A Metazoan ATAC Acetyltransferase Subunit That Regulates Mitogen-activated Protein Kinase Signaling Is Related to an Ancient Molybdopterin Synthase Component*

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Molybdopterin (MPT) synthase is an essential enzyme involved in the synthesis of the molybdenum cofactor precursor molybdopterin. The molybdenum cofactor biosynthetic pathway is conserved from prokaryotes to Metazoa. CG10238 is the Drosophila homolog of the MoaE protein, a subunit of MPT synthase, and is found in a fusion with the mitogen-activated protein kinase (MAPK) upstream protein kinase-binding inhibitory protein (MBIP). This fused protein inhibits the activation of c-Jun N-terminal kinase (JNK). dMoaE (CG10238) carries out this function as a subunit of the ATAC histone acetyltransferase complex. In this study, we demonstrate that Drosophila MoaE (CG10238) also interacts with Drosophila MoaD and with itself to form a complex with stoichiometry identical to the MPT synthase holocomplex in addition to its function in ATAC. We also show that sequence determinants that regulate MAPK signaling are located within the MoaE region of dMoaE (CG10238). Analysis of other metazoan MBIPs reveals that MBIP protein sequences have an N-terminal region that appears to have been derived from the MoaE protein, although it has lost residues responsible for catalytic activity. Thus, intact and modified copies of the MoaE protein may have been conscripted to play a new, noncatalytic role in MAPK signaling in Metazoa as part of the ATAC complex. *Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.015818, 90–99, 2012.

Histone acetyltransferase (HAT) complexes have been identified from multiple organisms. They are involved in nuclear events mainly related to chromatin biology (1). The Drosophila ATAC (Ada two A containing) histone acetyltransferase complex consists of 13 proteins including two distinct HATs (Gcn5/KAT2 and Atac2/KAT14), and this complex is conserved in humans (2–4). In addition to HAT activities, two histone-fold proteins within the Drosophila ATAC complex, CHRAC14 and NC2β, facilitate nucleosome remodeling catalyzed by ISWI and SWI/SNF families of nucleosome-remodeling complexes (4). The ATAC complex also serves as a cofactor for the c-Jun transcription factor at c-Jun N-terminal kinase (JNK) target genes (5). Osmotic stress causes the rapid phosphorylation and activation of JNK, which in turn phosphorylates c-Jun and enhances its transcriptional activity (6, 7). An ATAC subunit, dMoaE (CG10238), facilitates the recruitment of upstream kinases to JNK target genes and suppresses the levels of JNK activation in response to osmotic stress (5). Thus, ATAC governs the transcriptional response to MAPK signaling by serving as a positive coactivator of transcription while also suppressing further upstream signaling.

dMoaE (CG10238) is a two-domain protein that contains the Drosophila homolog of MoaE, the large subunit of molybdopterin synthase, in its N-terminal region fused to the sequence of MAPK upstream kinase (MUK)-binding inhibitory protein (MBIP) in the C terminus. Human MBIP was initially identified as a MUK-interacting protein by a yeast two-hybrid screen and was described as a negative regulator of MUK (8). The human ATAC complex was found to contain the MBIP protein, which appeared to be conserved in many Metazoa but was thought to lack any known similarity to other proteins (2). By contrast, the Drosophila ATAC complex was found to contain the MBIP derivative that has an added MoaE domain at the N terminus (5).

The MoaE protein is an essential enzyme involved in the synthesis of molybdenum cofactor (Moco) used by most organisms (9, 10). Mammalian and prokaryotic molybdopterin synthases are heterotetramers consisting of two MoaE and

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1 The abbreviations used are: HAT, histone acetyltransferase; MAPK, mitogen-activated protein kinase; MUK, MAPK upstream kinase; MBIP, MUK-binding inhibitory protein; Moco, molybdenum cofactor; JNK, c-Jun N-terminal kinase; TAP, tandem affinity purification.

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two MoaD subunits (9, 11). MPT synthase catalyzes the conversion of precursor Z to molybdopterin, a Moco precursor, by donating sulfur to form a dithiolene group, which will coordinate the molybdenum atom. MoaD is the sulfur donor for formation of the dithiolene group in molybdopterin (12). Moco deficiency in humans is a hereditary metabolic disorder that results in the pleiotropic loss of function of all the molybdenum enzymes and usually leads to death at an early age (13, 14). We have shown that dMoaE (CG10238) is a negative regulator of the JNK cascade stimulated by osmotic stress (5). In this study, we demonstrate that dMoaE (CG10238) homodimerizes with itself and heterodimerizes with dMoaD. We also show that the N-terminal region of vertebrate MBIPs may have been derived from the MoaE protein but must have lost enzymatic activity.

