Identification of New Subunits of the Multiprotein Mammalian TRRAP/TIP60-containing Histone Acetyltransferase Complex*‡

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The mammalian ATM/PI 3-kinase-related TRRAP protein was previously found to be a component of a multienzyme histone acetyltransferase (HAT) complex containing the HAT TIP60. In this report, we identify a previously uncharacterized protein encoded by the FLJ10914 ORF, which we designate MRGBP, as a new component of the TRRAP/TIP60 HAT complex. In addition, through purification of MRGBP and its associated proteins from HeLa cell nuclear extracts, we identify the thyroid receptor coactivating protein (TRCp120), DMAP1, and the related MRG15 and MRGX proteins as MRGBP-associated proteins, and we present biochemical evidence that they are previously unrecognized components of the TRRAP/TIP60 HAT complex. Taken together, our findings shed new light on the structure and function of the mammalian TRRAP/TIP60 histone acetyltransferase complex.

The ATM/PI 3-kinase-related TRRAP protein is an integral subunit of multiple distinct histone acetyltransferase (HAT) complexes that have critical roles in transcriptional regulation. To date, the best characterized TRRAP-containing HAT is the Saccharomyces cerevisiae SAGA complex (1–3). The SAGA complex includes the yeast TRRAP homolog Trap1, the Ada1, Ada2, and Ada3 proteins, the Spt3, Spt7, Spt8, and Spt20 proteins, several TAFs including TAF5, TAF6, TAF9, TAF10, and TAF12, and Gen5, the founding member of the Gen5-related N-acetyltransferase (GNAT) superfamily of HATs.

In contrast to yeast, mammalian cells contain multiple TRRAP-containing SAGA-like HATs including the STAGA (SPT3-TAF11/13-Gcn5-L acetyltransferase), PCAF (p300/CBP-associated factor), and TFTC (TATA-binding protein-free TAF/II complex) complexes. These GNAT HATs have overlapping but non-identical subunit compositions. The STAGA complex includes TRRAP, mammalian homologs of the yeast Ada1 (STAF242), Ada2, and Ada3 proteins, mammalian homologs of the yeast Spt3 and Spt7 proteins, TAF9 and TAF12, PAF65α, and PAF65β, which are similar to TAF6 and TAF5, respectively, the splicing factor SAP130, the UV-damaged DNA-binding protein DDB1, and a mammalian homolog of the yeast Gen5 HAT referred to as Gcn5L (4, 5). The PCAF complex includes TRRAP, mammalian homologs of the yeast Ada2, Ada3, and Spt3 proteins, TAF9 and TAF12, the TAF-like proteins PAF65α and PAF65β, and the Gen5-related HAT PCAF (6). Finally, the TFTC complex includes TRRAP, the mammalian Ada2 and Spt3 proteins, TAF2, TAF4, TAF5, TAF6, TAF7, TAF9, TAF10, and TAF12, the PAF65α and PAF65β proteins, SAP130, and Gcn5 (7–9).

In addition to its role as a subunit of the S. cerevisiae SAGA and mammalian SAGA-like complexes, the TRRAP protein is also a component of a structurally unrelated HAT referred to in yeast as the NuA4 complex. The S. cerevisiae NuA4 complex includes TRRAP homolog Tra1, the Enhancer of Polycomb protein, the chromodomain protein Eaf3, p33ING1 tumor suppressor homolog Yng2, actin-like protein Arp4, actin, and Esal, a HAT belonging to the MYST family (10–13).

A multiprotein NuA4-like HAT has also been identified in mammalian cells and, like the yeast NuA4 complex, was found to include TRRAP, the Enhancer of Polycomb protein, actin-like protein BAF53a, which is a homolog of yeast Arp4, actin, and TIP60, an Esal-related HAT belonging to the MYST family. In addition to these homologs of known yeast NuA4 subunits, the mammalian NuA4-like HAT includes the SNF2-related helicase p400 and the AAA ATPases TIF49a and TIF49b (14). In this report, we identify a previously uncharacterized protein encoded by the FLJ10914 ORF as a new component of the TRRAP/TIP60 HAT complex. In addition, through purification of FLJ10914 and its associating proteins from HeLa cell nuclear extracts, we identify the thyroid receptor-coactivating protein (TRCp120), DMAP1, and the related MRG15 and MRGX proteins as FLJ10914-interacting proteins, and we present biochemical evidence that these proteins are previously unrecognized components of the TRRAP/TIP60 HAT complex.

