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The yeast CLC chloride channel is proteolytically processed by the furin-like protease Kex2p in the first extracellular loop

Andrea Wächter, Blanche Schwappach*

Zentrum für Molekulare Biologie (ZMBH), Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany

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Abstract CLC chloride channels are a family of channel proteins mediating chloride transport across the plasma membrane and intracellular membranes. The single yeast CLC protein Gef1p is localized to the Golgi and endosomal system. Investigating epitope-tagged variants of Gef1p, we found that the channel is proteolytically processed in the secretory pathway. Proteolytic cleavage occurs in the first extracellular loop of the protein at residues KR136/137 and is carried out by the Kex2p protease. Fragments mimicking the N- and C-terminal products of the cleavage reaction are non-functional when expressed alone. However, functional channels can assemble when the two fragments are co-expressed.

Keywords: CLC chloride channel; Intracellular ion channel; Proteolytic processing; Furin

1. Introduction

CLC proteins are chloride ion channels associated with a broad spectrum of physiological functions [1]. Of the nine CLC homologues present in mammalian organisms five channels are mainly or exclusively localized to intracellular membranes, where they contribute to the ionic composition of the luminal compartment, particularly to endosomal and lysosomal acidification. Vesicle trafficking connects the different membrane-enclosed compartments. Thus, ion transport activities must be exquisitely regulated to maintain differences in ion composition between different organelles like the endoplasmic reticulum (ER), the Golgi apparatus, and the endo-lysosomal system. For CLC channels, this regulation is poorly understood but likely comprises high-fidelity sorting processes as well as functional regulation of these proteins.

We have chosen the single CLC protein present in the yeast *Saccharomyces cerevisiae* [2] as a model to investigate the sorting and regulation of CLC channels present in the Golgi and endosomal system. Inactivation of the CLC (*GEF1*) gene in yeast has been shown to result in two main phenotypes: loss of high affinity iron uptake and reduced resistance to toxic cations [2–4]. High-affinity iron uptake is lost because Fet3p (an oxidase involved in high affinity iron uptake at the cell surface) does not mature normally in Δ*gef1* strains. Copper loading to the active centre of Fet3p in the lumen of the late secretory pathway requires Cl^- ions that enter the compartment via the CLC channel [5]. The sequestration of toxic cations is presumably affected because this process depends on anions that can counterbalance the accumulation of positive charge on the luminal side. The subcellular localization of Gef1p to the Golgi and prevacuolar compartments [4,6] is consistent with the functions suggested by the knockout phenotypes. However, we report here that there is an additional level of complexity to consider, since the channel protein undergoes proteolytic processing in the secretory pathway.

2. Materials and methods

2.1. Yeast strains and media

Standard yeast media and genetic manipulations were as described [7]. LIM50 low iron selection medium was prepared as described [8]. Where indicated methionine was omitted from the media to allow for maximal expression from plasmids containing the *MET25* promoter [9]. The Δ*gef1* deletion strain has been described [4]. The BY4741-derived Δ*kex2* strain was obtained from the EUROSCARF consortium (www.uni-frankfurt.de/fb15/mikro/euroscarf). For comparison a Δ*gef1* strain from EUROSCARF was used for the experiment shown in Fig. 2C.

2.2. Molecular biology

Standard molecular biology protocols were adapted from [7]. Epitope-tagged, mutated versions, and GFP fusion constructs of Gef1p were created using the polymerase chain reaction. Constructs were verified by sequencing. GFP (S65T) was fused to the N- or C-terminus of Gef1p using an engineered *Not*I restriction site replacing the start or stop codon of the respective open reading frames with three codons encoding alanine. The 4 protein C (PC) epitope was fused to Gef1p using the same *Not*I sites. The sequence of the PC epitope (Roche) reads EDQVDPRLIDGK. For integrating constructs, plasmids pRS305 and pRS306 were used [10]. Plasmids containing the *MET25* promoter [9] were used when indicated.

