FEBS Letters 585 (2011) 1238–1242

journal homepage: www.FEBSLetters.org

Charged flanking residues control the efficiency of membrane insertion of the first transmembrane segment in yeast mitochondrial Mgm1p

Marie Österberg [a], Salomé Calado Botelho [a], Gunnar von Heijne [a,c,*], Hyun Kim [a,b,*]

[a] Center for Biomembrane Research, Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden
[b] School of Biological Sciences, Seoul National University, Seoul 151-747, South Korea
[c] Science for Life Laboratory Stockholm University, P.O. Box 1031, SE-171 21 Solna, Sweden

ARTICLE INFO

Article history:
Received 20 February 2011
Revised 24 March 2011
Accepted 24 March 2011
Available online 30 March 2011

Edited by Ulrike Kutay

Keywords:
Mgm1p
Mitochondria
TIM23
Transmembrane helix
Yeast
Saccharomyces cerevisiae

ABSTRACT

Mgm1p is a nuclearly encoded GTPase important for mitochondrial fusion. Long and short isoforms of the protein are generated in a unique "alternative topogenesis" process in which the most N-terminal of two hydrophobic segments in the protein is inserted into the inner mitochondrial membrane in about half of the molecules and translocated across the inner membrane in the other half. In the latter population, the second hydrophobic segment is cleaved by the inner membrane protease Pcp1p, generating the short isoform. Here, we show that charged residues in the regions flanking the first segment critically affect the ratio between the two isoforms, providing new insight into the importance of charged residues in the insertion of proteins into the mitochondrial inner membrane.

1. Introduction

Mitochondrial inner membrane proteins encoded in the nuclear genome are imported into the organelle through the TOM complex in the outer mitochondrial membrane and engage either the TIM23 or the related TIM22 translocons in the inner membrane. Some proteins follow a "conservative sorting" pathway, in which they are first fully translocated into the matrix and then inserted into the inner membrane from the matrix side [1,2]. Other proteins use a "stop-transfer" mechanism where the transmembrane segment(s) exit the TIM23 translocon laterally into the lipid bilayer [2].

Mgm1p, a dynamin-like GTPase involved in mitochondrial fusion, morphology, and genome integrity [3–5], is sorted into the inner membrane by the TIM23 complex through a unique process called alternative topogenesis [6], Fig. 1. Mgm1p has a classical N-terminal presequence followed by two weakly hydrophobic segments and a C-terminal globular domain. After cleavage of the presequence by the matrix processing peptidase MPP (either just before Asn^{70} [7] or Ile^{81} [8]), the first hydrophobic segment is inserted into the inner membrane in 30–40% of the molecules. This gives rise to a membrane-anchored long isoform of the protein (*l*-Mgm1p). In the remaining Mgm1p molecules, the first hydrophobic segment translocates through the TIM23 channel into the matrix whereupon the second hydrophobic segment is cleaved by the rhomboid-like protease Pcp1p in the inner membrane [8], releasing a truncated C-terminal part of Mgm1p (*s*-Mgm1p) to the intermembrane space (IMS). Both isoforms are required for proper function [9]. Although an earlier study [6] has shown that the hydrophobicity of the first transmembrane segment of Mgm1p influences the balance between the two isoforms, the precise sequence determinants that underlie the alternative topogenesis of Mgm1p are still ill defined.