EXPERIMENTAL PROCEDURES

Cell Lines, Extract Preparation, and Complex Purification—Drosophila S2 cells were stably cotransfected with the constructs containing pMT-Ada2a-TAP, pMT-TAP, or pMT-Atac2-FLAG,HA2, and pCoBlast, and the cells were selected by 25 μg/ml of blasticidin. The cell pellets were incubated with five volumes of Buffer I (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM PMSF, 1 mM DTT, 1 mM leupeptin, and 1 mM pepstatin) for 10 min followed by the addition of Nonidet P-40 to 0.9%. The cytoplasmic extracts were prepared by centrifugation at 5000 rpm for 5 min. The nuclear pellets were then incubated in the three volumes of Buffer II (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM PMSF, 1 mM DTT, 1 mM leupeptin, and 1 mM pepstatin) for 1 h, and the nuclear extracts were then collected after centrifugation at 14,000 rpm for 20 min. Thus, the nuclear or cytoplasmic extracts of these cell lines or parental wild-type S2 cells used as a control, were prepared as previously described (5). Tandem affinity purification (TAP) was performed as previously described (15). Briefly, extracts from 16 liters of cells were incubated with 500 μl of Ig-Sepharose and eluted with TEV protease. The eluant was incubated with 500 μl of calmodulin-Sepharose and eluted with EGTA. FLAG affinity purifications or FLAG-HA tandem affinity purifications were performed as previously described (5). A part of the affinity-purified complexes were subjected to SDS-PAGE, and the polyacrylamide gels were examined by silver staining. Another part of the complexes was examined by MudPIT analysis (see below).

Superose 6 Chromatography—Elution from the TAP purification was loaded on the Superose 6 HR size exclusion chromatography (Amersham Biosciences) containing 350 mM NaCl, 40 mM HEPES, pH 7.5, 10% (v/v) glycerol, 0.1% (v/v) Tween 20. The fraction profiles were analyzed by Western blotting. Parts of the fractions were subjected to MudPIT analysis. The Superose 6 column was calibrated with 2-MDa blue dextran 2000, 669-kDa thyroglobulin, 440-kDa ferritin, 232-kDa catalase, and 67-kDa BSA.

Ion Exchange (Mono Q) Chromatography—The S2 cell nuclear extracts were loaded on the Mono Q ion exchange column (Amersham Biosciences) running a gradient from buffer (A) containing 100 mM NaCl, 50 mM Tris-HCl, 10% glycerol, and 0.1% Tween 20 to buffer (B) containing 1 M NaCl, 50 mM Tris-HCl, 10% glycerol, and 0.1% Tween 20 to buffer. The fraction profiles were analyzed by Western blotting.

Expression and Purifications of Recombinant Proteins—S212 cells were cultured and infected with recombinant baculoviruses encoding the CG10238-F (1–367 amino acids)/CG10238-N (1–137 amino acids) with an N-terminal FLAG or HA tag or the CG13090 with an N-terminal FLAG tag as previously described (5). The lysates were prepared, and each tagged protein was individually purified using anti-FLAG M2 affinity gel (Sigma) or monoclonal anti-HA-agarose (Sigma). The individually purified recombinant proteins were coimmunoprecipitated using anti-FLAG M2 affinity gel (Sigma) or monoclonal anti-HA-agarose (Sigma), and bound proteins were analyzed by Western blotting as previously described (5).

Generation of Antibodies—To generate specific polyclonal antibodies, the complete CG13090 cDNA was cloned into pGEX-4T (Amersham Biosciences). The resulting GST-tagged protein was expressed in bacteria, purified following the manufacturer’s instruction, and then conjugated with keyhole limpet hemocyanin. The peptides of CG1749 (308–322 amino acids) and peptides of CG10238 (32–45 amino acids) were synthesized and purified by HPLC. Antibody production was done by Pocono Rabbit Farm & Laboratory. The antibodies against Atac3, Ada2a, NC2β, CHRAC14, and CG30390 were generated as previously described (4).

