Crystal structure of the DENR-MCT-1 complex revealed zinc-binding site essential for heterodimer formation

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The density-regulated protein (DENR) and the malignant T cell-amplified sequence 1 (MCT-1/MCTS1) oncoprotein support noncanonical translation initiation, promote translation reinitiation on a specific set of mRNAs with short upstream reading frames, and regulate ribosome recycling. DENR and MCT-1 form a heterodimer, which binds to the ribosome. We determined the crystal structure of the heterodimer formed by human MCT-1 and the N-terminal domain of DENR at 2.0-Å resolution. The structure of the heterodimer reveals atomic details of the mechanism of DENR and MCT-1 interaction. Four conserved cysteine residues of DENR (C34, C37, C44, C53) form a classical tetrahedral zinc ion-binding site, which preserves the structure of the DENR's MCT-1-binding interface that is essential for the dimerization. Substitution of all four cysteines by alanine abolished a heterodimer formation. Our findings elucidate further the mechanism of regulation of DENR-MCT-1 activities in unconventional translation initiation, reinitiation, and recycling.

protein synthesis regulation | translation initiation | translation reinitiation | translation recycling | zinc binding

Translation initiation is the most regulated step of the protein synthesis. In eukaryotes, it is coordinated by more than a dozen initiation factors (eIF) consisting of more than 30 proteins compared with only three IFs in bacteria. Initiation factors facilitate selection of the initiator tRNA (tRNA^{Met}), the recruitment of mRNA, and the scanning of its 5' untranslated region (UTR) to locate the start codon (AUG) of the main ORF, and, finally, the joining of the small (40S) and large (60S) ribosomal subunits, which results in the formation of the 80S ribosome primed for a protein synthesis (1, 2). Recent data have revealed that many 5' UTRs may have one or more AUG codons upstream of the main start codon. This may lead to a synthesis of peptides encoded by the upstream ORFs (uORFs) or a different isoform of the main protein if the upstream AUG is in-frame with the main AUG. uORFs may inhibit translation of the main ORF or regulate it by reinitiation (3, 4). Reinitiation may occur if the 40S ribosomal subunit does not dissociate from mRNA after translation termination, i.e., is not recycled and is able to reach the nearest AUG. Canonical translation initiation factors (eIF1, eIF1A, eIF2, eIF3, eIF4F) are likely involved in this process; however, the exact mechanism of reinitiation is still not well understood (5, 6).

The density-regulated protein (DENR) and the malignant T cell-amplified sequence 1 (MCT-1/MCTS1) oncoprotein were recently shown to promote reinitiation after short uORFs of a specific set of mRNAs, which are involved in cell proliferation and signaling in flies (7). The oncoprotein MCT-1 was identified in human T cell leukemia and lymphoma, and it has been associated with increased cell proliferation and genome instability (8, 9). Synthesis of DENR protein is up-regulated with increasing cell density, and it is also overexpressed in breast and ovarian cancers (10, 11). DENR forms a heterodimer with MCT-1, both in vivo and in vitro, and this heterodimerization is essential for the DENR-MCT-1 activity in mRNA translation initiation, reinitiation, and the ribosome recycling (12–16). Recently, we have characterized the DENR-MCT-1 interaction with the human 40S ribosomal subunit by determining the crystal structure

of the ribosomal complex with DENR-MCT-1 at 6-Å resolution (13). It showed that the structure of the C-terminal domain of DENR (C-DENR) is similar to that of the canonical translation initiation factor 1 (eIF1), which controls the fidelity of translation initiation and scanning. Moreover, C-DENR binds the 40S ribosomal subunit at the same site as eIF1. These data suggest a similar mechanism for DENR-MCT-1 and eIF1 in discriminating the initiator tRNA in the P site of the 40S subunit, which is crucial for translation initiation, reinitiation, and ribosomal recycling (13, 17). Binding of the MCT-1 to the 40S subunit is mutually exclusive with the binding of both eIF3a and 3b subunits of eIF3, which suggests that DENR-MCT-1 may function as the eIF3 sensor directing the posttermination 40S subunit either for reinitiation (if eIF3 is bound) or recycling (if eIF3 is dissociated) (13). The function of DENR-MCT-1 in translation is similar to that of the noncanonical translation initiation factor eIF2D (13, 15, 16, 18, 19). eIF2D is a single polypeptide, which shares domain architecture with DENR-MCT-1 (Fig. 1A). Recent X-ray crystallography and cryo-electron microscopy (cryo-EM) studies have revealed that the C-terminal domains of both eIF2D and DENR have the same SUI1 (eIF1-like) fold and binding site on the 40S ribosomal subunit (13, 15, 19). N-terminal domains of eIF2D and MCT-1 also have a similar fold and interact with the 40S subunit at the same site (13, 15, 20). Moreover, structure of the human 40S ribosomal subunit complex with eIF2D, the initiator tRNA, and the hepatitis C virus internal ribosome entry