2.3. Subcellular fractionation on sucrose gradients

Separation of organelles was performed according to Nass and Rao [11]. Briefly, cells were grown to mid-logarithmic phase, converted to spheroblasts and lysed by douncing in a hypotonic buffer (0.3 M sorbitol, 50 mM triethanol amine, 1 mM EDTA, and 2 mM Mg acetate, pH 8.9). The homogenate was layered on freshly prepared ten-step sucrose gradients and centrifuged for 2 h at 23 500 rpm in an SW28 rotor (Beckman). Membranes from the different fractions were pelleted by centrifugation (SW28, 30 min at 23 500 rpm). The sucrose concentration of each fraction was measured using a refractometer.

*Corresponding author. Fax: +49 6221 545894.
E-mail address: b.schwappach@zmbh.uni-heidelberg.de (B. Schwappach).

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2.4. Immunoblotting

Yeast extracts were prepared from 1.8 OD_{600} units of log-phase cells by alkaline lysis and precipitation of total cellular protein by trichloroacetic acid. Total extracts or purified membranes were separated by SDS–PAGE using 10% or 7% gels and transferred to nitrocellulose. Blots were blocked in TBS containing 5% milk powder and 0.02% NP-40. Primary antibodies (anti-PC mouse monoclonal HPC4, Roche, 0.25 μg/ml; anti-Pep12p, Molecular Probes, 0.5 μg/ml) and secondary antibodies (HRP-conjugated anti-mouse and anti-rabbit antibodies, Jackson, 1:3000) were diluted in TBS-blocking solution. Washes were in TBS-blocking solution and then in TBS, 0.02% NP-40. Detection was performed using the ECL system (Amersham).

3. Results

Published data addressing the subcellular localization of the yeast CLC protein employs overexpression of a C-terminally GFP-tagged variant [3,4,6,12]. Since localization to the Golgi and endosomal system can be saturated we wanted to study Gef1p expressed at endogenous levels. To this end, we created a number of strains with tagged versions of the open reading frame under the control of the endogenous promoter of *GEF1*. While it was impossible to detect GFP-tagged versions of Gef1p by Western blotting, immunoprecipitation, FACS analysis, or microscopy (not shown), these variants did complement the growth defect of the Δ*gef1* strain on iron-limited medium (Fig. 1A). The only tagged versions of Gef1p that we could detect by Western blotting were 4PC–Gef1p and Gef1p-4PC. Employing these N- or C-terminally PC epitope tagged variants, we addressed the localization of the protein by subcellular fractionation on sucrose gradients. To our surprise, we found that the Gef1p chloride channel is proteolytically processed giving rise to an N-terminal fragment of ≈30 kDa and a C-terminal fragment of ≈70 kDa (Fig. 1B). For the C-terminally tagged version most of the immunoreactivity was found in a band migrating at 70 kDa that was predominantly localized to the membranes containing strong Pep12p immunoreactivity, a marker for the pre-vacuole. A larger band of ≈100 kDa (presumably representing full-length Gef1p) was observed in the denser fractions of the gradient, where ER and plasma membrane marker proteins migrated (not shown). The N-terminally tagged variant of Gef1p was detected as a 100 and a 30 kDa band, presumably representing full-length Gef1p and the N-terminal processing product that is missing from the 70 kDa fragment observed for the C-terminally tagged form.

The recently solved three-dimensional structure of a bacterial CLC homologue [13] provides a basis for the assignment of transmembrane helices in the ClC-3,4,5 subfamily [14] as well as Gef1p. The first extracellular loop of Gef1p contains a consensus cleavage motif (KR136,137; Fig. 2A) for the furin-like Kex2p protease present in late Golgi compartments [15,16]. This di-basic motif is not conserved in the three closest mammalian homologues ClC-3,4,5 (Fig. 2B). Mutation of the two basic residues to alanines yielded a variant of Gef1p–4PC that migrated at around 100 kDa (Fig. 2C) as predicted for full-length Gef1p–4PC and as observed for a minor fraction of wildtype Gef1p–4PC (note that this minor species migrated in the dense fractions of the gradient as shown in Fig. 1B). Almost no protein with faster mobility was observed for the mutant form indicating that specific cleavage of the protein was in fact abolished by the mutation. Next, we investigated whether wildtype Gef1p is processed in a Δ*kex2* knockout strain. Again, only the 100 kDa form of the tagged protein was observed, migrating at the same position as the KR mutant of Gef1p from a wildtype extract (Fig. 2C). These findings demonstrate that specific proteolytic cleavage of the first extracellular loop of Gef1p requires Kex2p.

