Divergent Mitochondrial and Endoplasmic Reticulum Association of DMPK Splice Isoforms Depends on Unique Sequence Arrangements in Tail Anchors†

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Myotonic dystrophy protein kinase (DMPK) is a Ser/Thr-type protein kinase with unknown function, originally identified as the product of the gene that is mutated by triplet repeat expansion in patients with myotonic dystrophy type 1 (DM1). Alternative splicing of DMPK transcripts results in multiple protein isoforms carrying distinct C termini. Here, we demonstrate by expressing individual DMPKs in various cell types, including C2C12 and DMPK−−−−−−− cytoskel...
Amino acids common to all mDMPK isoforms are shown in bold, and residues identical in tail 1 and tail 2 are underlined.

FIG. 1. Mouse DMPK splice isoforms have distinct C termini. The domain organization and C-terminal amino acid sequences of mDMPK splice isoforms A, C, and E are shown. The N terminus, catalytic kinase domain, VSGGG segment, coiled-coil domain, and tail segments are indicated. Amino acids common to all mDMPK isoforms are shown in bold, and residues identical in tail 1 and tail 2 are underlined.

between the different coexpressed DMPK isoforms and with other members of the family of Rho kinases (26).

With the ultimate goal of understanding the biological function of DMPK, we focused in this study on a comparison between the subcellular localization of individual DMPK isoforms of mice and humans. We report that different DMPK splice forms either are associated with the endoplasmic reticulum (ER) or mitochondria or are cytosolic and that orthologous splice isoforms of humans and mice do not behave similarly. Use of confocal laser scanning microscopy and immunoelectron microscopy (immuno EM) in combination with transient transfection and mutation analysis of individual DMPK cDNAs in various cell types revealed new information on the sequence and structure of targeting signals required for localization to the mitochondrial outer membrane (MOM) or the ER. Furthermore, we report that anchoring of human DMPK A into the MOM affects mitochondrial distribution whereas mouse DMPK A clusters the ER. Based on our observations, we classify DMPK as the first kinase member of a family of Rho kinases (26).

MATERIALS AND METHODS

Cell culture and DNA transfection. COS-1 cells were cultured and transfected using the DEAE-dextran procedure as described previously (15). Neuro-2A (N2A), NIH 3T3, HeLa, and C2C12 cells were grown to subconfluence in Dulbecco minimal essential medium supplemented with 10% fetal calf serum and maintained at 37°C under a 5% CO2 atmosphere. N2A, NIH 3T3, HeLa, and C2C12 cells were transiently transfected with various expression plasmids by using Lipofectamine (Invitrogen) as specified by the manufacturer and maintained in culture for an additional 24 h prior to analysis. Alternatively, we used adenovirus vector-based DNA transduction for expression of single mDMPK isoforms in culture for an additional 24 h prior to analysis. Alternatively, we used adenovirus L cells grown and transfected on glass coverslips. The cells were fixed in phosphate-buffered saline containing 2% formaldehyde -24 h after transfection and permeabilized in phosphate-buffered saline containing 0.2% NP-40. Samples were processed for immunofluorescence microscopy using standard procedures. A rabbit anti-cytochrome c oxidase antibody was used to visualize mitochondria. Mouse monoclonal antibody (mAb) 12CA5 was used to detect the HA epitope tag, and mAb 414 was used as a nuclear envelope (NE) marker (57). Images of fixed cells were obtained with a Bio-Rad MRC1024 confocal laser-scanning microscope equipped with an argon/krypton laser, using a 60× 1.4 NA oil objective and LaserSharp2000 acquisition software. Images were further processed with Adobe Photoshop 7.0.

Live-cell imaging. N2A cells were grown and transfected with pEYFP-mDMPK A or pECFP-mDMPK C together with pEGFP-ER or were labeled with Rhodamine 123 in 35-mm glass-bottom dishes (Willeco Wells BV). They were washed twice in OptiMEM-I (Life Technologies), and live cells were imaged while placed on a temperature-controlled stage of a Zeiss LSM510-Meta confocal microscope (running software release 3.2) using the appropriate argon laser lines and a 63× 1.4 NA oil objective. In the case of overlapping GFP and cyan fluorescent protein signals, the metadetector in conjunction with the linear unmixing option in the software was used to generate the final images.