EXPERIMENTAL PROCEDURES

Materials—Anti-FLAG-agrose, anti-FLAG (M2) monoclonal antibodies, and anti-FLAG peptide were purchased from Sigma. Anti-TATA-binding protein-free TafII-containing; TRRAP, transformation/ transactivation-associated factor; TIP, Tat interactive 60 kDa protein; ORF, open reading frame; ING, inhibitor of growth; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DTT, dithiothreitol.
TRRAP (T-17) antibodies were obtained from Santa Cruz Biotechnology. Anti-TIP60 antibodies were from Upstate-Cell Signaling Solutions. Anti-MRGBP rabbit polyclonal antibodies were raised against the full-length bacterially expressed MRGBP protein (Cocalico Biologicals, Inc.)

Mass Spectrometry—Proteins were fractionated by SDS-polyacrylamide gel electrophoresis. Proteins in gel slices were subjected to in-gel reduction, S-carboxamidomethylation, 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, San Jose, CA). Identification of proteins present in gel slices was facilitated by the algorithm SEQUEST.

RESULTS AND DISCUSSION

We previously reported partial purification from rat liver nuclear extracts of a multiprotein Med8-containing Mediator complex with an apparent native molecular mass of more than 1000 kDa (15). Analysis of proteins present in the most highly enriched Med8-containing Mediator fractions by tandem mass spectrometry led to the identification of many known mammalian Mediator subunits, as well as a collection of potential Mediator subunits including the p28b, Surf5, HSPC296, and LCMR1 proteins, which we subsequently demonstrated are indeed bona fide Mediator subunits (16). Among the additional proteins present in the most highly enriched Med8-containing Mediator fractions and identified by mass spectrometry was a previously uncharacterized, 204-amino acid protein encoded by the FLJ10914 ORF. In further experiments, we did not, however, detect the FLJ10914 protein in the well characterized human TRAP/SMCC Mediator complex by Western blotting or mass spectrometry (data not shown), suggesting that it is not an integral subunit of the mammalian Mediator or that it associates only transiently with the complex under some conditions. Consistent with these findings, we observed that the bulk of the detectable FLJ10914 protein present in rat liver nuclear extracts fractionates away from the Med8-containing Mediator complex during chromatography, suggesting that the bulk of the FLJ10914 protein is not associated with the Mediator.

To shed light on the function of the FLJ10914 protein and to clarify its relationship with Mediator, we began by attempting to identify FLJ10914-associating proteins. As discussed in more detail below, these efforts led to the discovery that the FLJ10914 protein is associated with components of the TRRAP/TIP60 histone acetyltransferase complex and binds directly to the MORF4-related MRG15 and MRGX proteins. We henceforth refer to the FLJ10914 protein as MRG-binding protein, MRGBP.

To expedite isolation of MRGBP-associating proteins, we generated a HeLa cell line stably expressing the MRGBP protein containing an N-terminal FLAG tag. Nuclear extracts were prepared from parental and FLAG-MRGBP expressing HeLa cells and subjected to anti-FLAG-agarose immunoenrichment chromatography. As shown in the silver-stained SDS-polyacrylamide gel of Fig. 1A, anti-FLAG-agarose eluates from FLAG-MRGBP expressing HeLa cells included, in addition to FLAG-MRGBP, two abundant proteins of ~35 and ~39 kDa and a set of more than 10, less abundant polypeptides ranging in size from ~43 to more than 300 kDa (lane 3). As shown in lane 2, these polypeptides appear to be specifically associated with FLAG-MRGBP, since they are not apparent in anti-FLAG-agarose eluates prepared from parental HeLa cells.

