

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

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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, 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 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.

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Supplemental Table 1.

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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 mouse Mediator (7), TRAP¹ (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), TRAP80 (Srb4), p28b (Srb5), Surf5 (Srb6), Srb7, TRAP240 (Srb8), TRAP230 (Srb9), Cdk8 (Srb10), Cyclin C (Srb11), TRAP36 (Med4), Med6, Med7, Med8, HSPC296 (Med11), LCMR1 (Rox3), Nut2, Sur2 (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 a subunit of the mammalian Mediator complex. The protein encoded by the AK000411 ORF is 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.

¹ The abbreviations used are: TRAP, thyroid hormone receptor-associated 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; AEBFSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

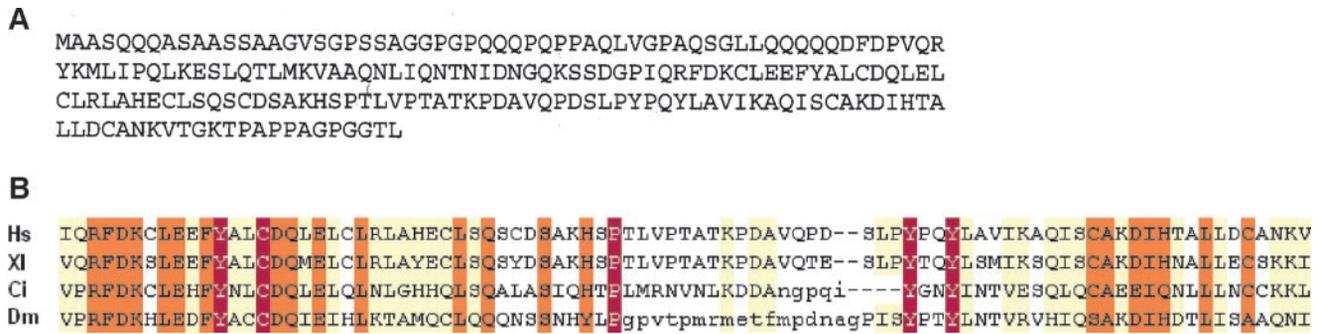


FIG. 1. The AK000411 protein shares significant sequence similarity with *Drosophila intersex*. A, amino acid sequence of the human AK000411 protein. B, multiple sequence alignment of higher eukaryotic and *D. melanogaster* Intersex proteins. Multiple sequence alignment was performed using the MACAW program (19). Accession numbers are AK000411, human (Hs); CB201403, *Xenopus laevis* (XI); BW175616, *Ciona intestinalis* (Ci); and NP_610677, *D. melanogaster intersex* (Dm).

EXPERIMENTAL PROCEDURES

Materials—Anti-FLAG (M2) monoclonal antibodies, anti-Myc (C-3956) rabbit polyclonal antibodies, anti-FLAG (M2)-agarose, and anti-FLAG peptide were purchased from Sigma. Anti-c-Myc (9E10) monoclonal antibodies were obtained from Roche Molecular Biochemicals. Anti-Med6 (E-20) and anti-TRFP (E-18) polyclonal antibodies were from Santa Cruz Biotechnology. Anti-Med8 rabbit polyclonal antibodies were raised against a peptide corresponding to Med8 residues 247–268 (Cocalico Biologicals, Inc.). Anti-TRAP80, anti-p28b, anti-SOH1, anti-HSPC296, and anti-hIntersex rabbit polyclonal antibodies were raised against recombinant proteins expressed in insect cells or *Escherichia coli* (Cocalico Biologicals, Inc.). Light chain-specific anti-mouse antibodies were from Bethyl Laboratories and labeled with Alexa Fluor 680 (Molecular Probes) according to the manufacturer's instructions.

