP (thryoid hormone receptor-associated proteins)/SMCC (Srb-Med-containing cofactor) (3), ARC (activator-recruited cofactor) (4), DRIP (vitamin D receptor-interacting proteins) (5), CRSP (cofactor required for Sp1 activation) (6), or rat Mediator (7, 8). Biochemical characterization of the mammalian Mediator complex has revealed that it is composed of apparent homologs of many of the S. cerevisiae Mediator subunits including TATA-binding protein related factor-proximal protein (TRFP) (Srb2), TRAP80 (Srb4), p28b (Srb5), Surf5 (Srb6), Srb7, TRAP240 (Srb9), Cdk8 (Srb10), CycC (Srb11), TRAP36 (Med4), Med6, Med7, Med8, HSPC296 (Med11), LCMR1 (Rox3), Nut2, Sur2 (Gal11), TRAP95 (Sin4), and Rgr1 (reviewed in Ref. 9).

In a previous study (7), we partially purified a mammalian Mediator complex from rat liver nuclei. Analysis of proteins present in our most highly purified rat Mediator fractions by tandem mass spectrometry led to the identification of a large fraction of the known mammalian Mediator subunits, as well as a collection of potentially new Mediator subunits. In this report, we identify one such protein encoded by the FLJ10193 open reading frame as a new subunit of the mammalian Mediator complex.

**EXPERIMENTAL PROCEDURES**

*Materials—Anti-FLAG (M2) monoclonal antibodies, anti-Myc (C-3956) rabbit polyclonal antibodies, anti-FLAG (M2) agarose, and FLAG peptide were purchased from Sigma. Anti-Myc (9E10) monoclonal antibodies were obtained from Roche Applied Science. Anti-TRAP220 (C-19), anti-Cdk8 (n-9), and anti-Med6 (E-20) antibodies were obtained from Santa Cruz Biotechnology. Anti-Med8 rabbit polyclonal antibodies were raised against a peptide corresponding to Med8 residues 247–268 (Cocalico Biologicals, Inc.). Light chain-specific anti-mouse antibodies were purchased from Bethyl Laboratories and labeled with Alexa Fluor 680 (Molecular Probes) according to the manufacturer’s instructions. SuperSignal West Dura extended duration substrate and SuperSignal West Femto Maximum Sensitivity Substrate were obtained from Pierce. Glutathione-Sepharose 4 Fast Flow and Glutathione-Sepharose 4B beads were from Amersham Biosciences.\(^*\)

**Anti-FLAG Agarose Chromatography—**Anti-FLAG agarose immuno-affinity chromatography was carried out essentially as described for purification of the TRAP-SMCC Mediator complex (10). HeLa cell nuclear extracts were prepared according to the method of Dignam et al. (11). Dialyzed nuclear extracts were incubated with anti-FLAG (M2) agarose beads in 10 mM Hepes-NaOH (pH 7.9), 1.5 mM MgCl\(_2\), 0.5 mM KCl, and 0.2% Triton X-100 for at least 4 h at 4°C. The beads were washed 5 times with a 50-fold excess of the same buffer and once with

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a 50-fold excess of 10 mM Hepes-NaOH (pH 7.9), 1.5 mM MgCl₂, 0.1 M NaCl, and 0.05% Triton X-100. Beads were then eluted with 10 mM Hepes-NaOH (pH 7.9), 1.5 mM MgCl₂, 0.1 M NaCl, and 0.05% Triton X-100, and 0.2 mg/ml FLAG peptide. All buffers contained 1:1000-fold dilution of Protease Inhibitor Mixture (Sigma catalog number P8340).

Mass Spectrometry—Identification of proteins was accomplished using a modification of the MudPIT procedure described by Washburn et al. (12). Aliquots of anti-FLAG agarose eluates were brought to 100 μl with 1 M Tris-HCl (pH 8.5) and H₂O to a final concentration of 100 mM Tris-HCl. Solid urea was added to 8 M. Proteins were reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Roche), alkylated with 20 mM iodoacetamide (IAM, Sigma), and digested with endoproteinase Lys-C (Roche) for at least 6 h at 37 °C. Proteins were then diluted 4 times 100 mM Tris-HCl (pH 8.5) and digested overnight at 37 °C with modified Trypsin (Roche), alkylated with 20 mM iodoacetamide (IAM, Sigma), and digested with endoproteinase Lys-C (Roche) for at least 6 h at 37 °C. Proteins were then diluted 4 times 100 mM Tris-HCl (pH 8.5) and digested overnight at 37 °C with modified Trypsin (Roche), alkylated with 20 mM iodoacetamide (IAM, Sigma), and digested with endoproteinase Lys-C (Roche) for at least 6 h at 37 °C.