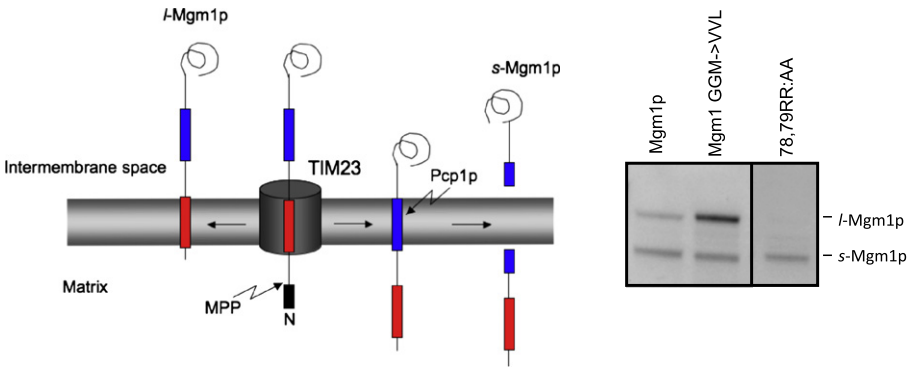
In a recent study [10] we replaced the first hydrophobic segment of Mgm1p by a model segment composed of varying numbers of Ala and Leu residues, and with different combinations of positively or negatively charged residues in the immediate flanking regions. A striking finding in this study was that positively charged residues (Arg, Lys) increase the efficiency of membrane insertion of the Ala-Leu segments when present as flanking residues either on the matrix side or the intermembrane space (IMS) side, while negatively charged residues (Asp, Glu) reduce the insertion efficiency when flanking the hydrophobic segment on the matrix side but have little effect when placed on the IMS side.

Here, we have analyzed the effects of naturally occurring charged residues flanking the inefficiently membrane-inserted first

* Corresponding authors. Addresses: Center for Biomembrane Research, Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden (G. von Heijne). School of Biological Sciences, Seoul National University, Seoul 151-747, South Korea (H. Kim).

E-mail addresses: gunnar@dbb.su.se (G. von Heijne), joy@snu.ac.kr (H. Kim).

0014-5793/$36.00
doi:10.1016/j.febslet.2011.03.056



ITR^{78}R^{79}SISH^{83}FPK^{86}IISK^{90} [IIR^{93}LPIYVGGGMAAAGSYIAYK^{112}M] E^{114}E^{115}ASSFTK^{121}D^{122}K^{123}LD

Fig. 1. Alternative topogenesis of Mgm1p in the mitochondrial inner membrane [6]. Mgm1p is imported through the TIM23 translocon. The presequence (black) is cleaved by the matrix processing peptidase (MPP). The first hydrophobic segment (H1, red) integrates into the membrane in 30–40% of the molecules, resulting in the membrane-anchored long isoform of Mgm1p (left, *l*-Mgm1p). In the remaining molecules, the first hydrophobic segment translocates into the matrix, leaving the second hydrophobic segment (blue) spanning the membrane (right). The second segment is cleaved by the inner membrane protease Pcp1p, giving rise to the short isoform of Mgm1p (*s*-Mgm1p). The gel shows Western blots of yeast cells expressing Mgm1p, the GGM → VVL mutant, and the R^{78}R^{79} → AA mutant; the long and short isoforms are indicated. The H1 hydrophobic segment of Mgm1p is shown at the bottom with all residues mutated in the study indicated.

hydrophobic segment of Mgm1p. The results are broadly consistent with the model sequence studies and show that charged residues on both sides of the transmembrane segment can serve to modulate the membrane-insertion efficiency over a wide range. We also find that the effect is sequence-position dependent in that neighboring charged residues do not affect membrane insertion to the same extent.

2. Materials and methods

2.1. Plasmid construction and yeast strains

Mutations of the flanking charged residues of the Mgm1p H1 segment were prepared by overlap PCR [11] using plasmid pHP84MGM1HA [10] as a template for the reaction. All plasmids carrying a mutation in the *MGM1* gene were constructed by homologous recombination [12] using a PCR amplified *MGM1* fragment and a *Sma I* digested pJK110 [10] with Δ*mgm1a* or α strains (*MATa* or α *his3Δ 1; leu2Δ0; ura3Δ0; mgm1::kanMX4*) [13]. Plasmids were isolated from yeast transformants and the correct sequences were confirmed by DNA sequencing. Correct constructs were then re-transformed into W303-1a (*MAT a, ade2, can1, his3, leu2, trp1, ura3*), selected on -Leu plates, and transformants were subjected to further analysis.