Immuno-EM. N2A cells were grown and transfected with plasmids YFP-mDMPK A or pECFP-mDMPK C together with pEGFP-ER or were labeled with Rhodamine 123 in 35-mm glass-bottom dishes (Willeco Wells BV). They were washed twice in OptiMEM-I (Life Technologies), and live cells were imaged while placed on a temperature-controlled stage of a Zeiss LSM510-Meta confocal microscope (running software release 3.2) using the appropriate argon laser lines and a 63× 1.4 NA oil objective. In the case of overlapping GFP and cyan fluorescent protein signals, the metadetector in conjunction with the linear unmixing option in the software was used to generate the final images.

 SDS-PAGE and Western blotting. Transfected COS-1 cells were lysed in RIPA buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF) and centrifuged for 10 min at 14,000 x g. Cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis.
The DMPK gene specifies different gene products. DMPK splice forms A and C carry distinct C termini, here referred to as tail 1 and tail 2, respectively, which are 96 and 95 amino acids in length and are identical in the N-terminal 16 amino acids (encoded by exon 13) (Fig. 1) (for details, see reference 15). The short isoform DMPK E is distinctly different and carries a short, truncated tail of 2 amino acids (designated tail 3). We previously reported that the splice modes leading to this C-terminal variation have been conserved between mice and humans and we determined the subcellular distribution of DMPK isoforms (54). Since this distribution was originally studied in ectopic host cells and since neither the conditions nor the exact mechanism for this targeted accumulation are well understood, we decided to focus first on the localization of individual splice products in authentic host cells. C2C12 myoblasts and immortalized myoblasts derived from DMPK−/− knockout mice harboring the H-2Kb-tsA58 transgene (33) were used for this purpose.

To analyze the specific distribution behavior of single isoforms, DMPKs were expressed as HA- or YFP-tagged versions from plasmid or adenovirus vectors and their localization was compared to that of various organelar markers by immunofluorescence microscopy. As shown in Fig. 2A and B, reticular structures revealed by staining of HA-tagged mDMPK A in C2C12 cells overlapped with the ER network stained by transient expression of the GFP-ER marker, containing the calreticulin ER targeting signal and KDEL ER retention signal. Since mDMPK A was confined solely to ER contiguous membranes (Fig. 2C), we tested colocalization with MAb414, a nuclear envelope (NE) marker, directed against the nuclear lamina (57). Also, this marker colocalized (see Fig. S2A to C in the supplemental material) showing that mDMPK A decorates all membranes of the ER, since the NE is a subdomain of, and continuous with, the ER (53). The intensely stained area observed close to the nucleus did not show any appreciable colocalization with a GFP-Golgi marker containing the transmembrane and stalk region of human glucosaminyl transferase I (47), suggesting that tail 1 is specific for ER and NE membranes and not for membranes in the

**RESULTS**

Membrane isolation and extraction. N2A cells were grown in 100-mm-diameter culture dishes and transfected with pSG8Eco-mDMPK expression plasmids. They were scraped into ice-cold 10% TES buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM NaCl) plus 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche), pelleted by centrifugation for 10 min at 3,000 × g, resuspended in 10% TES buffer, and incubated on ice for 15 min. Cell lysates were made using a glass-glass Dounce homogenizer and were cleared at 100,000 g for 30 min in a S55S rotor, and the supernatant fraction was collected (designated S1) for SDS-PAGE analysis. Membranes obtained were extracted on ice in (i) PBS, (ii) 1 M NaCl in PBS, (iii) 100 mM Na2CO3 (pH 11), (iv) 1% Triton X-100 in PBS and centrifuged for 30 min at 100,000 × g. Subsequent pellet (P) and supernatant (S) fractions were analyzed by SDS-PAGE followed by Western blotting using B79.