Significance

Protein synthesis or mRNA translation by ribosomes is essential for the cell's survival. A multitude of human diseases are the direct result of disruption of translation, specifically at the initiation step. The density-regulated protein (DENR) and the malignant T cell-amplified sequence 1 (MCT-1/MCTS1) oncoprotein support noncanonical translation initiation, reinitiation, and ribosome recycling linked to cancer, neurological disorders, and viral infections. Here, we present the crystal structure of a DENR-MCT-1 heterodimer, which reveals atomic details of DENR and MCT-1 interactions that are crucial for understanding their function in translation. Our results provide foundation for the future research of the mechanism of regulation of noncanonical protein synthesis and may potentially be used for antiviral, anticancer, and neurological drug design.

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The authors declare no conflict of interest.

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Data deposition: The crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID 6MS4).

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Fig. 3. Surface representation of DENR-MCT-1. (A) Hydrophobic interactions. The surface formed by hydrophobic amino acid residues is colored yellow. DENR is shown in coral and MCT-1 in green. (B) Electrostatic interactions. The electrostatic potentials were calculated by APBS software and mapped to the solvent-accessible surface. The intensity of color is proportional to the local potential. HP, hydrophobic patch; RBS, ribosome-binding site.

This structure and the one presented here are almost identical (rmsd of 0.694 for all residues; Fig. 1E and *SI Appendix, Fig. S2*). In the structure of the DENR-MCT-1 complex with the 40S ribosomal subunit, the PUA and DUF197 domains of MCT-1 are rotated toward each other even further, which may be due to an additional restraint provided by the binding of the C-terminal domain of DENR to the 40S subunit (Figs. 1E and 5B) (13, 15, 20). The small differences in the positions of the N and C termini of MCT-1 were attributed either to the effect of the crystal packing or the absence/presence of the six histidine amino acid residues at the C terminus.

The structure of the N-DENR bound to MCT-1 fits well the unbiased electron density assigned to the N-DENR in our recent map of the DENR-MCT-1 complex with the human ribosome (*SI Appendix, Fig. S3*) (13). It comprises the N terminus (amino acid residues 26–33), followed by the globular, Zn-binding domain (amino acid residues 34–69). The fold of this domain is stabilized by four Zn^{2+} -bound cysteine residues, which are conserved (Figs. 1D and 2). The N terminus of DENR binds to the DUF1947 domain of MCT-1 in the vicinity of its helix α_4 , while the Zn-binding domain of DENR interacts with the PUA domain of MCT-1 (Fig. 1D). The shape of the MCT-1-binding surface of DENR complements the shape of the interdomain cavity of MCT-1 (Fig. 24). The size of the DENR-MCT-1-binding surface is 840 Å² and is formed mostly by hydrophobic amino acid residues, which constitute two hydrophobic patches either on DENR or MCT-1 (Figs. 3 and 4). This agrees well with our observation that the DENR-MCT-1 heterodimer is stable even in a 1-M concentration of salt. In addition, binding interactions include hydrogen bonding between amino acid residues of the N terminus of DENR and the linker connecting DUF1947 and PUA domains of MCT-1 (N of Leu29 with O of Lys87), as well as the Zn-binding domain of DENR and the PUA domain of MCT-1 (Fig. 4 and *SI Appendix, Table S2*).