2.2. Western blot analysis

Yeast transformants carrying various *mgm1HA* mutants were grown overnight in 5 ml of -Leu medium at 30 °C. Whole-cell lysates were prepared as described [14]. Western blotting was carried out with an anti-HA antibody, blots were imaged on a Fuji LAS-3000 phosphoimager, and bands were quantified using the Image Gauge V 3.45 software. Selected *mgm1HA* mutants were also analysed in the temperature-sensitive strain *pam16-3* and in the *PAM16* isogenic wild-type strain [15].

2.3. Calculation of ΔG_{app}

Membrane integration of each H-segment was quantified from Western blots by measuring relative amounts of *l*-Mgm1p and *s*-Mgm1p isoforms. The relative amounts of the two isoforms were used to calculate an apparent equilibrium constant between the membrane integrated and non-integrated forms: $K_{app} = f_l/f_s$, where f_l is the fraction of membrane-integrated and f_s the fraction of membrane non-integrated isoforms. Finally, the membrane-insertion efficiency was expressed as an apparent free energy difference between the non-inserted and inserted states, $\Delta G_{app} = -RT \ln K_{app}$.

3. Results and discussion

The hydrophobicity of the inefficiently inserted N-terminal hydrophobic segment (H1) in Mgm1p is weak compared to typical transmembrane segments in single-spanning proteins from the mitochondrial inner membrane, Table 1. Previous studies [10,16,17] have shown that the presence of charged residues in the neighborhood of a transmembrane domain can affect membrane integration in the mitochondrial inner membrane. Consistent with this, statistical analysis on the distribution of charged residues flanking the transmembrane domains of mitochondrial inner membrane proteins that follow the 'stop-transfer' pathway has shown that positively charged Lys and Arg residues are abundant on both sides of the TM segments, whereas negatively

Table 1
Predicted free energy of membrane insertion (ΔG_{app}) for transmembrane segments from single-spanning mitochondrial inner membrane proteins as calculated by the ΔG-predictor [19]. The Mgm1p H1 segment is in italics.

YGDB identifier	Name	ΔG_{app} (kcal/mol)	Predicted TM sequence	Ref.
YDR393w	She9	−2.0	TWGTFILMGMNIFLFIVLQLLL	[16]
YIL111w	Cox5b	−1.4	AFITKGVFLGLGISFGLFGLVRLLA	[16]
YMR302c	Yme2	−1.1	TRIAIPVLFALLSIFAVLVF	[16]
YDR316w	Oms1	−1.0	MTKYMIGAYVIFLIYGLFFTKKLF	[16]
YPL132W	Cox11	−0.9	RTVAFYFSSVAVLFLGLAYAAVPLY	[20,21]
YGR174c	Cbp4	−0.5	LWVRWLKVYAIGGAIIGSGFLLFKY	[16]
YNL052w	Cox5a	−0.2	FIAKGVAAGLLFSVGLFAVVRMA	[16]
YOR065w	Cyt1	−0.0	RLGLKTVIILSSLYLLSIWV	[16]
YBR024w	Sco2	0.0	RWKATIALLLLSGGTYAYL	[16]
YPL063w	Tim50	0.4	YANWFYIFSLSALTGTAIYMAR	[16]
YBR037c	Sco1	0.5	FSTGKAIALFLAVGGALSYFF	[16]
YPR024w	Yme1	0.5	RWVKWLLVFGILTYSF	[16]
YKL195w	Mia40	0.5	TAGFIMGILSMAGALYFIA	[20,22]
YDL174c	Dld1	1.5	WLKYSVIASSATLFGYLFA	[16]
YER014w	Hem14	1.5	RAKVAVVGGGVSGLCFTYFLSKL	[16]
YER058w	Pet117	1.7	ITFAASCLITAATVVGVHYV	[20]
YOR211c	*Mgm1 H1*	*2.0*	*IIRLPIYVGGGMAAAGSYIAYKM*	[6]

charged residues seem to be under-represented on the matrix side [10]. Since the Mgm1p H1 segment is only inefficiently inserted into the inner membrane, we suspected that its membrane integration may be particularly sensitive to mutations in its charged flanking regions. We therefore replaced charged residues found in the N- and C-terminal flanking regions of the H1 segment by Ala and measured how the ratio of the *l*-Mgm1p and *s*-Mgm1p isoforms was affected.