Multidimensional Protein Identification Technology (MudPIT) Analysis—TCA-precipitated proteins were resuspended in 30 μl of 100 mM Tris-HCl, pH 8.5, 8 M urea, reduced with 5 mM thioredoxin, alkylated with 10 mM iodoacetamide (Sigma). As described in Ref. 16, a two-step digestion procedure was used. Endoprotease Lys-C (Roche Applied Science) was added to 0.5 μg for at least 6 h at 37 °C, and then the sample was diluted to 2 M urea with 100 mM Tris-HCl, pH 8.5. Calcium chloride was added to 2 mM, and the digestion with 0.5 μg of trypsin (Promega) was left to proceed overnight at 37 °C while shaking. The reaction was quenched by adding formic acid to 5%, and the peptide mixture was loaded on a 100-μM fused silica microcapillary column packed with 8 cm of reverse phase material (Aqua; Phenomenex), followed with 3 cm of 5-μM strong cation exchange material (Partisil SCX; Whatman), followed by 2 cm of 5-μM C₁₈ reverse phase (17). The loaded microcapillary column was placed 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 ~200–300 nl/min. Fully automated six- or seven-step chromatography runs were carried out. Three different elution buffers were used: 5% acetonitrile, 0.1% formic acid (Buffer A); 80% acetonitrile, 0.1% formic acid (Buffer B); and 0.5 M ammonium acetate, 5% acetonitrile, 0.1% formic acid (Buffer C). The peptides were sequentially eluted from the SCX resin by increasing salt steps, followed by an organic gradient. The last two chromatography steps consisted 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 (Thermo-Fisher Scientific). Full MS spectra were recorded on the peptides over a 400–1600 m/z range, followed by three MS/MS events sequentially generated in a data-dependent manner on the first to 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 (ThermoFisher Scientific). RAW files extracted into ms2 file format (18) using RAW Xtract v.1.0 (19). SEQUEST v.27 (20, 21) was used to match MS/MS spectra to peptides in a database of 35,050 amino acid sequences, consisting of 17,348 Drosophila melanogaster proteins (nonredundant entries from NCBI release of November 28, 2006), 177 usual contaminants such as human keratins, IgGs, and proteolytic enzymes, and, to estimate false discovery rates, 17,525 randomized sequences (keeping the same amino acid composition and length) for each nonredundant protein entry. MS/MS spectra were searched without specifying differential modifications, but +57 Da were added statically to cysteine residues to account for carboxamidomethylation. No enzyme specificity was imposed during searches, setting a mass...
tolerance of 3 atomic mass units for precursor ions and of $>0.5$ atomic mass unit for fragment ions. 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). DTASelect/CONTRAST (19) was used to select, sort, and compare peptide/spectrum matches. Spectra/peptide matches were only retained if they had a DeltCn of at least 0.08 and, minimum XCorr of 1.8 for singly, 2.0 for doubly, and 3.0 for triply charged peptide spectra. In addition, the peptides had to be fully tryptic and at least 7 amino acids long. Combining all runs, the proteins had to be detected by at least two such peptides or one peptide with two independent spectra. Proteins that were subsets of others were removed. Under these criteria, the final false discovery rates at the protein and peptide levels were on average 1.1 and 0.3%, respectively (supplemental Table 1). To estimate relative protein levels, spectral counts were normalized for each nonredundant protein detected in a particular MudPIT analysis, and distributed normalized spectral abundance factors (dNSAFs) were calculated as follows (22),

$$dNSAF_i = \frac{dSAF_i}{\Sigma_j dSAF_j} \quad (\text{Eq. 1})$$

with

$$uSpC_i + \frac{uSpC_i}{\Sigma_m uSpC_m} \times sSpC_i \quad (\text{Eq. 2})$$

in which shared spectral counts ($sSpC$) are distributed based on spectral counts unique to each protein ($uSpC$) divided by the sum of all spectral unique counts for the M protein isoforms that shared peptide $j$ with protein $i$.

To estimate the protein abundance, spectral counts were considered. To account for the fact that larger proteins tend to contribute more peptide/spectra, spectral counts are divided by protein length. Spectral abundance factor values are then normalized against the sum of all SAFs for a particular run (removing redundant proteins), allowing us to compare protein levels across different runs using NSAF values. The NSAFs are calculated as follows: $\text{NSAF} = (\text{spectral count/length})$ for protein $X/\text{sum (SpC/length)}$ for all nonredundant proteins in run. NSAF is used for approximation for protein levels in a sample. NSAF values range from 0 to 1, with values closer to 1 indicating higher protein levels.