Supplemental material. Supplemental material is available online. Information includes supplemental Materials and Methods, legends to supplemental figures, and expression characterization of DMPK mutants used in this work (Fig. S1), NE immunolocalization of YFP-mDMPK A mutants (Fig. S2), real-time analysis of YFP-mDMPK A associated with the ER (movies Fig. S3A and S3B), real-time analysis of CFP-mDMPK C associated with mitochondria (movie Fig. S4), comparative analysis of mouse and human DMPK C-termini (Fig. S5; Table S1), and expression of hDMPK A and C in C2C12 cells (Fig. S6).
Golgi apparatus (Fig. 2D to F; also see Fig. S2A to C in the supplemental material).

In contrast, YFP-mDMPK C in C2C12 cells was completely colocalized with the mitochondrial marker cytochrome c oxidase but not with the other organelar markers (Fig. 2G to I and data not shown). We noticed that in some cells weak YFP-mDMPK C staining also appeared at cytosolic locations. In these cases, mitochondria often appeared clustered in the perinuclear region (Fig. 2J) (see below).

To test whether the ER and mitochondrial localization of mDMPK A and C was uniquely dependent on the sequence arrangement in their tails and independent of intrinsic kinase activity, we tagged kinase-dead variants of mDMPK isoforms. The YFP-mDMPK A(K100A) and C(K100A) distributions at ER and mitochondria, respectively, were identical to those of their kinase-active counterparts (Fig. 2K and L).

Next, we tested if partitioning of DMPKs occurred similarly in myoblast host cells that were rendered completely DMPK deficient (19). Although these myoblasts lacked the normal mixture of endogenous DMPK isoforms completely, we expected them to provide an otherwise fully authentic host environment, closely mimicking the situation in vivo. Individual YFP-tagged mDMPK isoforms A or C were expressed by adenovirus gene delivery, the typical and selective targeting to either ER or mitochondria was observed (Fig. 2M and N).

Finally, we also tested other cell types with a zero or very low level of endogenous DMPK gene expression and hence “ectopic” host infrastructure, such as N2A and fibroblast (NIH 3T3) cells, and obtained identical results (data not shown). Since all these cell types were of mouse origin, we also studied mDMPKs in human HeLa cells and again found distributions at exact cognate locations (see below). In all cell types tested, the third shorter isoform, mDMPK E, adopted a diffuse cytosolic distribution (Fig. 2O and data not shown). Based on these findings, we studied other aspects of DMPK location mainly in one cell type, choosing N2A as the most amendable cell line for live cell microscopy, video imaging, and transfection analysis.

The ER and mitochondria are dynamic structures, which continuously move and rearrange shape by transport and fusion and fission events (10, 53). To evaluate the role or fate of mDMPK A and C in these events, we monitored the localization of YFP- or CFP-tagged mDMPK isoforms over time in individual YFP-tagged mDMPK isoforms A or C were expressed for live cell microscopy, video imaging, and transfection analysis. To study the mode of mDMPK membrane association. To assess binding, different buffer and solvent conditions were used to extract mDMPK from the total membrane fraction. As shown in Fig. 4, association of mDMPK A and C with membranes, i.e., their presence in the pellet fraction, was resistant to high salt conditions and alkaline sodium carbonate, indicating that these proteins are integrated into their target membranes. Only Triton X-100 extraction was able to release mDMPK A and C from the membrane fraction, shifting it into the soluble fraction. Previous studies have shown that mDMPK A, C, and E display doublet bands on immunoblots associated with autophosphorylation (Fig. 4, asterisks) (15, 54). In addition, we observed that certain lysis and buffer conditions induced nonenzymatic clipping of mDMPK with a long C terminus (Fig. 4, brackets; compare mDMPK A and C with mDMPK E), as occurred here also. Under the conditions used, the majority of mDMPK E was present in the first supernatant fraction (S1), confirming that its microscopic classification as a cytosolic protein was correct.