Peptide mixtures were pressure-loaded onto a 250 μm inner diameter (i.d.) fused-silica capillary packed first with 4 cm of 5 μm strong cation exchange material (Partisil SCX, Whatman), followed by 2 cm of 5 μm C18 reverse phase particles (Aqua, Phenomenex). Loaded and washed microcapillaries were connected via a 2 μm filtered union (UpChurch Scientific) to a 100 μm i.d. column, which had been pulled to a 5 μm i.d. tip using a P-2000 CO₂ laser puller (Sutter Instruments), then packed with 10 cm of reverse phase particles and equilibrated in 5% acetonitrile, 0.1% formic acid (Buffer A). This split-column was then installed in-line with a Quaternary Agilent 1100 series HPLC pump. Overflow tubing was used to decrease the flow rate from 0.1 ml/min to about 200–300 nl/min. Fully automated 6 step chromatography runs were carried out. Three different elution buffers were used: Buffer A; 80% acetonitrile, 0.1% formic acid (Buffer B); and 0.5M ammonium acetate, 5% acetonitrile, 0.1% formic acid (Buffer C). In such sequences of chromatographic events, peptides are sequentially eluted from the SCX resin to the reverse phase resin by increasing salt steps (increase in Buffer C concentration), followed by organic gradients (increase in Buffer B concentration). The last chromatography step consists in a high salt wash with 100% Buffer C followed by the acetonitrile gradient. The application of a 2.5 kV distal voltage electrosprayed the eluting peptides directly into a Deca-XP ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (ThermoFinnigan). Full MS spectra were recorded on the peptides over a 400 to 1,600 m/z range, followed by three tandem mass (MS/MS) events sequentially generated.
in a data-dependent manner on the first, second, and third most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFinnigan). The software algorithm ZYGA (13) was used to determine charge state and to delete spectra of poor quality. SEQUEST (14) was used to match MS/MS spectra to peptides in a data base containing 82,422 human, mouse, and rat protein sequences downloaded from NCBI on October 23, 2003. The validity of peptide/spectrum matches was assessed using the SEQUEST-defined parameters, cross-correlation score (XCorr) and normalized difference in cross-correlation scores (DeltCn). Spectra/peptide matches were only retained if they had a DeltCn of at least 0.05 and minimum XCorr of 1.8 for +1, 2.5 for +2, and 3.5 for +3 spectra. In addition, the peptides had to be at least 7 amino acids long. DTASelect (15) was used to select and sort peptide/spectrum matches passing this criteria set. Peptide hits from multiple runs were compared using CONTRAST (15).

RESULTS AND DISCUSSION

We previously described partial purification from rat liver nuclear extracts of a multiprotein Mediator complex exhibiting an apparent native molecular mass by gel filtration of more than 1000 kDa (7). Our most highly purified rat Mediator contained many previously characterized mammalian Mediator subunits, as well as a collection of potential Mediator subunits including the p28b, Surf5, LCMR1, HSPC296, and mammalian Intersex proteins, which we subsequently demonstrated are bona fide subunits of the mammalian Mediator complex (8, 16). Among the collection of additional proteins present in our most highly purified rat Mediator fractions and identified by mass spectrometry was a 142-amino acid protein encoded by the FLJ10193 open reading frame (Fig. 1). PSI-BLAST searches of the NCBI protein data base indicates that the FLJ10193 protein has diverged substantially during evolution, with obvious FLJ10193 orthologs in insects, but not in more distantly related species such as Caenorhabditis elegans and yeast. The N-terminal ~50 amino acids of FLJ10193 diverge significantly even between human and mouse; in contrast, the C-terminal ~80 amino acids is highly conserved from mammals to insects, suggesting that it may represent the functional core of the protein.