An “in-house” algorithm to refine spectral counts (hence NSAF) is implemented to deal with peptides shared between multiple proteins. Therefore, there are additional columns for each run: sS, spectral counts for peptides shared between protein isoforms; uS, spectral counts for peptides uniquely matching the protein; dS, spectral counts for peptides shared between proteins are counted only once and distributed according to the spectral count contribution of peptides unique to each isoform (as a way to estimate the relative proportion between isoforms); and dNSAF, NSAF are calculated based on distributed spectral counts (with shared spectral counts distributed among isoforms).

RESULTS

dMoeA (CG10238) Is a Bona Fide Subunit of the ATAC Complex—Previously we have shown that the ATAC complex governs the transcriptional response to MAPK signaling by functioning both as a downstream transcription cofactor and as a suppressor of upstream signaling. We found that the ATAC subunit dMoeA (CG10238, NP_651340.1) associates with the protein components of the JNK signaling pathway and inhibited JNK activation in response to osmotic stress (5). dMoeA (CG10238) is annotated in Flybase as encoding the $Drosophila$ homolog of the molybdopterin synthase large subunit. MoaE is an essential component of the Moco biosynthetic pathway, which is conserved in all kingdoms of life. In $Drosophila$, MoaE is translationally fused to MBIP, a protein that is implicated in the regulation of MAPK signaling in mammals (8).

Given its role as both a transcriptional cofactor and an inhibitor of JNK activation, we wondered whether distinct versions of the ATAC complex might exist for these two functions. Hence, we subjected nuclear extracts to ion exchange chromatography to test whether there was more than one version of ATAC. We examined the chromatographic profile of the endogenously expressed ATAC subunit, Ada2a, which is required for both the coactivator and JNK inhibitory activities of ATAC (5). Nuclear extracts from $Drosophila$ S2 cells were subject to anion exchange chromatography (Mono Q) using a gradient from 100 mM NaCl to 1 M NaCl buffer. Fractions were analyzed by Western blots probing with antibody against Ada2a (NP_001014637.1) (Fig. 1A). The fraction profile of Ada2a revealed a single peak (Fraction 7). We also confirmed that a single peak of Ada2a existed in the fractions from a Mono Q column running a shallower gradient, 100–600 mM NaCl buffer (data not shown). These data suggested that Ada2a exists in a single complex, ATAC, in S2 cell nucleus.

In addition to proteins considered to be bona fide subunits of ATAC, additional proteins can be found associated with it during standard single-step FLAG affinity purification (4, 5). Given its annotation as a subunit of molybdopterin synthase, we sought to confirm that dMoeA (CG10238) was constitutively present in more highly purified preparations of ATAC. Thus, ATAC was subjected to multiple affinity purification steps. We generated S2 cell lines, which stably expressed Ada2a-TAP as a bait for ATAC and a TAP tag cassette alone as a control. The nuclear extracts from these cell lines were subjected to TAP, and the final elution was analyzed by SDS-PAGE and silver staining (Fig. 1B). The TAP-purified ATAC complex was further fractionated by a size exclusion column (Superose 6 HR) (Fig. 1C). Western blot analysis of fractions from this column revealed that dMoeA (CG10238, NP_651340.1) cofractionated with Atac2/KAT14 (NP_609889.1), an acetyltransferase subunit of ATAC (Fig. 1D) (4). Both proteins eluted in broad peaks from fractions 11–18 corresponding to sizes ranging from 1.8 to 1.3 MDa. We individually examined fractions 11 and 12 (data not shown) and fractions 16 and 17 by MudPIT analysis (Fig. 1E and supplemental Table 1). All of the proteins found in fractions 16 and 17 were also found in fractions 11 and 12. Fractions 11 and 12 contained additional peptides that are enriched in the control purifications and may be nonspecific. We conclude