To identify the MRGBP-associating proteins, anti-FLAG-agarose eluates were further fractionated and concentrated by adsorption to a TSK SP-NPR HPLC column at 0.1 M KCl, followed by step elution with buffer containing 1 M KCl. Proteins present in the peak MRGBP-containing fraction were resolved by preparative SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining (Fig. 1B). Proteins present in gel slices were subjected to in-gel reduction, S-carboxamidomethylation, and digestion with trypsin. The sequences of tryptic peptides, which are presented in Table I of
Markers are indicated on the positions and relative molecular masses in kilodaltons of protein size in electrophoresis, and proteins were visualized by silver staining. The proteins were pre-equilibrated in buffer containing 20 mM Hepes-NaOH (pH 7.9), 0.5 M NaCl, 0.5 mM EDTA, 1 mM DTT, and 5% (v/v) glycerol, and 0.1 mM KCl. The column was eluted at 0.2 ml/min with a 6-ml linear salt gradient from 0.1 to 0.6 M KCl. The positions and relative molecular masses in kilodaltons of protein size markers are indicated on the left. Catalase and chymotrypsinogen standards were eluted from Superox 6 column in peaks centered at fractions 32 and 41, respectively.

Supplemental Material were determined in a single run by microcapillary reversed-phase chromatography coupled to the nanospray ionization source of a quadrupole ion trap mass spectrometer.

Among the MRGBP-associated proteins identified by mass spectrometry in the TSK SP-NPR eluate were the AMF family member TRRAP, the SNF2-related p400 helicase, the Enhancer of Polycomb protein, the histone acetyltransferase TIP60, actin-related protein BAF53a, actin, tubulin, and the AAA ATPases TIP49a and TIP49b, which were all previously shown to be subunits of the multiprotein TRRAP/TIP60 histone acetyltransferase complex, suggesting that the MRGBP protein associates with components of the TRRAP/TIP60 complex. The mammalian MRGBP protein had not been identified previously as a subunit of the TRRAP/TIP60 complex nor had it apparent S. cerevisiae ortholog encoded by the YNL136W ORF (E-value 3e^-4) been identified as a subunit of the homologous yeast NuA4 HAT complex.

In addition to the known subunits of the TRRAP/TIP60 complex, the TSK SP-NPR eluate contained a set of additional MRGBP-associated proteins not previously recognized as components of the complex (Fig. 1B). These proteins included the thyroid hormone receptor-activating protein TRCP120, DMAP1, ING, MRG15, MRGX, Gnas41, and the protein encoded by the FLJ11730 ORF, which were present in ~120-, ~60-, ~45-, ~40-, ~35-, ~29-, and ~27-kDa protein bands, respectively (Fig. 1B); the presence of the TRRAP, DMAP1, TIP60, and MRGBP proteins in anti-FLAG eluates from FLAG-MRGBP expressing but not parental HeLa cells was confirmed by Western blotting (Fig. 1C).

TRCP120 protein (thyroid receptor-coactivating protein p120) was originally identified in a yeast two-hybrid screen as a thyroid hormone receptor-interacting protein that is capable of stimulating thyroid hormone receptor-dependent transcription (17). TRCP120 includes a C-terminal bromodomain, and reiterative PSI-BLAST searches of the S. cerevisiae protein data base identified the bromodomain-containing proteins Rsc2, a subunit of the RSC chromatin remodeling complex, and Bdf1 as its two most likely yeast orthologs. Although neither Rsc2 nor Bdf1 had been shown to be components of the yeast NuA4 HAT complex, deletion of Bdf1 is lethal in yeast when combined with mutations in the NuA4 HAT Esa1 or with non-acetylable variants of histone H4 (18).

The DMAP1 protein was originally identified in a yeast two-hybrid screen as a DNA methyltransferase 1 (DNMT1)-associated protein and shown to be capable of acting as a transcriptional corepressor in some contexts (19). The DMAP1 protein shares significant sequence similarity with the protein encoded by the S. cerevisiae YGR002c ORF (E-value 4e^-10), which had not been previously identified as a component of the yeast NuA4 HAT complex.