The observation that mDMPK A and C are strongly associated with membranes via their C termini places them in a protein family classified as C-terminally anchored or TA proteins, a recently discovered group of proteins which anchor preferentially in membranes of the ER or MOM (reference 4 and references therein). This notion prompted us to analyze in more detail the amino acid composition of tails 1 and 2, with emphasis on their overall hydrophobicity and positions of known relevant amino acid residues. With these proteins are integrated into their target membranes. Only Triton X-100 extraction was able to release mDMPK A and C from the membrane fraction, shifting it into the soluble fraction. Previous studies have shown that mDMPK A, C, and E display doublet bands on immunoblots associated with autophosphorylation (Fig. 4, asterisks) (15, 54). In addition, we observed that certain lysis and buffer conditions induced nonenzymatic clipping of mDMPK with a long C terminus (Fig. 4, brackets; compare mDMPK A and C with mDMPK E), as occurred here also. Under the conditions used, the majority of mDMPK E was present in the first supernatant fraction (S1), confirming that its microscopic classification as a cytosolic protein was correct.

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TMD (Fig. 5A). We did identify a stretch of amino acids of moderate hydrophobicity, excluding any charged amino acids, between positions 597 and 616. Next, C termini of TA proteins known to bind membranes of the ER or MOM were compared with mDMPK tail sequences (Fig. 5B). One evident similarity between mDMPK tails and ER/MOM targeting sequences of other TA proteins is that basic residues (i.e., lysine and arginine) are highly prevalent in the regions flanking the 17- to 23-residue long hydrophobic stretches. Since no significant other similarity emerged from this comparison, these results suggest that amino acid content as well as amino acid distribution specifies mDMPK anchoring.

A hydrophobic domain in mDMPK tail 1 is responsible for ER/NE targeting. Previous studies using mutagenesis approaches have shown that the nature of the TMD and positioning of flanking lysine and arginine residues determine ER or MOM localization of TA proteins (5, 18, 21). Therefore, we examined which particular amino acid residues in tails 1 and 2 were responsible for ER and MOM targeting and whether basic residues contributed to the targeting specificity of mDMPK anchors.

**FIG. 3.** Mouse DMPK A and C localize to ER membranes or the MOM. Immuno-EM of YFP-mDMPKs A, C, or E in transiently transfected N2A cells visualized with an anti-GFP antibody followed by incubation with protein A complexed to 10-nm gold particles is shown (A). YFP-mDMPK A localized to ER and NE structures. (B and C) Overexpression caused abnormal ER membrane stacks (B) next to dilated ER and NE structures (B and C, asterisks). (D and E) YFP-mDMPK C localized exclusively to the MOM (D), whereas a cytosolic localization was observed for YFP-mDMPK E (E). M, mitochondrion; N, nucleus. Bars, 100 nm.

**FIG. 4.** Mouse DMPK A and C are tail-anchored proteins. Western blots of pellet (P) and supernatant (S1 and S2) fractions of N2A cells transfected with mDMPK A, C, or E after phosphate-buffered saline (PBS), high-salt, alkaline Na2CO3, or Triton X-100 extraction of 100,000 × g pellets. N2A cells were grown, transfected, and fractionated into supernatant and pellet fractions before extraction as described in Materials and Methods. DMPK was visualized with an anti-DMPK antibody, inp., input; S1/S2 and P, supernatant and pellet fractions after 100,000 × g centrifugation. Asterisks mark doublet DMPK signals, and brackets indicate C-terminally truncated mDMPK products as a result of nonenzymatic breakdown (see the text).
mDMPK. Deletion mutants of mDMPK A and C or the separate tails were fused to YFP, and the subcellular localization of the chimeric proteins was examined during transient expression in N2A cells (Fig. 6A). Proper expression of YFP fusions was assessed via immunoblotting (see Fig. S1 in the supplemental material). Tail 1 alone was sufficient to target YFP, normally mainly a cytosolic/nuclear protein (Fig. 6B), to the ER (Fig. 6C) and the NE (see Fig. S2D to F in the supplemental material). Analysis of additional mutants showed that all information was present in amino acids 579 to 631 (Fig. 6D and E; see Fig. S2G to I in the supplemental material). This confirmed that, as predicted from comparison with other TA proteins, the ER targeting signal is contained within the ultimate C-terminal part of tail 1. Involvement of basic residues was investigated by further truncating tail 1 to amino acids 585 to 631, thereby removing R581 and R584 but leaving R591 in place. Fusion protein YFP-mDMPK A(585–631) still localized to ER networks (Fig. 6F) as well as to the NE (see Fig. S2J to L in the supplemental material), indicating that R581 and R584 are not required for ER targeting. Also, an additional R591A mutation did not affect the ER/NE association (Fig. 6G; see Fig. S2M to O in the supplemental material). This supports the idea that positively charged amino acids are not required for proper ER targeting of tail 1. Hence, the ER targeting signal for mDMPK A is confined within the ultimate 40 amino acids of tail 1, encompassing a putative TMD.