Anti-FLAG-Agarose Chromatography—Anti-FLAG-agarose immunoaffinity chromatography was carried out essentially as described for purification of 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 KCl for at least 4 h at 4 °C. 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 0.1 M NaCl. Bound proteins were eluted from the beads with 10 mM Hepes, 0.1 M NaCl, 1.5 mM MgCl₂, 0.05% Triton X-100, and 2 mg/ml FLAG peptide.

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 µg/ml streptomycin. Sf21 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 M NaCl, 5 mM MgCl₂, 0.2% Triton X-100, 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 benzamide. Lysates were centrifuged 100,000 × *g* for 30 min at 4 °C.

Purification of Recombinant Proteins—FLAG-hIntersex was expressed in baculovirus-infected Sf21 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-NPR HPLC column (Tosoh-BioSep) pre-equilibrated in buffer C (40 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol) containing 0.1 M NaCl. FLAG-hIntersex was recovered in the flow-through fraction. His-Myc-TRFP was expressed in baculovirus-infected Sf21 cells and purified batchwise by nickel chromatography using HIS-Select™ HC nickel affinity gel (Sigma). Following incubation of Sf21 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 benzamide) containing 0.5 M NaCl and 10 mM imidazole, and bound proteins were eluted with buffer B containing 0.3 M NaCl and 250 mM imidazole.

Mass Spectrometry—Proteins were fractionated by SDS-polyacrylamide gel electrophoresis. Proteins in gel slices were subjected to in-gel reduction, *S*-carboxyamidomethylation, 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

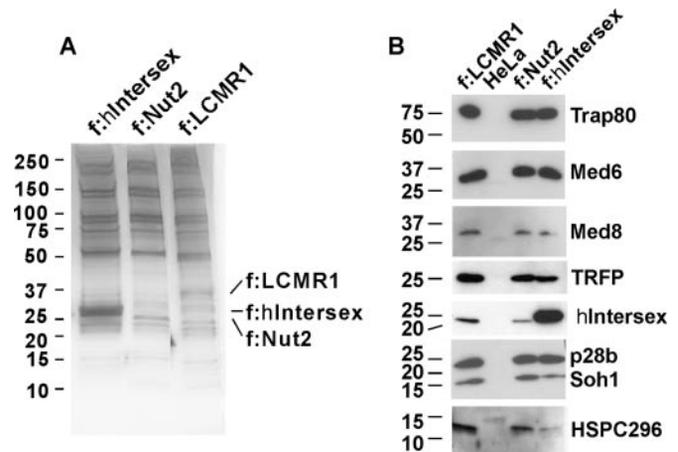


FIG. 2. The hIntersex protein is present in the purified TRAP/SMCC mammalian Mediator complex. A, comparison of proteins present in the TRAP/SMCC Mediator complex and anti-FLAG-agarose eluates from FLAG-hIntersex and FLAG-LCMR1-expressing HeLa cells by SDS-polyacrylamide gel electrophoresis. Anti-FLAG-agarose immunoaffinity chromatography of nuclear extracts from FLAG-hIntersex, FLAG-Nut2, and FLAG-LCMR1-expressing HeLa cells was carried out as described under "Experimental Procedures." Aliquots of anti-FLAG-agarose eluates were analyzed by SDS-polyacrylamide gel electrophoresis in a 4–20% gradient gel, and proteins were visualized by silver staining. The positions and relative molecular masses (in kilodaltons) of protein size markers are indicated on the left. f:hIntersex, FLAG-hIntersex; f:Nut2, FLAG-Nut2; f:LCMR1, FLAG-LCMR1. B, the hIntersex protein copurifies with subunits of the mammalian mediator complex. Anti-FLAG-agarose immunoaffinity chromatography of nuclear extracts from FLAG-hIntersex-, FLAG-Nut2-, and FLAG-LCMR1-expressing HeLa cells was carried out as described under "Experimental Procedures." Aliquots of anti-FLAG eluates were fractionated by SDS-polyacrylamide gel electrophoresis in a 4–15% gradient gel, and proteins were analyzed by Western blotting with the antibodies indicated in the figure. Western blots were developed using horseradish peroxidase-labeled secondary antibodies and Super-Signal West Dura extended duration substrate (Pierce). The positions and relative molecular masses (in kilodaltons) of protein size markers are indicated on the left. f:hIntersex, FLAG-hIntersex; f:Nut2, FLAG-Nut2; f:LCMR1, FLAG-LCMR1.