Zn-Binding Site. Upon building the model of the N-DENR polypeptide chain, we observed that four cysteine residues—Cys34, -37, -44, and -53—surround a strong peak of additional electron density, which corresponds to a Zn^{2+} ion as we confirmed later (Fig. 1D and *Materials and Methods*). Zn-binding proteins are the

most abundant metalloproteins in nature (22). Zn can constitute a catalytic center in enzymes and be the center of the structural foundation governing a protein's fold and binding interfaces (23). Among Zn^{2+} -binding amino acid residues such as cysteine, histidine, aspartate, and glutamate, cysteine is the most common coordinating ligand in proteins. The presence of four coordinating ligands is required for the minimal stable Zn coordination sphere. As it is also observed here, four cysteines in a tetrahedral geometry represent one of the most common Zn^{2+} coordinations (23, 24). The four conserved cysteine amino acid residues of the N-DENR—Cys34, -37, -44, and -53—are bound to Zn^{2+} and constitute the Zn-binding domain of N-DENR (amino acid residues 33–60), which interacts with the PUA domain of MCT-1 mostly through hydrophobic (patch 1) and electrostatic interactions (Figs. 2 and 3). Simultaneous mutations of these four cysteine residues for alanine residues abolished the binding of DENR to MCT-1 (Fig. 1C, lane 7). This demonstrates that proper folding of the DENR-MCT-1 heterodimer is crucial for the formation of the DENR-MCT-1 heterodimer. Interestingly, the C37Y mutation was identified in a patient with autism spectrum disorder (25). However, coimmunoprecipitation experiments performed in HEK293T cells showed that this mutation does not prevent DENR-MCT-1 heterodimer formation (12). In agreement with this, MCT-1 was copurified with N-DENR(C37Y) when both were coexpressed in *Escherichia coli*. However, a large portion of the expressed N-DENR and MCT-1 was insoluble, and only about 30% of soluble MCT-1 was retained bound to N-DENR (Fig. 1C, lanes 8–11). These data suggest that the C37Y mutation affects DENR's stability and the conformation of the MCT-1-binding site, which likely perturb the heterodimer equilibrium in the cell and may limit its availability for interaction with the 40S ribosomal subunit. Our structure analysis may explain this observation. We propose that mutation C37Y may slightly disturb the conformation of the

Fig. 4. The DENR-MCT-1 binding. N-DENR (colored in coral in cartoon representation; only amino acid residues 26–53 are shown) is bound to MCT-1 (colored in green, surface representation). Atomic details are shown in magnified panels. Zn^{2+} ion, O, N, and S are shown in gray, red, blue, and yellow, respectively. Some amino acid residues are removed for a better view. Hydrogen bonds (2.6–3.8 Å) are shown by yellow dotted lines.

37–43 region of DENR, but the folding of the Zn-binding domain can be partially preserved because proline 40 and tyrosine 43 will provide sufficient rigidity for the DENR-MCT-1-binding site (Fig. 2*A*). Both Pro40 and Tyr43 are deeply buried in the hydrophobic pocket of the PUA domain of MCT-1 (patch 1, Fig. 3*A*), and Tyr43 makes hydrogen bond interaction with the backbone carbonyl oxygen of His141 of MCT-1 (Fig. 4 and *SI Appendix, Table S2*). In addition, the C37Y mutation may disturb the interaction of DENR-MCT-1 with the P-site tRNA on the ribosome, which would change heterodimer activity in translation. We have proposed recently that this solvent-exposed region of the Zn-binding domain of DENR, together with the PUA domain of MCT-1, constitutes the surface that may interact with the acceptor stem of the P-site tRNA (13), although the precise mechanism of the interaction between DENR-MCT-1 and tRNA remains unclear. Thus, the mutation C37Y may affect the rate of the tRNA^{Met} accommodation for the initiation or reinitiation steps, as well as the rate of the deacylated P-site tRNA dissociation at the stage of ribosomal recycling. However, our study does not exclude the possibility that the C37Y mutation may change activities of DENR or DENR-MCT-1 outside of the protein synthesis pathway. Elimination of one cysteine residue from the four-cysteine Zn-binding site will decrease the DENR's affinity to the Zn²⁺. This may lead to complete Zn²⁺ removal from DENR in the case of zinc deficiency inside the cell because DENR cannot compete for Zn²⁺ now with the majority of the other cellular Zn-binding proteins. Therefore, the mutation C37Y may cause the collapse of the Zn-binding domain of DENR when the cellular concentration of Zn is too low and temporarily deactivate DENR and the DENR-MCT-1 heterodimer.