We wondered whether the non-cleavable mutant form would complement a Δ*gef1* knockout strain with respect to the defect in high-affinity iron uptake (Fig. 3A). This was indeed the case

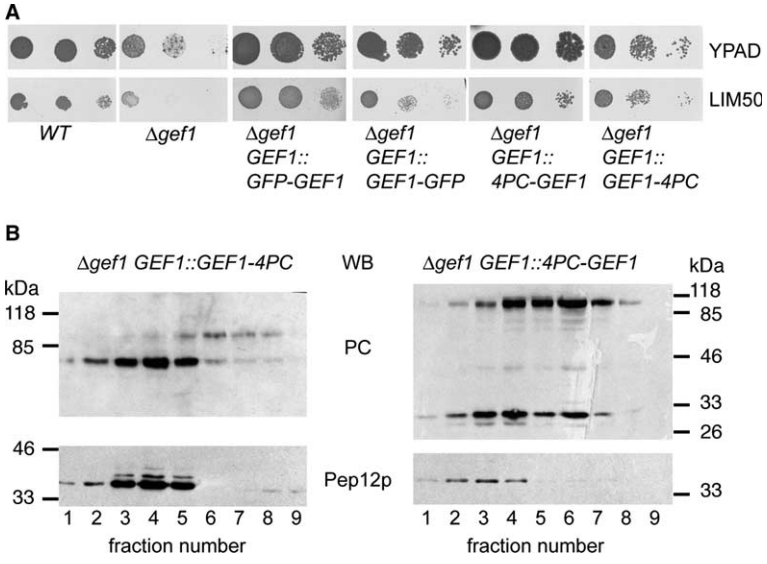

Fig. 1. Gef1p is proteolytically processed. (A) Four tagged versions of Gef1p complement the growth defect on iron-limiting medium when expressed under control of the *GEF1* promoter. All strains were created using integrating plasmids containing the *GEF1* 5′ and 3′ untranslated regions. Serial dilutions of cultures grown to the same density in YPAD are shown on YPAD and LIM50 plates. (B) Subcellular fractionation of yeast cells expressing Gef1p–4PC and 4PC–Gef1p in the Δ*gef*1 background under the control of the *GEF1* promoter. Homogenates were prepared from spheroblasts and loaded on ten-step sucrose gradients. Membranes were sedimented from the fractions indicated and 5 μg of membrane protein was loaded in each lane followed by Western blotting analysis using the indicated antibodies.