First, we investigated the effects of N-terminal, matrix-facing charged flanking residues of the Mgm1p H1 segment on its membrane insertion. As seen in Fig. 2A (see Supplementary Fig. S1 for Western blots), mutation of the positively charged residue Arg at position 79 (R^{79}), but not at position 78 (R^{78}), to Ala significantly increased the apparent free energy of membrane insertion (ΔG_{app}) of the H1 segment by 0.5 kcal/mol, meaning reduced membrane insertion of the H1 segment. A further increase in ΔG_{app} was observed when R^{78} and R^{79} were simultaneously mutated to AA, and when combined with a third H^{83} to A mutation. Changing R^{78} to D also led to a significant decrease in the membrane insertion of the H1 segment, while replacing $R^{78}R^{79}$ with KK did not lead to a significant change in insertion, indicating that it is the positive charge on R^{78} and R^{79} that is critical. The simultaneous mutation of K^{86}, K^{90}, and R^{93} in the N-terminal flanking region to AAG also led to a significant increase in ΔG_{app}, whereas the individual mutations had little effect.

As shown before [6], increasing the hydrophobicity of H1 leads to a significant decrease in ΔG_{app} (mutation GGM → VVL; underlined in Fig. 1), but again membrane insertion of this mutant is critically dependent on flanking charged residues as changing R^{78} and R^{79} to Ala increases ΔG_{app} from −0.7 to 1.2 kcal/mol.

It has been suggested that R^{78} and R^{79} could be part of the MPP consensus cleavage site [8], but more recent work has identified a MPP cleavage site at Asn^{70} [7]. We have not investigated this further, but since we did not detect any band corresponding to the presequence-containing precursor form of Mgm1p in our Western blot analysis we assume that single or double mutations of R^{78} and R^{79} do not influence presequence recognition and cleavage by MPP.

To study the roles of charged residues in the C-terminal, IMS-facing flanking region of H1, we systematically mutated charged residues within 10 residues from the end of H1 segment to Ala, Fig. 2B. Individually mutating K^{112}, E^{114}, or E^{115} to A did not give rise to a statistically significant change in ΔG_{app}. However, when E^{114} and E^{115} were simultaneously mutated to A (with or without including the $K^{112} \rightarrow A$ mutation), ΔG_{app} was significantly increased. Mutation of three charged residues further away from the H1 segment, $K^{121}D^{122}K^{123}$ to A did not increase ΔG_{app}. Unexpectedly, we observed a nearly complete absence of membrane insertion of H1 segment when E^{114} and E^{115} were simultaneously mutated to K. Schäfer et al. [18] have recently investigated the importance of charged residues flanking the H2 segment in