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

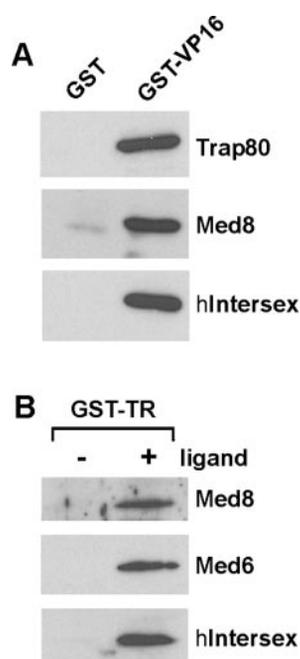


FIG. 3. hIntersex associates with the VP16 and TR transcriptional activation domains. *A*, GST-VP16 was prebound to glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences) in 10 mM Hepes-NaOH (pH 7.9), 0.5 M KCl, 1.5 mM MgCl₂, 0.5 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride), and 0.1% Triton X-100 and incubated for at least 4 h at 4 °C with undialyzed HeLa cell nuclear extracts prepared according to the method of Dignam *et al.* (16). Following incubation with HeLa cell nuclear extracts, the glutathione-Sepharose beads were washed seven times with a 50-fold excess of the same buffer. Bound proteins were eluted from the beads with GST elution buffer (50 mM glutathione, 100 mM Tris-HCl (pH 7.9), 120 mM NaCl, and 0.1% Triton X-100). *B*, GST-TR was prebound to glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences) in GST-TR buffer (100 mM NaCl, 20 mM Hepes-NaOH (pH 7.9), 0.2 mM EDTA, 0.05% Triton X-100, 0.5 mM AEBSF, and 1 mM dithiothreitol) with and without 100 μM ligand for TR (T3, 3,3',5-triiodo-L-thyronine). Following incubation with HeLa cell nuclear extracts at 4 °C for 4 h, the beads were washed five times with a 10-fold excess of GST-TR buffer containing a final concentration of 0.1% Triton X-100. Bound proteins were eluted from the beads with the same buffer containing 0.1% Triton X-100 and 26 μM TRAP220 peptide. Aliquots of glutathione-Sepharose eluates were fractionated by 10% SDS-polyacrylamide gel electrophoresis, and proteins were analyzed by Western blotting with the antibodies indicated in the figure. Western blots were developed using horseradish peroxidase-labeled secondary antibodies and Super-Signal West Dura extended duration substrate (Pierce).

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 data base revealed that the AK000411 protein bears significant sequence similarity (E -value $2e^{-13}$) to the 188-amino acid *D. melanogaster* intersex 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 anti-FLAG-agarose immunoaffinity purification of the transcriptionally active human TRAP/SMCC Mediator complex (15). Nuclear extracts prepared from parental, FLAG-Nut2,