Members of the mammalian ING (inhibitor of growth) protein family resemble the S. cerevisiae Yng2 protein, which had been previously identified as a subunit of the yeast NuA4 HAT complex (12), suggesting that the ING protein could be a subunit of the TRRAP/TIP60 complex. The chromodomain-containing MRG15 and MRGX proteins share significant sequence similarity (E-value e^-106) and are members of a larger family that includes the MORF4 protein, which was originally identified as a protein capable of inducing a senescent phenotype in a subset of immortal human cell lines (20). Members of the MRG protein family have been implicated in both transcriptional activation (21, 22) and repression (23). The human MRG15 and MRGX proteins share significant sequence similarity with the S. cerevisiae chromodomain-containing protein Eaf5 (E-values 7e^-33 and 5e^-22, respectively), which had been previously identified as a subunit of the yeast NuA4 HAT complex (24), suggesting that one or both of the MRG15...
and MRGX proteins could be subunits of the TRRAP/Tip60 complex.

The Gas41 protein shares significant sequence similarity with the S. cerevisiae Yaf9 protein (E-value $e^{-24}$), which was very recently identified as a new subunit of the yeast NuA4 HAT complex (25). Reiterative PSI-BLAST searches of the S. cerevisiae protein data base suggest that the FLJ11770 protein is related to the S. cerevisiae Eaf6 protein, which has been identified as a possible Esa1-associated protein in the NuA4 complex.2

Since neither the mammalian TRCp120 and DMAP1 proteins nor their potential yeast orthologs had been previously identified as subunits of the TRRAP/Tip60 or NuA4 HAT complexes, we sought to determine whether these proteins were previously unrecognized components of the TRRAP/Tip60 complex. As a means of addressing this question, we subjected anti-FLAG-agarose eluates from FLAG-MRGBP expressing HeLa cells to further purification by TSK SP-NPR HPLC. As a means of addressing this question, we subjected anti-FLAG-agarose eluates from FLAG-MRGBP expressing HeLa cells to further purification by TSK SP-NPR HPLC with a peak of anti-FLAG eluate from FLAG-MRGBP expressing HeLa cells (Fig. 2A). In addition, when fractions from TSK SP-NPR HPLC were analyzed by SDS-polyacrylamide gel electrophoresis, and proteins were visualized by silver staining, bands corresponding in size to TRCp120 were observed to coelute from the column with TRRAP. Based (i) on the stoichiometries of MRGBP, MRG15, MRGX, and other components of the TRRAP/Tip60 complex and (ii) on our observation that MRGBP binds directly to MRG proteins (data not shown), the anti-FLAG eluate from FLAG-MRGBP expressing HeLa cells is likely a mixture of the MRG-associated TRRAP/Tip60 complex and MRGBP-MRG15 and MRGBP-MRGX heterodimers, which are resolved by TSK SP-NPR HPLC with a peak of MRGBP-MRG15 heterodimers eluting from the column in fractions 16 and 17 and with a peak of MRGBP-MRGX heterodimers eluting from the column in fractions 20 and 21 (Fig. 2B).

To characterize further the MRGBP-associated proteins and to confirm the association of TRCp120 and DMAP1 with the TRRAP/Tip60 complex, an anti-FLAG-agarose eluate that had been concentrated by stepwise elution from a TSK SP-NPR column (Fig. 1B) was subjected to analytical gel filtration on Superose 6. Proteins eluting from the column were visualized by staining with Coomassie Blue following SDS-polyacrylamide gel electrophoresis. The identities of all proteins labeled in the figure were confirmed by tandem mass spectrometry (data not shown). As shown in Fig. 3, a discrete peak of TRCp120, DMAP1, and MRGX eluted from the column in fractions 33 and 34 with other components of the TRRAP/Tip60 complex, including TRRAP, p400, Tip49a, Tip49b, Baf53, and actin. Whereas a small fraction of the MRGX protein is clearly observed to cofractionate with the TRRAP/Tip60 complex during Superose 6 gel filtration, it is not possible to determine unequivocally whether MRG15 also associates with the TRRAP/Tip60 complex, since a peak containing approximately stoichiometric amounts of MRG15 and MRGBP overlaps the peak of the TRRAP/Tip60 complex. Finally, we note that the TRRAP/Tip60 complex elutes reproducibly from the Superose 6 gel filtration column with a smaller than predicted Stokes radius, similar to that of catalase. Because TRRAP and p400

2 D. Cronier, S. Allard, and J. Cote, S. cerevisiae genome data base entry.