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Research Article

Identification of functional domains of Mid1, a stretch-activated channel component, necessary for localization to the plasma membrane and Ca^{2+} permeation

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Abstract

The *Saccharomyces cerevisiae* MID1 gene product (Mid1) is a stretch-activated Ca^{2+}-permeable channel component required for Ca^{2+} influx and the maintenance of viability of cells exposed to the mating pheromone, α-factor. It is composed of 548-amino-acid (aa) residues with four hydrophobic segments, H1 (aa 2–22), H2 (aa 92–111), H3 (aa 337–356) and H4 (aa 366–388). It also has 16 putative *N*-glycosylation sites. In this study, sequentially truncated Mid1 proteins conjugated with GFP were expressed in *S. cerevisiae* cells. The truncated protein containing the region from H1 to H3 ($Mid1^{1-360}$-GFP) localized normally in the plasma and endoplasmic reticulum (ER) membranes and complemented the low viability and Ca^{2+}-uptake activity of the mid1 mutant, whereas $Mid1^{1-133}$-GFP containing the region from H1 to H2 did not. $Mid1^{\Delta 3-22}$-GFP lacking the H1 region failed to localize in the plasma membrane. Membrane fractionation showed that $Mid1^{1-22}$-GFP containing only H1 localized in the plasma membrane in the presence of α-factor, suggesting that H1 is a signal sequence responsible for the α-factor-induced Mid1 delivery to the plasma membrane. The region from H1 to H3 is required for the localization of Mid1 in the plasma and ER membranes. Finally, trafficking of Mid1-GFP to the plasma membrane was dependent on the *N*-glycosylation of Mid1 and the transporter protein Sec12.

Keywords: MID1; Yeast; Mechanosensitive channel; Ca^{2+} channel; *N*-glycosylation; Plasma membrane; GFP; Subcellular localization; Sec

Introduction

A variety of Ca^{2+}-permeable, stretch-activated channels (SACs) have been detected electrophysiologically in prokaryotes and eukaryotes [1]. However, only a limited number of genes encoding stretch-activated channels have been cloned, including yeast *MID1* encoding a channel component responsible for cell survival upon mating pheromone treatment [2,3]; bacteria *MscL*, a mechanosensitive channel of large conductance [4]; bacteria *MscS*, a mechanosensitive channel of small conductance [5]; *Drosophila nompC*, a channel for mechanoreceptor potential [6]; and mammalian *TRPV2*, a vanilloid receptor homologue [7]. *MID1* is a yeast *Saccharomyces cerevisiae* gene encoding a plasma membrane protein required for Ca^{2+} influx induced by the mating pheromone, α-factor [8]. When exposed to this pheromone, cells of the *mid1* mutant will die because of low Ca^{2+} uptake, showing that the Mid1 protein has a crucial role in supplying Ca^{2+} during the

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mating process. Mid1 is composed of 548-amino-acid residues with four hydrophobic regions named H1, H2, H3 and H4. Although Mid1 has no overall sequence similarity to known ion channels, the amino acid sequence of its putative transmembrane segments (H2 and H4) is similar to those of the S2/H2 and S3/H3 membrane-spanning domains of voltage-sensitive ion channels according to computer analysis [2,8,9]. Single channel current recordings of Mid1 expressed in mammalian cells suggest that Mid1 is a Ca^{2+}-permeable SAC [2,3]. After translation, Mid1 is *N*-glycosylated and transported to the plasma membrane, where it would be activated by membrane stretch during the polarized growth of cells exposed to α-factor [8]. However, the details of transport to the plasma membrane and the functional domains of Mid1 crucial for the channel activity are largely unknown.

Trafficking of ion channels to the plasma membrane has been studied in various eukaryotes [10,11]. The trafficking process is dependent on a signal sequence [12], glycosylation of particular regions of channel proteins [13] and transporters known as Sec family proteins [14]. Specific proteins and co-factors that control distinct steps including budding, transport, docking and fusion with target membranes regulate vesicle biogenesis. Budding requires an assembly of a coat protein complex on the membrane, membrane deformation and subsequent cleavage of the nascent vesicle from the donor membrane. Sec proteins bind to other factors and form COPI and COPII complexes [15]. These complexes are responsible for the transportation of proteins between the endoplasmic reticulum (ER) and trans Golgi network. In addition, channel subunits, such as the β-subunit associated with Ca^{2+} channels, are necessary for the trafficking of ion channels [16].

