A Mammalian Homolog of *Drosophila melanogaster* Transcriptional Coactivator Intersex Is a Subunit of the Mammalian Mediator Complex*

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From the Stowers Institute for Medical Research, Kansas City, Missouri 64110, the Division of Biology, California Institute of Technology, Pasadena, California 91125, the Department of Biochemistry and Molecular Biology, Kansas University Medical Center, Kansas City, Kansas 66160, and the **Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190.

The multiprotein Mediator complex is a coactivator required for transcriptional activation of RNA polymerase II transcribed genes by DNA binding transcription factors. We previously 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, L., 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 enriched Mediator fractions by tandem mass spectrometry led to the identification of several new mammalian Mediator subunits, as well as several potential Mediator subunits. Here we identify one of these proteins, encoded by the previously uncharacterized AK000411 open reading frame, as a new subunit of the mammalian Mediator complex. The AK000411 protein, which we designate hIntersex (human Intersex), shares significant sequence similarity with the *Drosophila melanogaster* intersex protein, which has functional properties expected of a transcriptional coactivator specific for the *Drosophila* doublesex transactivator. In addition, we show that hIntersex assembles into a subcomplex with Mediator subunits p28b and TRFP. Taken together, our findings identify a new subunit of the mammalian Mediator and shed new light on the architecture of the mammalian Mediator complex.

The *Drosophila melanogaster* doublesex (*dsx*) gene encodes a transcription factor that is necessary for somatic sexual development in male and female flies (1, 2). Male- and female-specific forms of the doublesex proteins, DSX^M^ and DSX^F^, are encoded by alternatively spliced forms of the *dsx* transcript. DSX^M^ and DSX^F^ bind to an enhancer in the promoter of the *yolk protein 1* and *protein 2* genes and repress or activate their transcription in male and female flies, respectively (3, 4). Activation of the *yolk protein 1* and *protein 2* genes by DSX^F^ also requires the product of the *intersex* (*ix*) gene. Although the precise mechanism of action of the *intersex* protein has not been determined, it appears to function as a coactivator for DSX^F^, since it binds specifically to DSX^F^ in vitro and is required for activation of a reporter gene driven by the *yolk protein* enhancer in flies (5).

The Mediator of RNA polymerase II transcription is a multiprotein coactivator that regulates eukaryotic messenger RNA synthesis through direct interactions between DNA bound transcriptional activators and RNA polymerase II and the general initiation factors. The Mediator was first identified and characterized in *Saccharomyces cerevisiae*, where it was found to be composed 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 (6).

Structurally and functionally similar mammalian Mediator complexes were subsequently identified in several laboratories and designated Mediator (7), TRAP (thyroid hormone receptor-associated proteins)/SMCC (Srb-Med-containing cofactor) (8), ARC (activator-recruited cofactor) (9), DRIP (vitamin D receptor-interacting proteins) (10), CRSP (cofactor required for Sp1 activation) (11), and rat liver Mediator (12, 13). Characterization of these mammalian Mediator complexes revealed that they are composed of apparent homologs of many of the *S. cerevisiae* Mediator subunits including TRFP (Srb2), TRAP50 (Srb4), p28b (Srb5), Surf5 (Srb6), TRAP240 (Srb8), TRAP230 (Srb9), Cdk8 (Srb10), Cyclin C (Srb11), TRAP36 (Med4), Med6, Med7, Med8, HSPC296 (Med11), LCMR1 (Rox3), Nut2, Surf2 (Gal11), TRAP95 (Sin4), and Rgr1 (for a review, see Ref. 14).

We recently partially purified a Med8-containing Mediator complex from rat liver nuclei (12). Analysis of proteins present in our most highly enriched Mediator fractions by tandem mass spectrometry led to the identification of a large number of the known mammalian Mediator subunits, as well as a collection of proteins not previously recognized as subunits of the mammalian Mediator complex. In this report, we identify one such protein encoded by the AK000411 ORF as an apparent homolog of *Drosophila* intersex (5), raising the possibility that direct contacts between the *Drosophila* DSX^F^ and intersex proteins could recruit the Mediator complex to promoters activated by DSX^F^ in flies.

### Notes

The abbreviations used are: TRAP, thyroid hormone receptor-associ- ated protein; ARC, activator-recruited cofactor; CRSP, cofactor required for Sp1 activation; DRIP, vitamin D receptor-interacting protein; GST, glutathione S-transferase; Med, Mediator; HPLC, high pressure liquid chromatography; ORF, open reading frame; SMCC, Srb-Med-containing cofactor; Srb, suppressor of RNA polymerase B; TRFP, TATA-binding protein-related factor-proximal protein; TR, thyroid hormone receptor; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