Heterodimer Interface and tRNA Binding. It was shown that DENR forms a heterodimer with MCT-1 *in vivo*, and, as a heterodimer, they interact with the ribosome (14, 26). Without DENR bound, the solvent-exposed surface of MCT-1 has a positively charged region stretched from the PUA to the DUF1947 domain, which may interact nonspecifically with the negatively charged tRNA or rRNA backbone (Fig. 3*B*). Formation of the DENR-MCT-1 heterodimer may prevent these nonspecific interactions and ensure that the DENR-MCT-1 heterodimer is positioned specifically for the interaction with the tRNA bound to the P-site of the 40S ribosomal subunit. This model proposes that the C-terminal domain of DENR interacts with the tRNA directly in the P-site of the 40S ribosomal subunit (13, 15). However, the mode of DENR's N-terminal

domain and MCT-1 interaction with the acceptor stem and the CCA tail of the tRNA remains elusive. Indeed, insufficient resolution of the cryo-EM map of the DENR-MCT-1 translation initiation complex did not allow a structural model to be built. Nevertheless, M. Weisser et al. proposed that the P-site tRNA is located near the SWIB/MDM2 and PUA domains of DENR and MCT-1, respectively, which is similar to that in the translation initiation complex with eIF2D (Fig. 5*A*) (15). In that complex, the β 2-loop of the eIF1-like domain of eIF2D interferes with the position of the D-stem of the tRNA, the SWIB/MDM2 domain binds the acceptor stem, and the PUA domain interacts with the CCA end of the tRNA, keeping tRNA tilted toward the ribosomal E site in the hybrid P/E-like state (Fig. 5*A*) (15).

Two key structural features of eIF2D that are crucial for the interaction with the P-site tRNA are absent in DENR: first, the β 2-loop of C-DENR is small and may not interact with the D-stem of the P-site tRNA (Fig. 5*B*) (13). Second, the structure of N-DENR bound to MCT-1 presented here is different from that of the SWIB/MDM2 domain of eIF2D. In addition, electron density connecting C- and N-DENR (a region between amino acid residues 70 and 110, which is less than half of the size of the SWIB/MDM2 domain of eIF2D) is not seen in the structures of the DENR-MCT-1 complex with the 40S subunit or N-DENR-MCT-1, which suggests that this region is flexible or unstructured. Similarly, no connection between DENR and MCT-1 were reported for the low-resolution cryo-EM map of the complex with the tRNA, whereas the SWIB/MDM2 domain of eIF2D remains structured regardless of the tRNA presence (15, 19). Therefore, the absence of the interactions between DENR-MCT-1 and the P-site tRNA that force tRNA to tilt toward the E site, as proposed for the eIF2D complex, may rather favor our recent model for the DENR-MCT-1 interaction with the P-site tRNA (13). In this model, the β 2-loop of C-DENR does not interfere with the position of the tRNA on the ribosome, the N-DENR-MCT-1 binding provides the interface for the interaction with the CCA tail of the P-site tRNA, and tRNA would rather assume a conformation similar to the eP/I state in the canonical translation initiation complex (Fig. 5*B*) (13, 27). However, high-resolution structures of DENR-MCT-1 translation initiation complexes are needed to distinguish between these models or to build a new one.

Conclusion. We report that the N-terminal domain of DENR^{26–98} binds MCT-1 through interactions between its unfolded N terminus and α 4 helix of the DUF1947 domain of MCT-1 and

Fig. 5. Model of eIF2D and DENR-MCT-1 interactions with the P-site tRNA. (*A*) Superposition of the structures of DENR-MCT-1, the eIF2D reinitiation complex (PDB ID 5OA3), and C-DENR (PDB ID 5VYC). (*B*) As in *A*, with MCT-1-like and SWIB/MDM2 domains of eIF2D removed and proposed P-site tRNA (in gold) stabilized by DENR-MCT-1 added. The 40S ribosomal subunit is shown as a gray surface, tRNA in the eIF2D reinitiation complex is in blue, eIF2D is in violet, MCT-1 is in green, and DENR is in coral. Spheres show Zn²⁺ ion (gray) and MCT-1 phosphorylation site (Ser118, red). Dashed line connects N- and C-terminal domains of DENR.

between its globular domain and the PUA domain of MCT-1. Our structure revealed that the globular domain of N-DENR includes four cysteine amino acid residues (C34, C37, C44, C53), which are bound to the Zn^{2+} ion. The Zn^{2+} is tetrahedrally coordinated, and it is crucial for stabilizing the tertiary structure of the DENR's MCT-1-binding domain because substitution of all for cysteines by alanines abolished DENR MCT-1 binding. Based on our structure, we proposed an explanation for the single C37Y mutation in the Zn-binding domain of DENR, which was recently linked to the autism spectrum disorder (25). Mutation C37Y may partially destabilize the DENR-MCT-1 heterodimer and/or affect the conformation of the P-site tRNA. These will influence the dynamics of translation initiation, reinitiation, or ribosomal recycling. Our data provide insights into the mechanism of the non-canonical translation initiation, reinitiation, and ribosomal recycling by demonstrating that Zn-ion binding regulates the interaction between DENR and MCT-1 and between the DENR-MCT-1 heterodimer and the P-site tRNA in the context of the ribosomal complex. Additional structural and functional studies will be needed to determine whether other proteins are involved in this regulation and to elucidate the mechanism further.