The N-terminal 33-amino-acid (aa) residues of mammalian RGS4 (a GTPase-activating protein for the α-subunits of G-proteins) constitute a signal sequence [17]. Deletion of this sequence results in a loss of plasma membrane localization, while the fusion of this sequence with GFP is sufficient for the transport of GFP to the plasma membrane. The signal sequence is important for membrane-targeted delivery of *S. cerevisiae* proteins. The H1 region of Mid1 is a potential signal sequence according to our computer analysis [8,18].

N-glycosylation plays a crucial role in the trafficking of various membrane proteins to the cell surface [19], and in the recognition of mis-folded proteins in the ER [20]. Mutational analysis has shown that *N*-glycosylation is required for proper membrane trafficking and/or the functioning of some G-protein-coupled receptors (GPCRs) [21]. Mid1 is also modified by *N*-glycosylation [8], but the role of this modification in the Mid1 function has not been studied.

Mid1 has two cysteine-rich regions at the C-terminal (C1 and C2) and 16 potential *N*-glycosylation sites [8]. We made a series of deletion mutants of Mid1 fused with GFP (Mid1-GFP) based on the protein's hydrophobic segments and cysteine-rich regions and analyzed their ability to complement the *mid1* mutation. We also investigated their subcellular localizations and Ca^{2+} permeability to analyze the responsible domains for the trafficking of Mid1-GFPs and Ca^{2+} permeation, respectively. Finally, we examined the significance of the *N*-glycosylation of Mid1 and the role of Sec transport proteins in the trafficking and function of Mid1.

Materials and methods

Yeast strains and media

The yeast strains used in this study are listed in Table 1. Rich and synthetic media (SD) were prepared as described previously [8]. SD medium contained 680.2 μM $CaCl_2$ and 0.8 μM calcium pantothenate. Ca^{2+}-deficient medium (SD-Ca) contained nominally free $CaCl_2$, and sodium pantothenate instead of calcium pantothenate. SD.Ca100 medium was prepared by adding 100 μM $CaCl_2$ to SD-Ca medium. Wild-type cells, but not *mid1* cells, survive in a low-Ca^{2+} medium (SD.Ca100) when incubated with α-factor [8]. Temperature-sensitive *sec6* (ANS6-2D), *sec7* (SF821-8A) [22] and *sec12* (MBY10-7A) [23] mutants were incubated at 30°C for propagation and at a restrictive temperature (37°C) for 2 h for the expression of mutant properties.

Determination of mid1 complementing activity using the methylene blue liquid method

To examine the complementing activity of a series of deletion mutants of Mid1-GFP, we employed the methylene blue liquid method [8]. A portion (0.1 ml each) of exponentially growing culture in SD.Ca100 medium or that treated with α-factor for 0, 4 and 8 h was mixed with an equal volume of 0.01% methylene blue/2% sodium citrate solution, and the viable white cells and inviable blue cells were enumerated under a differential interference-contrast microscope.

Table 1
Saccharomyces cerevisiae strains used in this study

Strain	Genotype	Source
H207	*MATa his3-Δ1 leu2-3,112 trp1-289 ura3-52 sst1*-2H301	8
H301	*MATa his3-Δ1 leu2-3,112 trp1-289 ura3-52 sst1-2 mid1*-1MBY10-7A	8
ANS6-2D	*MATa sec6-4 ura3-52 leu2-3,112 trp1-289 his3/4*	Gift from A. Nakano and M. Sato
SF821-8A	*MATa sec7-1 ura3-52 leu2-3,112 trp1-289 his4-580a* ANS6-2D	22
MBY10-7A	*MATa sec12-4 ura3-52 leu2-3,112 trp1-289 his3/4 suc gal2*SF821-8A	23