Multiple sequence alignments of the FLJ10193 protein with yeast Mediator subunits identified two short regions of FLJ10193 with some similarity to S. cerevisiae Mediator subunit Cse2 (Fig. 1B), raising the possibility that FLJ10193 and Cse2 may be related. Notably, like the sequence of the FLJ10193 protein, the sequence of Cse2 has diverged substantially during evolution, with apparent Cse2 orthologs in yeasts closely related to S. cerevisiae, but not in more distantly related yeasts such as Schizosaccharomyces pombe, Candida, and Neurospora. Below we present direct biochemical evidence that the FLJ10193 protein, which we designate Med25, is a previously unrecognized subunit of the mammalian Mediator complex with features similar to those of yeast Mediator subunit Cse2.

To begin to address the possibility that the Med25 protein is a component of the mammalian Mediator complex, we sought to identify Med25-associating proteins. To accomplish this, we took advantage of four HeLa cell lines stably expressing either human Mediator subunit Nut2 (10), mouse Mediator subunit LCMR1 (16), human Mediator subunit Intersex (16), or the mouse Med25 protein, all with N-terminal FLAG epitope tags. The FLAG-Nut2 expressing cell line has been used extensively for preparation of the transcriptionally active TRAP/SMCC Mediator complex (10), and we have previously used HeLa cell
lines expressing FLAG-tagged mouse LCMR1 and FLAG-tagged human Intersex as sources for anti-FLAG immunoaffinity purification of the mammalian Mediator complex (16).

Nuclear extracts prepared from equivalent numbers of parental, FLAG-Nut2, FLAG-LCMR1, FLAG-Intersex, and FLAG-Med25 expressing HeLa cells were subjected to anti-FLAG agarose chromatography as described previously for purification of the TRAP\textsubscript{SMCC} Mediator complex (10). As shown in the silver-stained SDS-polyacrylamide gel of Fig. 2, anti-FLAG agarose eluate from FLAG-Med25 expressing HeLa cells appeared to include a very similar set of proteins as the TRAP\textsubscript{SMCC} Mediator complex purified from FLAG-Nut2, FLAG-LCMR1, or FLAG-Intersex expressing HeLa cells. In addition, analysis of Med25-associating proteins present in the anti-FLAG agarose eluate from FLAG-Med25 expressing HeLa cells by tandem mass spectrometry (Table I in Supplemental Material) and by Western blotting (Fig. 3) identified nearly all of the mammalian Mediator subunits known to be present in the TRAP\textsubscript{SMCC} Mediator complex, arguing that Med25 is a previously unrecognized subunit of the mammalian Mediator complex. The Med25 protein was detected by Western blotting only in anti-FLAG agarose eluates from FLAG-Med25 and FLAG-Intersex expressing HeLa cells. Med25 was, however, detected by tandem mass spectrometry in anti-FLAG-agarose eluates from all four HeLa stable cell lines, but not from parental HeLa cells (Table II in Supplemental Material). These observations suggest that the Med25 protein is present at varying levels in these different Mediator preparations. Finally, and consistent with previous findings indicating that the mammalian Mediator complex binds to and can be purified through interactions with the VP16 transcriptional activation domain (4, 10), we observe that the Med25 protein can be purified together with other Mediator subunits from nuclear extracts of FLAG-Med25 expressing HeLa cells by GST-VP16 chromatography. As shown in the Western blot of Fig. 4, the Med25 protein and representative Mediator subunits Cdk8, Med8, Med6, and TRAP36 are copurified from HeLa cell nuclear extracts using immobilized GST-VP16, but not GST. Thus, the Med25 protein copurifies with the mammalian Mediator complex by several independent methods, arguing that it is a \textit{bona fide} Mediator subunit.

To obtain additional evidence supporting assignment of the Med25 protein as a new subunit of the mammalian Mediator complex, we sought to identify pair-wise binding partners of Med25 among the known mammalian Mediator subunits. To accomplish this, we began by exploiting a convenient screen (8).

**Fig. 4.** The Med25 protein associates with the VP16 transcriptional activation domains. GST-VP16 or GST alone was prebound to glutathione-Sepharose 4B beads in 10 mM Hepes-NaOH (pH 7.9), 0.3 M KCl, 1.5 mM MgCl\textsubscript{2}, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, and 0.1% Triton X-100 and incubated for at least 4 h at 4°C with undialyzed nuclear extracts prepared from FLAG-Med25 expressing HeLa cells according to the method of Dignam et al. (11). The glutathione-Sepharose beads were then washed 7 times with a 50-fold excess of the same buffer containing 0.5 M KCl, and bound proteins were eluted from the beads with 100 mM Tris-HCl (pH 7.9), 0.12 M NaCl, 0.1% Triton X-100, and 20 mM glutathione. Aliquots of glutathione-Sepharose eluates were analyzed by 10% or 13% SDS-PAGE, and proteins were visualized by Western blotting with antibodies recognizing the Mediator subunits indicated in the figure. Western blots were developed using horseradish peroxidase-labeled secondary antibodies and Super-Signal West Dura extended duration substrate or SuperSignal West Femto Maximum Sensitivity Substrate.