Metazoan MBIP Is Derived from MoaE
**Fig. 1.** dMoaE (CG10238) is a subunit of ATAC complex. A, nuclear extracts from S2 cells were fractionated on an anion exchange column (Mono Q) with a gradient from 0.1 to 1 M NaCl. The fractions were subjected by Western blot probing with Ada2a antibody. B, nuclear extracts from TAP-tagged Ada2a or TAP tag only stably expressing *Drosophila* S2 cells were used for the TAP. Silver-stained gels show the TAP-purified Ada2a complexes (Ada2a-CBP) and CBPs as a control purification. C, TAP-purified dAda2a complex from B was examined by Superose 6 HR size exclusion chromatography. The fractions were analyzed by Western blots probed with antibodies against anti-Atac2/KAT14 and anti-dMoaE (CG10238). The estimated molecular mass was determined by calibration with size markers under the same conditions. Fractions 11 and 12 and fractions 16 and 17 were examined by MudPIT analysis. The resulting NSAF values from the MudPIT analysis of this sample and from a biological repeat are represented as a heat map in E. The numbers indicate the fraction number. In indicates input. D, nuclear extracts from FLAG2HA2-tagged Atac2/KAT14 or parental *Drosophila* S2 cells were used for FLAG-HA tandem affinity purification. Silver-stained gels show the FLAG-HA-purified Atac2/KAT14 complexes (Atac2-FH) and the control purification (Control). The elutions from the beads
that the proteins found in fractions 16 and 17, including dMoaE (CG10238), as ATAC complex subunits. These are listed in Fig. 1E and supplemental Table 1. To further confirm the presence of dMoaE (CG10238) in ATAC, we carried out a distinct tandem affinity purification of the complex using FLAG and HA. Atac2/KAT14 is one of the catalytic subunits of ATAC. We generated the Atac2-FLAG$_2$HA$_2$ stable S2 cell line and purified Atac2/KAT14 by FLAG-HA tandem affinity purification from nuclear extracts. As a control, nuclear extracts from cells not expressing the tagged protein were subjected to the same affinity purification steps as the Atac2 purification. The final elution was examined by SDS-PAGE and silver staining (Fig. 1D) and also analyzed by MudPIT (Fig. 1E). The heat map shown in Fig. 1E illustrates the dNSAF values for peptides of ATAC subunits found in the TAP-Superose purification shown in Fig. 1B, the FLAG/HA affinity purification shown in Fig. 1D, and biological repeats of each. The Atac2/KAT14 purifications revealed peptides from all the subunits of the ATAC complex including dMoaE (CG10238) (Fig. 1E and supplemental Table 1). Thus, MudPIT analysis of the two highly purified ATAC preparations (TAP-Ada2a purification followed by gel filtration and tandem FLAG-HA affinity purification of Atac2-FLAG$_2$HA$_2$) demonstrated consistent copurification of dMoaE (CG10238) with other ATAC complex subunits (Fig. 1E).

We also confirm this result by dMoaE (CG10238) purifications. We generated stable S2 cell lines that expressed FLAG-HA-tagged dMoaE (CG10238) and took cytoplasmic and nuclear extracts, which were used for affinity purification by FLAG beads. As a control, the parental S2 cell cytoplasmic or nuclear extracts were treated with the same procedure. Each elution was examined by silver staining (supplemental Fig. 1A) and Western blot probing with antibodies against HA (supplemental Fig. 1, B and C) and ATAC subunits, D12 and Atac1 (supplemental Fig. 1C). We confirmed that only the nuclear CG10238 purifications contained the ATAC subunits (supplemental Fig. 1C). Hence, we conclude that dMoaE (CG10238) functions as a subunit of the ATAC complex.

To confirm that dMoaE (CG10238) is an ATAC subunit, we tested for interactions of ATAC subunits with dMoaE (CG10238) in vivo by coimmunoprecipitation of endogenous proteins. S2 cell nuclear extracts were immunoprecipitated with the antibodies against ATAC subunits, Atac3 (CG32343, NP_728520.1), Ada2a, NC2β (NP_609736.1), CHRAC14 (NP_476647.2), and CG30390 (ortholog of Sgf29, NP_726051.1) (Fig. 1F). As controls, nuclear extracts were immunoprecipitated with rat IgG and rabbit IgG (Fig. 1F). Western blots of the immunoprecipitates were probed with antibody against dMoaE (CG10238) and indicated that it co-immunoprecipitated with all of the tested ATAC subunits and was not detected in the control immunoprecipitates (Fig. 1F). Thus, endogenous CG10238 is associated with ATAC subunits in S2 cell nuclei.