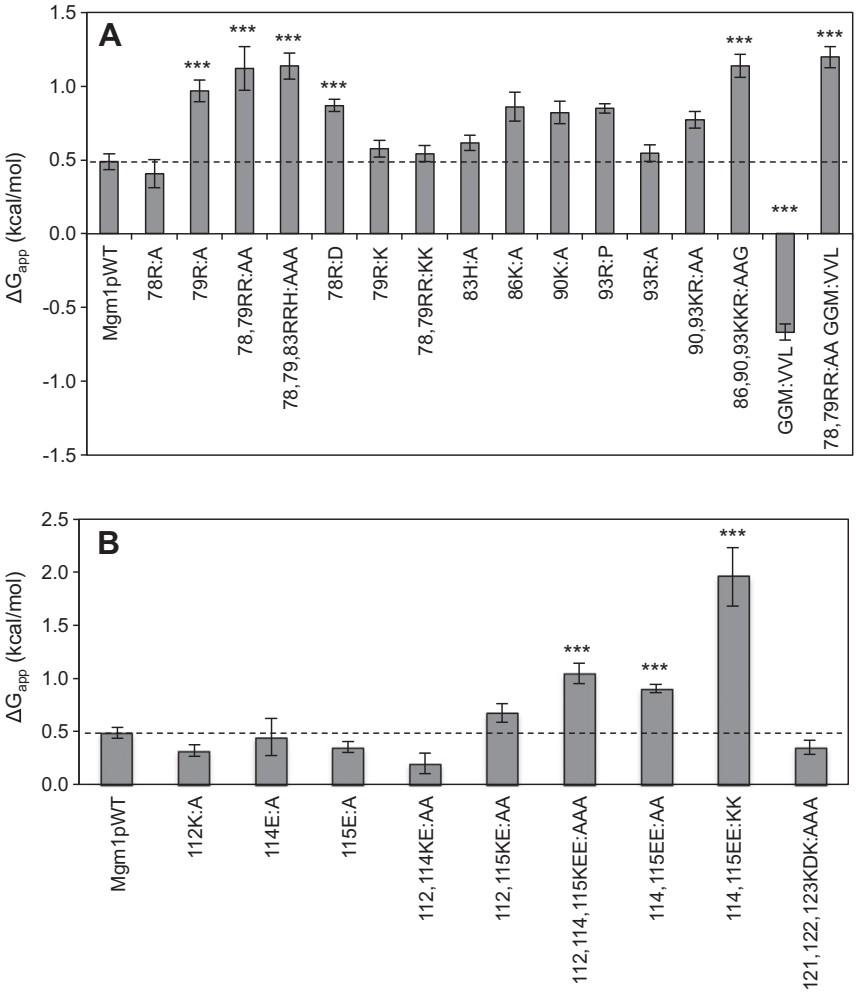


Fig. 2. Apparent free energy of membrane insertion (ΔG_{app}) of the H1 segment in different Mgm1p mutants. (A) Mutations in the N-terminal matrix-facing flanking region. (B) Mutations in the C-terminal IMS-facing flanking region. Averages from at least three independent experiments and standard deviations are shown. Statistically significant differences between Mgm1p wild type (wt) and mutants are indicated by ***(two-tailed *t*-test, 0.1% level).

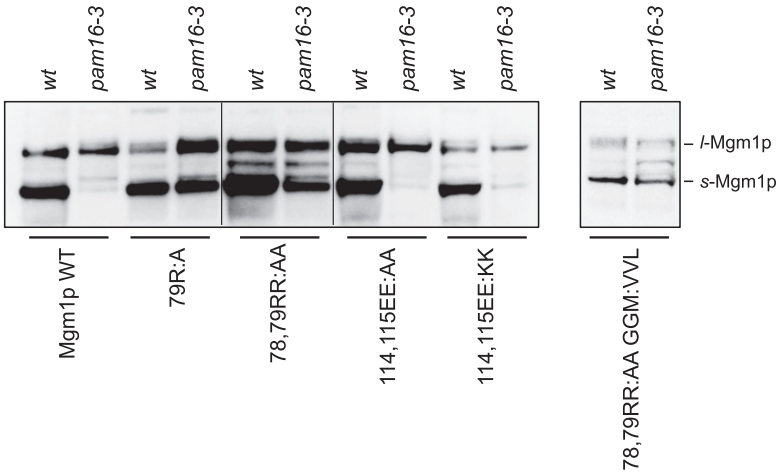


Fig. 3. Western blot analysis of Mgm1p mutants expressed in the *pam16-3* strain and in the isogenic *PAM16* wildtype (*wt*) strain. Lanes within the same panel are from the same gel.

Mgm1p for Pcp1 recognition and cleavage during import. The residues we have mutated are 40 to 80 amino acids upstream of the Pcp1 cleavage site and the fact that we see either no change in the ratio between *l*-Mgm1p and *s*-Mgm1p or an increase in the relative amount of *s*-Mgm1p show that mutations around the H1 segment do not impair the intrinsic ability of the H2 segment to be recognized and cleaved by Pcp1.