Materials and Methods

All DENR's deletion mutants were made by PCR using the primers encoded the BamHI restriction site, the tobacco etch virus protease cleavage site at the 5'-region in frame with the DENR-encoding sequence, and the HindIII site at the 3'-region. PCR fragments were cloned in pET28a expression vector (Novagen). For coexpression with DENR, the MCT-1-coding region was amplified by PCR using a 5'-region primer with an encoded XhoI site, a T7 promoter and the ribosome-binding site from pET33-MCTS1 (13), and a 3'-region primer with the XhoI site. PCR fragment was then cloned in the pET28a-TEV-N-98-98) plasmid using the XhoI site. Cysteine-to-alanine mutations were introduced using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Protein expression, purification, and in vitro-binding assay were performed as described previously (13).

Crystals were grown in 24-well sitting-drop plates using the vapor diffusion technique. Three microliters of DENR²⁶⁻⁹⁸-MCT-1 (20 mg/mL) were mixed with 3 μ L of reservoir solution (50 mM Tris-HCl, 20% PEG 4000, pH 8.6). Plates were incubated at 20 °C for 14–19 d. Crystals were stabilized by soaking for 15 min in the following buffer: 0.1 M of NaCl, 0.05 M of Tris-HCl,

pH 8.6, 30% of PEG 4000, and 30% of glycerol. After stabilization, crystals were frozen in liquid nitrogen.

X-ray diffraction data collection was performed at the Advanced Photon Source in the Argonne National Laboratory at beamline 24ID-C. A complete dataset was collected from a single crystal to a 2.0-Å resolution. A single-wavelength anomalous diffraction dataset for DENR-MCT-1 was collected at the Zn absorption peak wavelength of 1.2822 Å. Diffraction data were processed and scaled using X-ray Detector Software (SI Appendix, Table S1) (28).

The structure was solved by molecular replacement using PHASER from the CCP4 program suite and the structure of human MCT-1 as a model (PDB ID 3R90, chain A) (20, 29). Program Coot was used for model building and Refmac from the CCP4i suite was used for the model refinement (29, 30). The final cross-validated R_{free} after model refinement was 25.8% (SI Appendix, Table S1).

Analysis of the DENR-MCT-1-binding interface was done using PDBePISA service at www.ebi.ac.uk/pdbe/prot_int/pistart.html (31). The electrostatic potentials were calculated by APBS software and mapped to the solvent-accessible surface (32). The intensity of color is proportional to the local potential.

Alignment of the amino acid sequences of the Zn-binding domain of N-DENR was generated by Clustal Omega (www.clustal.org/omega) algorithm with default settings and visualized by the Jalview program (www.jalview.org). The sequences from the following organisms were used: *Homo sapiens* (NP_003668), *Mus musculus* (NP_080879), *Gallus gallus* (NP_001072973), *Anolis carolinensis* (XP_003222792), *Xenopus tropicalis* (NP_001006814), *Danio rerio* (NP_001002697), *Drosophila melanogaster* (NP_573176), *Caenorhabditis elegans* (NP_499450), *Schistosoma japonicum* (AAW27105), *Amphimedon queenslandica* (XP_003385869), *Hydra vulgaris* (XP_002165948), *Dictyostelium discoideum* (XP_641593), *Entamoeba invadens* (NP_004259902), *Trypanosoma cruzi* (EFK32067), *Plasmodium vivax* (XP_001617181), *Schizosaccharomyces pombe* (NP_596803), *Saccharomyces cerevisiae* (NP_012548), *Neurospora crassa* (XP_965370), *Physcomitrella patens* (XP_024386006), and *Arabidopsis thaliana* (NP_196751). Accession numbers are shown in parentheses.

Figures showing atomic models were generated using PYMOL [Delano Scientific, The PyMOL Molecular Graphics System, Version 1.8 Schrödinger (<https://www.pymol.org>)].

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