CG10238 Homodimerizes and Interacts with MoaD—Sequence analysis of dMoaE (CG10238) suggests that the N-terminal region is homologous to the MoaE family of enzymes broadly conserved in many species of Bacteria, Archaea, and Eukaryota (including animals, plants, fungi, and some protists) and involved in the synthesis of Moco, which is required for activity of a diverse group of oxidoreductases (Fig. 2A). However, its function as a subunit of the nuclear ATAC HAT complex (above) and its role in regulating MAPK signaling (5) raised the question as to whether dMoaE (CG10238) really does function as MoaE in Drosophila. Interestingly, all of the amino acid residues that are involved in the substrate interactions and catalysis in MoaE are highly conserved in dMoaE (CG10238), as are the residues mediating MoaD-MoaE interaction (Fig. 3) (11).

The presence of putative catalytic residues in dMoaE (CG10238), a protein found in a fusion with dMBIP, and the lack of other apparent MoaE orthologs in the Drosophila genome indicate that dMoaE (CG10238) may not only be part of the ATAC acetyltransferase complex that regulates JNK signaling but also functions as a bona fide subunit of molybdopterin synthase (Fig. 2A) (9, 11). We tested whether dMoaE (CG10238) displayed properties of MoaE. In prokaryotes, two copies of MoaE dimerize, and each MoaE molecule interacts with a copy of the MoaD protein to form the heterotetrameric molybdopterin synthase enzyme (Fig. 2B) (23). To verify whether dMoaE (CG10238) has interaction properties similar to prokaryotic MoaE, we tested binding of purified recombinant dMoaE (CG10238) to recombinant Drosophila MoaD. In Drosophila, there are two candidate MoaD proteins, CG13090 (NP_609240.2) and CG1749 (NP_572722.2) (Fig. 2A). Full-
length dMoaE (CG10238) bound to MoaD (CG13090) in vitro (Fig. 2C, lane 3). Furthermore, the MoaE domain of dMoaE (CG10238) alone was able to homodimerize (Fig. 2D, lane 5) and dimerize with MoaD (Fig. 2D, lane 4). Thus, the N-terminal domain of dMoaE (CG10238) binds to MoaD and to itself, like its well studied prokaryotic ortholog. We further confirmed the interaction of endogenously expressed dMoaE (CG10238) and MoaD in Drosophila Schneider's S2 cells by coimmunoprecipitation. Both MoaD orthologs, CG13090 and CG174, coimmunoprecipitated with endogenous dMoaE (CG10238) (Fig. 2E and G).

The N-terminal Domain of Vertebrate MBIP Appears to Have Evolved from MoaE—dMoaE (CG10238) consists of two protein domains: its N-terminal region is homologous to MoaE and its C-terminal domain is homologous to MBIP. This suggests that the N-terminal domain of dMoaE (CG10238) binds to MoaD and to itself, like its well studied prokaryotic ortholog. We further confirmed the interaction of endogenously expressed dMoaE (CG10238) and MoaD in Drosophila Schneider's S2 cells by coimmunoprecipitation. Both MoaD orthologs, CG13090 and CG174, coimmunoprecipitated with endogenous dMoaE (CG10238) (Fig. 2E and G).

**Fig. 2.** dMoaE (CG10238) interacts with dMoaD proteins and assembles into molybdopterin synthase. A, homology domains of dMoaE (CG10238), CG13090, and CG1749. MoaE indicates MoaE domain, MBIP indicates MBIP protein sequence, ThiF MoeB Hesh family indicates the family of E1-like adenylyltransferases involved in molybdopterin and thiamine biosynthesis, and RHOD indicates rhodanese homology domain. B, the illustration of the MoaE-MoaD heterotetramer referred from crystal structure of bacteria MoaE-MoaD (11). C, the direct interaction of dMoaE (CG10238) with Drosophila MoaD in vitro. The individually purified recombinant HA-tagged dMoaE (CG10238)-FL and FLAG-tagged Drosophila MoaD (CG10390) from Sf21 cells were coimmunoprecipitated using FLAG beads. D, the direct interaction of MoaE domain of dMoaE (CG10238) with MoaD and homodimerization of the MoaE domain. The individually purified recombinant FLAG-tagged dMoaE CG10238-N/MoaE domain or FLAG-tagged MoaD and HA-tagged CG10238-N/MoaE domain were immunoprecipitated using HA beads. E–G, the dMoaE associated with MoaD (CG10390 and CG1749) in S2 cells. MoaD (CG13090) and MoaD (CG1749) were immunoprecipitated with dMoaE (CG10238) (E); dMoaE (CG10238) was immunoprecipitated with MoaD (F and G) by using nuclear extracts from Drosophila S2 cell. As a control, S2 cells were immunoprecipitated with IgG. IP, immunoprecipitation; In, input.
MOCS2B (human MoaE), and the C-terminal region is homologous to the human MBIP (Fig. 2A). Thus, MoaE and MBIP exist as separate proteins in humans but are fused in Drosophila (Fig. 4B). Analysis of the domain structure in MoaE and MBIP homologs encoded by various genomes indicates that many other arthropods with completely sequenced genomes, i.e. various insects (excluding mosquitoes), as well as a crustacean Daphnia pulex, encode the two domains as the same translational fusion as dMoaE (CG10238) (Figs. 3 and 4B). By contrast, mosquitoes, nematodes, plants, most fungi and protists, and all prokaryotes are missing the MBIP domain but (except for parasitic bacteria) have a MoaE homolog (Fig. 4B). MoaE proteins in these organisms are encoded as a stand-alone gene or fused to other domains, most often also involved in Moco biosynthesis. The MBIP homologs in vertebrate animals have an N-terminal extension of similar size as the MoaE domain of dMoaE (CG10238) (Fig. 4B).