**Fig. 5.** Screening for Med25 binding partners among the known mammalian Mediator subunits. \textsuperscript{35}S-labeled Mediator subunits were synthesized in the rabbit reticulocyte lysate TNT T7 Quick Coupled Transcription/Translation System (Promega) programmed with pcDNA3.1 expression vectors encoding the mammalian Mediator subunits and other proteins indicated in the figure. Reticulocyte lysates containing the indicated \textsuperscript{35}S-labeled proteins were mixed with bacterially expressed and purified GST-Med25 or GST alone prebound to glutathione-Sepharose 4 Fast Flow beads in phosphate-buffered saline and incubated for 2 h with rocking at 4°C. The beads were washed 2 times with 40 mM Tris-HCl (pH 7.5), 0.25 M NaCl, and 0.2% Triton X-100. The beads were then washed once with Tris-buffered saline, and proteins were eluted from the beads with Tris-buffered saline containing 10 mM glutathione. Aliquots of glutathione-Sepharose eluates were fractionated by 12% SDS-PAGE, and proteins were visualized using a Typhoon 8600 PhosphorImager (Amersham Biosciences).
to assess the ability of Med25 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 and additional proteins indicated in Fig. 5 were used to program rabbit reticulocyte lysates for translation of 35S-labeled Mediator proteins. Binding of 35S-labeled Mediator proteins to a GST-Med25 fusion protein was assayed in GST pull-down experiments using glutathione-agarose beads, and bound proteins were visualized by autoradiography. The results of this screen revealed significant and reproducible binding of Med25 to Mediator subunit TRAP36 (Fig. 5), the mammalian ortholog of yeast Mediator subunit Med4. Notably, in a previous study, S. cerevisiae Mediator subunit Cse2 was found to interact directly only with yeast Mediator subunit Med4 (17), suggesting that Med25 and Cse2 may be functionally related.

To confirm and extend this finding, we sought to reconstitute the Med25-TRAP36 interaction detected in in vitro translation experiments in transiently transfected human 293T cells and with recombinant proteins expressed in SF21 insect cells and E. coli. As shown in Fig. 6A, the TRAP36 protein could be coinmunoprecipitated with the Med25 protein from lysates of 293T cells cotransfected with pcDNA3.1 expression vectors encoding TRAP36 and Med25. In addition, direct pairwise binding of Med25 to TRAP36 was detected following their coexpression in SF21 cells (Fig. 6B) and in E. coli (Fig. 6C), arguing that Med25 binds directly to TRAP36 and may be recruited to the Mediator complex through this interaction.

In summary, in this report we identify the previously uncharacterized FLJ10193 protein as a new mammalian Mediator subunit designated Med25. Although lower eukaryotic orthologs of the Med25 protein have not yet been unequivocally identified, our findings have brought to light structural and functional similarities between Med25 and yeast Mediator subunit Cse2. First, Med25 exhibits limited sequence similarity with Cse2. Second, like Cse2, the Med25 protein forms a heterodimer with Mediator subunit Med4 (or TRAP36 in mammals) and thus may be recruited to the “middle module” of the mammalian Mediator complex through its interaction with Med4. Whether Med25 fulfills a similar role as Cse2 in Mediator, however, remains to be determined.

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(Sigma), and 20 μM imidazole. Proteins bound to the Ni-NTA agarose were eluted in the same buffer containing 0.3 M imidazole. Ni-NTA eluates were then further fractionated by glutathione-Sepharose chromatography using glutathione-Sepharose 4B pre-equilibrated in Ni-NTA elution buffer lacking imidazole, and proteins were eluted with the same buffer containing 20 μM glutathione and 1 μM (2-aminoethyl)benzenesulfonyl fluoride. Proteins present in aliquots of glutathione-Sepharose eluates were analyzed by 10% SDS-PAGE and visualized by Coomassie staining. IP, immunoprecipitation; FL, Med25, FLAG-Med25; his, His-tag; Trx, thioredoxin; glut, glutathione.