Mitochondrial targeting by mDMPK tail 2 requires basic residues flanking a putative TMD and presence of the coiled-coil domain. We also examined requirements for mDMPK C anchoring in more detail. Unlike for mDMPK A, just the tail region of mDMPK C appeared insufficient for targeting YFP to mitochondria, since a YFP-mDMPK C(536–632) fusion protein was found in the cytosol and to some extent also in the nucleus (Fig. 6H). When the coiled-coil domain was included, as in fusion protein YFP-mDMPK C(421–632), mitochondrial localization was nearly completely restored (Fig. 6I). To determine whether a leucine zipper motif in the mDMPK N terminus participated in the binding of DMPK C to mitochondria, this domain was deleted. Its absence did not affect normal mitochondrial distribution (Fig. 6J). Although this does not necessarily exclude any targeting role for the motif, it makes a direct role in mitochondrial targeting unlikely. In contrast, the C-terminal 45-amino-acid domain plays a dominant role, since removal of the polypeptide stretch between positions 588 and 632 resulted in a cytosolic localization (Fig. 6K). Thus, the targeting signal of mDMPK C must be contained within the C-terminal ~45 amino acids, similar to the situation in mDMPK A. When R624 positioned at the C-terminal side of the putative TMD in tail 2 was mutated to alanine, a small but significant fraction of the YFP-tagged protein shifted to a location in the cytosol, and the mitochondria to which the majority of protein still bound appeared rounded and fragmented (Fig. 6L). This suggests that R624 plays an important role in determining targeting specificity for the MOM (see also below). Finally, introduction of mutations R588A and R589A completely abolished mitochondrial localization (Fig. 6M), indicating that positively charged residues preceding the TMD are important in the targeting signal of tail 2. Taken together, our results demonstrate that MOM targeting by mDMPK tail 2 requires the presence of the coiled-coil domain as well as critical basic residues flanking the putative TMD in the 45-amino-acid tail domain.

The human DMPK A orthologue associates with mitochondria instead of the ER and causes mitochondrial clustering. Although the amino acid sequences of DMPK isoforms are highly homologous between humans and mice (15), we found it conspicuous that the percent sequence identity and similarity for the C termini fell far below the overall values for the whole tail region (see Table S1 in the supplemental material). Between the two species, strong differences exist mainly in the presence and arrangement of essential basic residues, in particular for tail 1. The positioning of basic residues in the human sequence suggested that hDMPK A would localize to mitochondria and not to the ER, like mDMPK A (see Fig. S5 in the supplementary material).
supplemental material). To test this hypothesis, we compared the localization of YFP-hDMPK A and C with that of their mouse orthologues (Fig. 7). In our analyses, we included HeLa cells in order to exclude cross-species effects caused by membrane composition in combination with features in the DMPK sequences. As anticipated, we observed that not only hDMPK C but now also hDMPK A localized to mitochondria in both N2A and HeLa cells (Fig. 7A to F and G to L). Note that mitochondria devoid of hDMPK A remain unclustered (H). Bars, 10 μm.