When we searched sequence databases with the PSI-BLAST program (24) using the human MBIP (gi 119586267) as a query, we detected the MBIP homologs in vertebrates and invertebrates, including sea anemone Nematostella vectensis and placzoan Trichoplax adhaerens, as well as recently sequenced protist Capsaspora owczarzaki, and in select fungi. A MBIP homolog, however, appears to be missing from nematodes. Interestingly, in the same search, starting from the third iteration, we saw matches between the N-terminal portion of MBIP proteins, which had an unknown provenance in vertebrates, and stand-alone MoaE proteins from various organisms with the e values of $10^{-4}$ (Fig. 3). These matches were retrieved after insect MBIPs were already included in the checkpoint profile, yet inspection of the alignments of the vertebrate and insect MBIPs revealed that the region of similarity between them aligned by PSI-BLAST covered both the C-terminal MBIP domain and the N-terminal regions of the human and fly proteins (supplemental Fig. 2). Thus, it appears that not only the C-terminal MBIP domains of vertebrate and insect MBIP/dMoaE (CG10238) proteins are related, but so are their N-terminal parts. Thus, vertebrate N-terminal regions of MBIP proteins are distantly related to MoaE. We could not

**Fig. 3. The evolutionary emergence of MBIP/dMoaE (CG10238)-like architecture in Animals.** Multiple alignments of the N-terminal domains in dMoaE (CG10238), MBIPs, and MoaE-like proteins. GenBank™ identification numbers or Protein Data Bank identification numbers are shown for each sequence, followed by the number representing distance, in amino acids, from the N-terminal residue. dMoaE (CG10238) is 21356259_drome. In the secondary structure line, s stands for strand, l stands for loop, and h stands for helix. Yellow shading indicates conserved bulky hydrophobic residues (Ile, Phe, Leu, Met, Val, Tyr, or Trp), red type indicates conserved negatively charged residues (Asp, Glu, Asn, or Gln), blue type indicates conserved positively charged residues (Lys or Arg), red type shaded in green indicates conserved histidine, and gray-shaded purple type indicates conserved small or kingly side chains (Ala, Gly, Ser, or Pro). In the interactions line, asterisks indicate the residues implicated in cofactor binding or catalysis (11), and dollar signs indicate the residues that are involved in interaction with MoaD, as computed directly from the structure of Staphylococcus aureus Moco biosynthesis enzyme (Protein Data Bank code 2Q5W). Shading and color in this line indicate the residues shared by MoaE and MBIPs. The alignment was constructed using the MACAW program (28).
confirm the presence of such MoaE-like moiety in the predicted MBIP-like proteins from fungi or protists, even though some of them also contain putative N-terminal extensions that are similar in length to the MoaE-like domain in dMoaE (CG10238).

Multiple alignment and analysis of amino acid substitutions indicate significant differences between N-terminal domains of metazoan MBIPs (MoaE-like) and dMoaE (CG10238) (Fig. 3) (25). The putative second α-helix in the α-β hammerhead is missing in the N-terminal domains of vertebrate MBIPs, and β-strands in these atypical MoaE-like regions are not as well defined as in dMoaE (CG10238) and may be remodeled. Moreover, of seven residues (Phe-31, Arg-36, His-100, Arg-101, Ile-112, Lys-116, and Lys-123) implicated in binding molybdopterin and catalysis (11), all seven are conserved in dMoaE, but only three (Phe-31, Arg-36, and Lys-123) are preserved in other MBIP proteins (Fig. 3). On the other hand, 10 residues that mediate MoaD-MoaE interaction in the bacterial heterodimer, seven are conserved in MoaE-like moieties of MBIPs. These observations suggest that the N-terminal regions of MoaE-like protein sequences in vertebrate animals are unlikely to be catalytically active but may have retained the ability to interact with MoaD.