The formation of the *s*-Mgm1p isoform depends on the so-called import motor in the matrix [5]. Since many of the mutations around the H1 segment described above give rise to an increase in *s*-Mgm1p and hence more efficient translocation of the H1 segment across the inner membrane, we decided to also study their behavior in *pam16-3* cells where the import motor is not fully functional [15]. As expected, very little *s*-Mgm1p was seen in *pam16-3* cells expressing wildtype Mgm1p, Fig. 3. Low levels of *s*-Mgm1p were also evident for the $E^{114}E^{115} \rightarrow AA$ and $E^{114}E^{115} \rightarrow KK$ mutations. Interestingly, however, when R^{79} or R^{78} together with R^{79} were mutated to Ala (with or without the additional GGM → VVL change in H1), the *pam16-3* mutation had a much smaller effect on the amount of *s*-Mgm1p, suggesting that the $R^{79} \rightarrow A$ mutation may reduce the membrane-insertion efficiency of the H1 segment to such an extent that even a functionally impaired import motor can pull it across the inner membrane.

In summary, the alternative topogenesis of Mgm1p is critically dependent not only on the hydrophobicity of the H1 segment, but also on flanking charged residues. As suggested by our previous study with model transmembrane segments [10], mutations of positively charged residues to Ala or Glu in the N-terminal, matrix-facing flanking region reduce membrane insertion (increased ΔG_{app}), but not all residues contribute equally. In particular, R^{79} has a much stronger effect than R^{78}, which is only one residue further away from the end of the H1 segment. Replacing R^{78} and R^{79} by lysines has no effect on membrane insertion, but replacing R^{78} with a negatively charged Asp reduces insertion. It is also noteworthy that the $R^{78}R^{79} \rightarrow AA$ mutation increases ΔG_{app} by as much as 1.7 kcal/mol for an H1 segment of increased hydrophobicity (the GGM → VVL mutant). These results suggest that the two positively charged residues at positions 78 and 79 are critical determinants for the balanced production of the two Mgm1p isoforms. Further, the results show that the effect of the $R^{78}R^{79} \rightarrow AA$ mutation overrides the effect of increased hydrophobicity of the H1 segment.

In the C-terminal, IMS-facing flanking region, the two negatively charged residues E^{114} and E^{115} seem particularly important for membrane insertion of the H1 segment. When these two residues were simultaneously mutated to A, ΔG_{app} increased by 0.4 kcal/mol, and when mutated to Lys the effect was even stronger and almost no membrane-anchored, long isoform of Mgm1p was detected. In our previous study [10] we found that positively charged flanking residues promote membrane insertion of model hydrophobic segments when placed either on the matrix or IMS side of the inner membrane, however replacement of $E^{114}E^{115}$ in the C-terminal IMS-facing flanking region of the H1 segment by KK completely inhibits membrane insertion of the H1 segment and promotes translocation into the matrix. Further studies will be required to resolve these conflicting observations.

We conclude that flanking charged residues can have a major effect on the insertion of transmembrane segments into the mitochondrial inner membrane. However, in the context of the Mgm1p H1 segment the effects are not always additive, but can depend on the precise location of the residue in the flanking segment. Among the charged residues on the N-terminal, matrix-facing side of the Mgm1p H1 segment the $R^{78}R^{79}$ pair shows the most significant influence on membrane insertion, whereas at the C-terminal, IMS-facing side the $E^{114}E^{115}$ pair is important for proper sorting of the Mgm1p H1 segment. While the hydrophobicity of the Mgm1p H1 segment correlates directly with the efficiency of membrane insertion [6,10], our results here point to a more complicated influence on insertion from the regions flanking the hydrophobic stretch.

Acknowledgements

This work was supported by grants from the Swedish Research Council, the Swedish Cancer Foundation, the Swedish Foundation for Strategic Research, and the European Research Council (ERC-2008-AdG 232648) to G.v.H. and from the National Research Foundation of Korea (NRF0409-20100093) to H.K. S.C.B. is a recipient of a graduate student fellowship (SFRH/BD/36107/2007) from Fundação para a Ciência e a Tecnologia, Portugal. We thank Dr. Nikolaus Pfanner, Freiburg, for kindly providing the *pam16-3* strain and the isogenic wildtype strain.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.03.056.

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