FIG. 7. Human DMPK A and C localize to mitochondria and alter mitochondrial distribution. Confocal fluorescence microscopy analysis of YFP-hDMPK A and C in transiently transfected N2A (A to F) and HeLa (G to L) cells. YFP-hDMPK A localized at mitochondria, stained by an anti-cytochrome c oxidase marker, which accumulated in the nuclear periphery. YFP-hDMPK C also localized to mitochondria (D to F and J to L) but did not show the accumulation near the nucleus as observed for hDMPK A (B and H). Note that mitochondria devoid of hDMPK A remain unclustered (H). Bars, 10 μm.

DISCUSSION

In the present study, we analyzed parameters involved in the subcellular distribution of protein products of DMPK, the gene mutated in DM1 (16). We demonstrate that distinct mouse DMPK splice isoforms specifically associate with either the ER membrane or the MOM, guided by unique sequence information in their C-terminal tail. Human DMPK forms show no divergence in localization and target to mitochondria only. To our knowledge, DMPK is the first protein kinase that can be classified into the recently established group of TA proteins (reference 4 and references therein).

DMPK is a tail-anchored serine/threonine protein kinase. TA proteins constitute a group of proteins that specifically insert into intracellular membranes, using a single membrane-spanning region located close to the COOH terminus (reference 4 and references therein). Similar to what was found for
the location preference of most tail-anchored proteins (4, 55), mDMPK localization is confined to either membranes of the ER or NE (for tail 1) or to the MOM (for tail 2). Since no appreciable colocalization with Golgi markers or the plasma membrane was observed for mDMPK A, we conclude that the protein, after translation and insertion into the ER membrane, is not further sorted into the vesicular transport route. How this is regulated, via lipids or through active retention, is at present unknown.

Anchoring of mDMPK A and C into their target membranes must be strong, since they cannot be liberated by high-salt or alkaline sodium carbonate treatment. Since the membrane anchors of mDMPK A and C are positioned within the last 40 C-terminal amino acids, we consider it most likely that membrane insertion occurs posttranslationally in an \( N_{\text{out}}-C_{\text{in}} \) orientation. Exactly how mDMPK is anchored in the membrane is not clear, however, and hypothetical models in which mDMPK tails span the membrane or attach to the lipid bilayer are still open (Fig. 10, modes I and II). Further mechanistic studies are therefore required to decide between possible modes of membrane association.

Structural features of ER- and MOM-targeting signals of DMPK isoforms. Little is known about the molecular machinery and steps involved in partitioning of TA proteins into the ER or MOM, except that conventional import pathways do not seem to apply (3, 4). A specific role for the signal recognition particle for targeting of TA proteins to the ER was reported (1), but detailed insight in the mechanism of membrane insertion is currently lacking. Data from various studies suggest that shared structural elements in C termini reflect the only critical features implicated in membrane selectivity. To discriminate between various mechanistic hypotheses, we started here by making a comparison between elements in the tail sequences of DMPK A and C and other members of the group of TA proteins. Length and hydrophobicity of the TMD, together with flanking basic amino acids, have been proposed as the most crucial determinants of membrane selectivity (5, 18, 21, 22).
Positively charged amino acids preceding the TMD may be essential for ER localization, as demonstrated for VAMP-1A, VAMP-2, and VAMP-8 (22). We observed here, however, that an mDMPK A mutant lacking all three basic residues preceding the TMD remained associated with ER and NE membranes. We can exclude compensatory replacement effects of amino acids placed immediately upstream of the TMD since all basic residues derived from the C-terminus of YFP and a short stretch of amino acids specified by the multiple-cloning site in the vector were removed (see the supplemental information). Previous reports showed that a reduction in the overall positive charge C-terminally of the TMD results in higher affinity for ER membranes (18, 24). The fact that amino acids following the TMD in the mDMPK A tail are all neutral is consistent with this observation and may determine binding strength. Taking these data together, we conclude that it is the strong hydrophobic character of the mDMPK A TMD (average hydrophobicity of 2.1) that constitutes the ER and NE targeting signal. Our findings are compatible with other studies showing that artificially engineered stretches of hydrophobic amino acids of different lengths can be sufficient for ER targeting (56, 58).