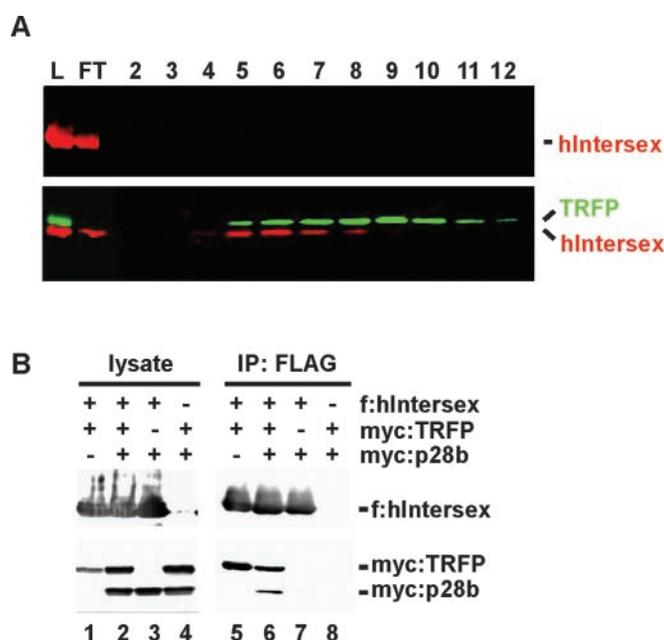


FIG. 4. Interaction of hIntersex with mammalian Mediator subunit TRFP. *A*, ~100 μg of purified FLAG-hIntersex was incubated for 1 h on ice alone or with ~70 μg of His-Myc-TRFP and applied to a 0.6-ml TSK DEAE-NPR HPLC column pre-equilibrated in buffer C containing 0.1 M NaCl. The column was eluted with a 6-ml linear gradient from 0.1 to 0.5 M NaCl in buffer C, and 0.2-ml fractions were collected. Aliquots of the indicated fractions were analyzed by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting. FLAG-hIntersex was detected with anti-FLAG (M2) monoclonal antibodies and Alexa Fluor 680-labeled anti-mouse IgG (α light chain-specific) secondary antibodies (red); Myc-TRFP was detected with rabbit anti-cMyc antibodies and IR Dye™ 800-labeled goat anti-rabbit IgG secondary antibodies (green). Fluorescently labeled secondary antibodies were detected using a Li-Cor Odyssey infrared imaging system. *B*, interaction of hIntersex with a TRFP-p28b Heterodimer. Lysates from Sf21 cells coinfecting with baculoviruses encoding FLAG-hIntersex, Myc-TRFP, or Myc-p28b in the combinations indicated in the figure were prepared as described under "Experimental Procedures." Immunoprecipitations were carried out with the antibodies indicated in the figure. Bound proteins were eluted with 150 ng/μl FLAG peptide, analyzed by SDS-polyacrylamide gel electrophoresis, and detected by Western blotting as described for *A*.

FLAG-LCMR1, and FLAG-hIntersex HeLa cell lines were subjected to anti-FLAG-agarose chromatography as described previously for purification of the human FLAG-Nut2-containing TRAP/SMCC Mediator complex. As shown in the silver-stained SDS-polyacrylamide gel of Fig. 1A, anti-FLAG-agarose eluates from the FLAG-hIntersex (lane 1)-, FLAG-Nut2 (lane 2)-, and FLAG-LCMR1 (lane 3)-expressing HeLa cells appeared to include remarkably similar sets of proteins. Analysis of proteins present in the FLAG-Nut2-containing TRAP/SMCC Mediator complex and anti-FLAG-agarose eluates from FLAG-LCMR1-expressing HeLa cells by tandem mass spectrometry confirmed that they contained similar sets of proteins and that each included most of the known mammalian Mediator subunits (see Table I in the Supplemental Material). Importantly, the hIntersex protein was detected in both the FLAG-Nut2- and FLAG-LCMR1-containing Mediator complexes by mass spectrometry (see Table I in Supplemental Material) and by Western blotting with anti-hIntersex rabbit polyclonal antibodies raised against full-length human hIntersex, arguing that the hIntersex protein is a previously unrecognized subunit of the mammalian Mediator complex. In addition, anti-FLAG-agarose eluates from FLAG-hIntersex-expressing HeLa cells were found in Western blotting experiments to contain many of the known mammalian Mediator subunits (Fig. 2B), again arguing that hIntersex is associated with the mammalian Mediator

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 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 coinfecting 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 coimmunoprecipitated 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 DSX^F (5), our results raise the possibilities (i) that direct contacts between the *Drosophila* DSX^F and intersex proteins could recruit the Mediator complex to promoters activated by DSX^F 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* DSX^F. 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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