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EXPERIMENTAL PROCEDURES

Materials—Anti-FLAG (M2) monoclonal antibodies, anti-Myc (C-3956) rabbit polyclonal antibodies, anti-FLAG-agarose, and anti-FLAG peptide were purchased from Sigma. Anti-c-Myc (9E10) monoclonal antibodies were obtained from Roche Molecular Biochemicals. Anti-Med8 (E-20) and anti-TRFP (E-18) monoclonal antibodies were from Santa Cruz Biotechnology. Anti-FLAG-Agarose Chromatography—Anti-FLAG-agarose immunoaffinity chromatography was carried out essentially as described for the TRAP/SMCC Mediator complex (15). HeLa cell nuclear extracts were prepared according to the method of Dignam et al. (16). Undialyzed nuclear extracts were incubated with anti-FLAG (M2)-agarose beads in buffer A (10 mM Hepes-NaOH (pH 7.9), 1 mM MgCl₂, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 0.1% Triton X-100) containing 0.3 M NaCl and 250 mM imidazole. The beads were washed five times with a 50-fold excess of buffer A containing 0.3 M NaCl and once with a 50-fold excess of buffer A containing 1.0 M NaCl. Bound proteins were eluted from the beads with 10 mM Hepes, 1.5 mM MgCl₂, 0.05% Triton X-100, and 2 mg/ml FLAG peptide.

Expression of Recombinant Proteins in Insect Cells—S2F1 cells were cultured at 27 °C in SF-900 II SFM (Invitrogen) with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. S2F1 cells were infected at a multiplicity of infection of 10 or 20 with the appropriate recombinant baculoviruses. Forty-eight hours after infection, cells were collected and lysed in ice-cold buffer containing 50 mM Hepes-NaOH (pH 7.9), 0.5 mM MgCl₂, 0.2% Triton X-100, 20% (v/v) glycerol, 0.28 mg/ml leupeptin, 1.4 mg/ml pepstatin A, 0.17 mg/ml phenylmethylsulfonyl fluoride, and 0.33 mg/ml benzamidine. Lysates were centrifuged 100,000 × g for 30 min at 4 °C.

Purification of Recombinant Proteins—FLAG-hIntersex was expressed in baculovirus-infected S2F1 cells and purified by anti-FLAG-agarose chromatography as described above. The eluate from anti-FLAG-agarose chromatography was adjusted to a conductivity equivalent to that of 0.05 M NaCl and applied to a 0.6 ml of TSK DEAE-NPB HPLC column (Tosoh-Biosep) pre-equilibrated in buffer C (40 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM diithiothreitol, and 1% (v/v) glycerol) containing 0.1 mM NaCl. FLAG-hIntersex was recovered in the flow-through fraction. His-Myc-TRFP was expressed in baculovirus-infected S2F1 cells and purified batchwise by nickel chromatography using His-Select™ IC nickel affinity gel (Sigma). Following incubation of S2F1 lysates containing His-Myc-TRFP with nickel affinity gel, the gel was washed with buffer B (40 mM Hepes-KOH (pH 7.6), 20% (v/v) glycerol, 0.28 μg/ml leupeptin, 1.4 μg/ml pepstatin A, 0.17 mg/ml phenylmethylsulfonyl fluoride, and 0.33 mg/ml benzamidine) containing 0.5 mM NaCl and 10 mM imidazole, and bound proteins were eluted with buffer B containing 0.3 mM NaCl and 250 mM imidazole.

Mass Spectrometry—Proteins were fractionated by SDSPolyacrylamide gel electrophoresis. Proteins in gel slices were subjected to in-gel reduction, S-carboxymethylation, and tryptic digestion. Peptide sequences were determined in a single run by microcapillary reversed-phase HPLC coupled to the nanospray ionization source of a quadrupole ion trap mass spectrometer (Finnigan LCQ DECA XP™PLUS, San Jose, CA). Tandem mass spectrometry spectra were interpreted using the SEQUEST algorithm run in the BioWorks 3.0 software package from ThermoFinnigan.

RESULTS AND DISCUSSION

We previously reported partial purification from rat liver nuclear extracts of a multiprotein Mediator complex with an apparent native molecular mass by gel filtration of more than 1000 kDa (12). Analysis of proteins present in the most highly enriched Mediator fractions by tandem mass spectrometry led to the identification of many previously characterized mammalian Mediator subunits, as well as a collection of potential...
Mediator subunits including the LCMR1, p28b, Surf5, and HSPC296 proteins, which we subsequently demonstrated are bona fide Mediator subunits (13). Among the additional proteins present in the most highly enriched Mediator fractions and identified by mass spectrometry was a previously uncharacterized, 200-amino acid protein encoded by the AK000411 ORF (Fig. 1A). PSI-BLAST searches of the NCBI protein database revealed that the AK000411 protein bears significant sequence similarity (∼E-value 2e−15) to the 188-amino acid D. melanogaster hIntersex protein (Fig. 1B). BLAST searches revealed no obvious hIntersex orthologs in lower eukaryotes including Caenorhabditis elegans and yeast.