For the MOM targeting of mDMPK C, more elaborate structural information is required, as can be inferred from the cytosolic localization of YFP-mDMPK C(1–587) and YFP-mDMPK C(536–632). Although the coiled-coil domain by itself confers no targeting properties, as is evident from the cytosolic localization of mDMPK E, the amino acid information in this domain appeared a critical component for correct MOM association of mDMPK C. This could indicate that, apart from providing the appropriate sequence context, the coiled-coil domain could also play a role in protein-protein interactions that determine the specificity and strength of MOM binding (Fig. 10, hypothetical binding modes III and IV). Alternatively, close proximity of coil segments could exert intramolecular effects on the tail conformation necessary for determining MOM anchoring specificity. Whether we can draw any structure-function analogy to proteins with a similar arrangement of membrane-proximal coils and membrane-inserted domains, like the SNARE proteins, remains a question for future study (32). Previous work indicated that basic residues flanking a TMD dictate MOM targeting of TA proteins (4, 21). Consistent with this observation, we report here that mutations R588A and R589A render mDMPK C a cytosolic protein. Remarkably, reducing the basic amino acid content of the very C-terminal end of the tail, by mutating Arg at position 624 to Ala, did not abolish the specificity of the MOM signal. This observation and the finding that its C-terminal end has a net negative charge gives mDMPK C a unique position in the group of MOM anchored proteins.

Interestingly, the sequence arrangements that differ between human and mouse DMPK A tails confer differential properties to the protein, in that hDMPK A is targeted to the MOM whereas mDMPK A associates with the ER. This finding was unanticipated, since there is a remarkable conservation of gene structure and alternative splice sites between the two DMPK genes (15). Perhaps preference for MOM anchoring of hDMPK A is determined by the distinct positioning of basic residues within its tail. Neutral amino acids in the C-terminal stretch of mDMPK A are replaced by basic residues in hDMPK A (see Fig. S5 in the supplement material). More probably, the Arg at position 600 in the human protein breaks up the hydrophobic stretch that was identified as the minimal ER-targeting signal in the mouse form. As a consequence, the resulting tail has a TMD with a lower hydrophobicity, probably too low to promote ER binding. Indeed, an R600A mutation in the human tail I sequence resulted in an ER-localized protein, but deletion of the final 10 C-terminal residues, including 3 arginines, did not alter mitochondrial localization (data not shown). Variation in the guidance motifs in tail anchors may provide an adaptive mechanism for matching intracellular location to function during evolution. On the one hand, we

![FIG. 10. Hypothetical modes of membrane association of DMPK isoforms. A schematic representation of the putative association of DMPK isoforms with their target membrane is shown. C-terminal DMPK anchors can either pass the membrane entirely (mode I) or give close surface binding to the outer leaflet of the lipid bilayer (mode II). A requirement of the coiled-coil domain for proper MOM localization of mDMPK C suggests that either heterologous protein interactions may stabilize mDMPK localization at the membrane (mode III) or multimerization of DMPK via the coiled-coil domain may promote binding specificity and/or strength (mode IV). Possible intramolecular effects of proximity of the coiled-coil segment on folding of the hydrophobic domain can also be envisaged but are not included in the figure.](image)
should therefore consider the possibility that the difference in location preference rendered the functions of mouse and human DMPK. A orthologues completely divergent. On the other hand, it is conceivable that for proper function DMPK needs to be positioned close to sites of ER-MOM contact, which can be specified by association with either the ER or MOM side of the membranous interface. No such speculation about an evolutionary shift in function for the DMPK C isoforms is needed, since both DMPK C isoforms show an identical distribution at the MOM in both human and mouse cells.

Under conditions of high expression, human and mouse DMPK C both adapted a cytosolic localization. This suggests that the MOM interaction of DMPK C can become saturated, as was also shown for VAMP-1B (27). It is important to note here that such an effect was never observed for hDMPK A. Our interpretation of this finding is that, besides the stronger hydrophobicity of the TMD, the net positive charge of +3 of the C-terminal region of the hDMPK A tail, as opposed to the net negative charge found in human and mouse DMPK C, contributes to its more efficient anchoring into the MOM. Species differences in lipid composition of the MOM may influence the requirements of the targeting signal, as shown for the TOM5 protein in yeast and mammalian cells (18). Our observation that the specificity of location is retained between human and mouse DMPK isoforms in cultured cells of human and mouse origin implies, however, that species effects of membrane lipid composition on the location of tail-anchored proteins are of no or only minor concern when compared between mammals.