The MoaE-like proteins in eukaryotes must have been derived from a bacterial homolog, the taxonomic position of which cannot be established with certainty (insect MoaE have Bacteroidetes, Bacillales, and Chloroflexi as their nearest, albeit very distant, neighbors; data not shown). The occurrence of both subunits of molybdopterin synthase in all lineages of life suggests that the emergence of MoaE domain predates
Metazoan MBIP Is Derived from MoaE

the origin of eukaryotes (Fig. 4A). The provenance of the C-terminal region of MBIP protein (i.e. MBIP homology domain in the proper sense) is more recent: it may have evolved in a common ancestor of animals and fungi, if not in an earlier ophistokont, judging by its presence in C. owczarzaki, but it is missing from other known eukaryotes and from prokaryotes (Fig. 4). The MBIP domain may have fused to a copy of MoaE in early Metazoa (Fig. 4). The fusion enabled the interaction of the MBIP C-terminal region with other proteins. In most lineages, there was no selection for maintaining the MoaE catalytic activity, because a separate active copy of this domain was also present. However, in insects that lacked a second copy, the MoaE activity of the fusion protein was preserved (Fig. 4).

**DISCUSSION**

Previously we have shown that the dMoaE-MBIP fusion protein CG10238 regulates the MAPK cascade in Drosophila. The MBIP domain is required for the incorporation of dMoaE into the ATAC complex, whereas MoaE domain is critical for the JNK inhibitory activity of dMoaE (5). In this work, we show that less well conserved, catalytically inactive MoaE-like domains appear to be present in vertebrate MBIP homologs. Based on the analogy with the fruit fly proteins, they may also be important for regulating JNK activation. MoaE is broadly conserved from prokaryotes through eukaryotes. In Drosophila and many other arthropods, the only copy of MoaE is found in a fusion with MBIP. Other Metazoa contained a separate gene encoding MoaE, allowing the extensive remodeling of the MoaE-like domain of MBIPs, which only needed to function in ATAC and MAPK signaling and not in molybdopterin synthesis.

In this study, we also demonstrated that dMoaE retains the ability to homodimerize and to interact with the dMoaE subunit of molybdopterin synthase. It is known that humans catalytically active MoaE, MOC52B, is localized in the cytoplasm of HeLa cells (26). We have shown that the MoaE domain of dMoaE localized largely in the cytoplasm when the C-terminal MBIP domain was truncated. On the other hand, the full-length dMoaE is also found in the nucleus as a component of the ATAC complex (Fig. 1 and supplemental Fig. 1C) (5). Therefore, the dMoaE-MBIP fusion may play dual roles as a subunit of molybdopterin synthase and as a nuclear subunit of the ATAC complex. We examined the MBIP activity of dMoaE (CG10238) in inhibiting activation of both cytoplasmic and nuclear JNK (supplemental Fig. 3). Indeed, dMoaE (CG10238) inhibited the presence of activated JNK in response to osmotic stress in both the cytoplasm and the nucleus. Interestingly, under conditions of osmotic stress, the overexpressed dMoaE (CG10238) was primarily localized in the nucleus (supplemental Fig. 3, lanes 3–5 and 8–10), and the active form/phosphorylated JNK was enriched in the nucleus (supplemental Fig. 3, lanes 6 and 7). It would be interesting to address whether inhibition of JNK activation by dMoaE (CG10238) primarily occurs in the nucleus and whether dMoaE (CG10238) functions as a shuttle for JNK into the nucleus upon stress signaling.

Osmotic stress signaling via MAPK cascades is a phylogenetically ancient signaling pathway conserved in yeast, animals, and plants (27). By contrast, the ATAC complex has thus far only been found in flies and mammals and is not present in plants and yeast, which also lack the MBIP sequences (above). This raises the intriguing possibility that in eukaryotes lacking MBIP, MoaE itself may play some role in regulating MAPK signaling.

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