To begin to address the possibility that hIntersex is a bona fide subunit of the mammalian Mediator complex, we took advantage of three HeLa cell lines stably expressing either Mediator subunit Nut2, Mediator subunit LCMR1, or hIntersex, all with N-terminal FLAG tags. The FLAG-Nut2-expressing HeLa cell line has been used extensively as a source for bona fide HSPC296 proteins, which we subsequently demonstrated are Mediator subunits including the LCMR1, p28b, Surf5, and HSPC296 proteins, which we subsequently demonstrated are bona fide Mediator subunits (13). Among the additional proteins present in the most highly enriched Mediator fractions and identified by mass spectrometry was a previously uncharacterized, 200-amino acid protein encoded by the AK000411 ORF (Fig. 1A). PSI-BLAST searches of the NCBI protein database revealed that the AK000411 protein bears significant sequence similarity (∼E-value 2e−15) to the 188-amino acid D. melanogaster hIntersex protein (Fig. 1B). BLAST searches revealed no obvious hIntersex orthologs in lower eukaryotes including Caenorhabditis elegans and yeast.

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complex. Finally, and consistent with previous findings indicating that the mammalian Mediator complex binds to and can be purified through interactions with the VP16 and thyroid hormone receptor (TR) transcriptional activation domains, we observe that hIntersex can be purified along with other Mediator subunits from HeLa cell lysates by GST-VP16 or GST-TR chromatography. As shown in Fig. 3, hIntersex, as well as the Mediator subunits TRAP80 and Med8, can be purified from HeLa cell lysates using immobilized GST-VP16, but not GST. Similarly, hIntersex and other Mediator subunits bind immobilized GST-TR in a ligand-dependent fashion. Thus, we observe that the hIntersex protein copurifies with the mammalian Mediator complex by several independent methods, arguing that it is a bona fide subunit of the Mediator.

To obtain additional evidence supporting assignment of the hIntersex protein as a subunit of the mammalian Mediator complex, we sought to identify pairwise binding partners of the hIntersex protein among the known Mediator subunits. To accomplish this, we carried out initial screens to assess the ability of hIntersex to interact with known mammalian Mediator subunits coexpressed with hIntersex in transiently transfected 293T cells or baculovirus-infected insect cells and to assess the ability of in vitro translated Mediator subunits to bind bacterially expressed GST-hIntersex. The results of these experiments identified the TRFP, TRAP25, and Surf5 Mediator subunits as potential hIntersex binding partners (data not shown), with TRFP exhibiting the best binding in all assays. The hIntersex-TRFP interaction could be reconstituted by mixing FLAG-hIntersex and His-Myc-TRFP, which had been expressed independently in baculovirus-infected insect cells and used as shown), with TRFP exhibiting the best binding in all assays. The hIntersex-TRFP interaction could be reconstituted by mixing FLAG-hIntersex and His-Myc-TRFP, which had been expressed independently in baculovirus-infected insect cells and purified. As shown in Fig. 4A, hIntersex alone flowed through a TSK DEAE-NPR column at 0.1 M NaCl, whereas the reconstituted hIntersex-TRFP complex bound to the column and eluted with ~0.15 M NaCl.

In light of our previous observation that TRFP interacts directly with the p28b Mediator subunit to form a stable heterodimer (13), we investigated the possibility that hIntersex can interact with the TRFP-p28b complex. To accomplish this, insect cells were cotransfected with baculoviruses encoding various combinations of FLAG-hIntersex, Myc-TRFP, and Myc-p28b. As shown in Fig. 4B, Myc-TRFP and Myc-p28b could be communoprecipitated with FLAG-hIntersex. Binding of p28b to hIntersex depended on the presence of TRFP, indicating that TRFP bridges hIntersex and p28b in the complex and raising the possibility that the TRFP-p28b module may serve to recruit the hIntersex protein into the Mediator complex.

In summary, in this report we identify the previously uncharacterized mammalian hIntersex protein as a new subunit of the mammalian Mediator complex. Our data indicates that hIntersex is likely to be located in or adjacent to the Mediator head-domain (also known as the Srb4 subcomplex), since we have shown that it forms a heteromeric complex with TRFP and p28b, mammalian homologs of the S. cerevisiae head-domain subunits Srb2 and Srb5 (14, 17). Whether the Drosophila intersex protein is also a subunit of the Drosophila Mediator complex remains to be determined; however, it seems most likely that it is given the strong similarity between other mammalian and Drosophila Mediator subunits. Because the Drosophila intersex protein functions as a coactivator for the sex-specific DNA binding transactivator DSXF (5), our results raise the possibilities (i) that direct contacts between the Drosophila DSXF and intersex proteins could recruit the Mediator complex to promoters activated by DSXF in flies and (ii) that hIntersex, analogous to its Drosophila homolog, functions as an adaptor molecule between the human Mediator complex and one or more DNA binding transactivators. At the present time, we have not identified transactivators that function through interactions with hIntersex, and we have been unable to detect a direct interactions between hIntersex and Drosophila DSXF. Although a number of mammalian transcription factors of the Doublesex family have been identified (18), they share little homology with Drosophila doublesex outside of their DNA binding domains. Consequently, more work will be required to determine which, if any, transactivators function through direct interactions with hIntersex and whether hIntersex, like Drosophila intersex, plays a critical role in specific developmental pathways.

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