Perinuclear clustering of mitochondria and OSER formation by DMPK A. An interesting and important difference between DMPK isoforms from mice and humans was that hDMPK A preferentially clusters mitochondria near nuclei while mDMPK A can form OSER structures. Perinuclear clustering and aggregation of mitochondria has also been observed in cultured cells when expressing proteins like mitofusin 1 and 2, as well as with hFIS1 (42, 44, 59), OMP25 (36), or OPA1 (46). Except for OMP25, these proteins are involved in fusion or fission of the mitochondrial membrane, contain a coiled-coil domain, and have targeting properties in common with TA proteins (42, 43, 59). One possible explanation for the observed aggregation effects could be that cytosolic exposure of the coiled-coil domain of hDMPK A induces complex formation and brings together opposing mitochondrial membranes, similar to what was reported recently (23). Interactions between coiled-coil regions contribute to DMPK multimerization (8; R. E. M. A. van Herpen et al., unpublished data), and it is therefore likely that these segments just upstream of the tails play an active role in the clustering behavior. We can virtually rule out low-affinity dimer-binding effects of the YFP-tag since this moiety was present in both constructs but mitochondrial aggregation ability differed profoundly between hDMPK C and A isoforms (compare Fig. 7K and H). To explain why the effect was much weaker with DMPK C, we will need more information about the topology of membrane insertion for each of the DMPK variants. Current evidence also indicates that low-affinity protein interactions at the cytosolic side of the ER membrane are sufficient to promote OSER formation (48).

Coiled-coil regions of mDMPK A may therefore have contributed to the effects seen at the ER membrane as well.

Perinuclear clustering of mitochondria can play diverse roles, including the initiation of apoptosis (50) or local modulation of intracellular Ca\(^{2+}\) transients, as observed in parotid acinar cells (7). Fission and fusion behavior and movement of mitochondria are dependent on the integrity of the cytoskeletal network, and we know that DMPK may play a role in actomyosin dynamics by controlling myosin phosphatase activity via MYPT1 phosphorylation (2, 35, 54). Thus, a functional link between DMPK activity on the cytosolic face of the MOM and interaction with the actin cytoskeleton could well affect mitochondrial distribution. Whether similar associations also could play a role in OSER formation is not known.

Results presented here, combined with preliminary findings in a previous study (54), now suggest a model where distinct locations by insertion into ER or MOM membranes via unique tail anchors determine DMPK’s functional selectivity of activity. Organelles themselves appear to serve as organizing platforms for DMPK function and its subsequent role in cell signaling. Elucidation of possible reciprocal effects of multimORIZATION and kinase activity on organelle membrane dynamics ultimately will be essential for understanding the function of DMPK. Parallel study of DMPK isoforms from different species may be helpful in these studies.

Finally, it is important to note that our findings were confirmed by results obtained with various types of cells, including skeletal muscle-derived myoblasts which, before and after fusion into myotubes, provide the normal host environment for DMPK A and C. Strict control of isoform ratios of C-tailed DMPKs and their levels and location may very well be important for the well-being and viability of muscle cells and other cell types in which the DMPK gene is expressed. In view of the cytopathological effects reported here, this may be especially true for the human DMPK A and C gene products. Just recently, our group has published results showing that cumulative distress of overexpression of hDMPK in a transgenic-mouse model translates into increased muscle workload intolerance, hypertrophic cardiomyopathy with dysrhythmia, myotonic myopathy, and hypertension (37). Further research is thus warranted to see if an imbalance in the mitochondrial targeting and the associated aggregation behavior demonstrated here could have contributed to these DM1-like traits.

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REFERENCES

DMPK C-TERMINAL ANCHORS BIND ER OR MITOCHONDRIA