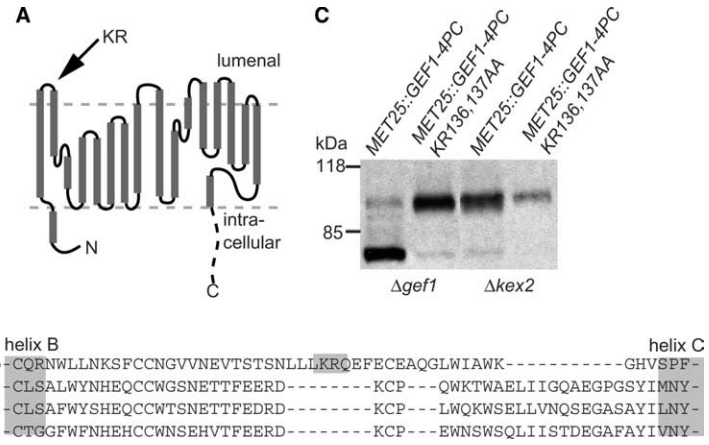

Fig. 2. Gef1p is cleaved by the Kex2p protease in the first extracellular loop. (A) Schematic representation of the structural organization of the membrane-embedded part of CLC channels (adopted from [13]). The putative Kex2p cleavage site (KR) is marked with an arrow. (B) Alignment of the first extracellular loop for Gef1p and the three most related mammalian CLC proteins ClC-3,4,5. Assignment of helices derived from [13] was performed according to Gentzsch et al. [14]. (C) Mutation of residues KR136,137 in Gef1p to alanines or absence of Kex2p abolish cleavage. Strains were transformed with plasmids encoding the indicated constructs and grown in SD medium without methionine for induction. Total protein extracts from identical amounts of cells were resolved by SDS–PAGE and analyzed by anti-PC immunoblotting.

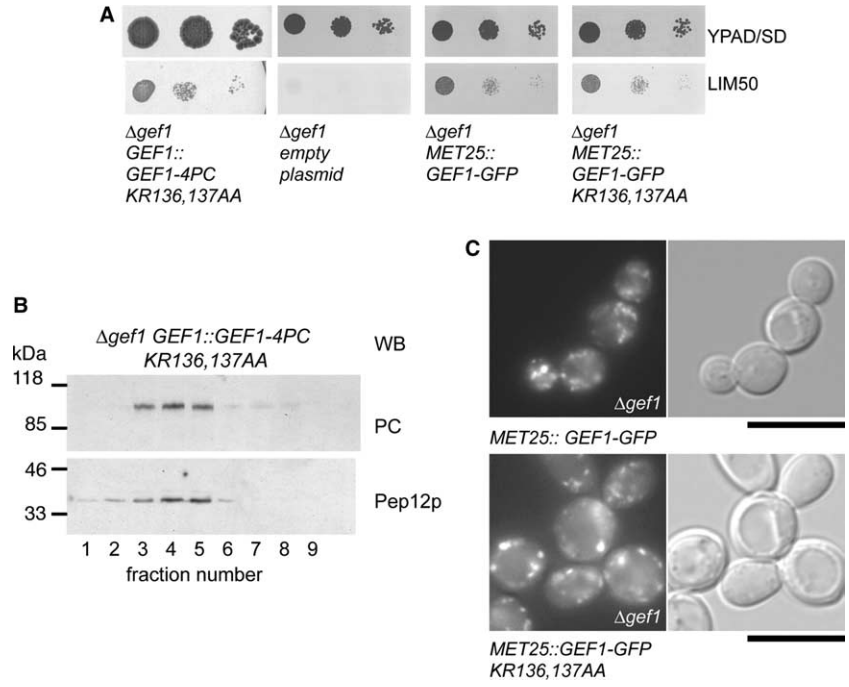

Fig. 3. A non-cleavable mutant of Gef1p is functional. (A) The mutated version of Gef1p–4PC complements the growth defect on iron-limiting medium when expressed under control of the *GEF1* or the *MET25* promoter. The *GEF1::GEF1–4PC(KR136,137AA)* strain was created using integrating plasmids containing the *GEF1* 5′ and 3′ untranslated regions. Serial dilution of a culture grown in YPAD is shown on YPAD and LIM50 plates. Δ*gef1* cells were transformed with the empty plasmid p416MET25 or the same plasmid containing the indicated constructs. Transformants were grown in SD medium lacking uracil to the same density and serial dilutions were spotted on SD and LIM50 plates. (B) Subcellular fractionation of yeast cells expressing the non-cleavable mutant form of Gef1p–4PC in the Δ*gef*1 background under the control of the *GEF1* promoter. Everything else as described in Fig. 1C. (C) GFP fluorescence patterns of cells transformed with plasmids encoding Gef1p–GFP fusion proteins as indicated. Nomarski images are shown on the right. Size bar is 10 μm.

for the mutant Gef1p–4PC expressed at endogenous levels as well as mutant Gef1p–GFP overexpressed from an exogenous promoter. We addressed the sub-cellular localization of the non-cleavable protein by the fractionation of organelles on a sucrose gradient (Fig. 3B). Like the products of the cleavage reaction (Fig. 1), the non-cleavable variant accumulated in the prevacuole as marked by Pep12p indicating that sorting of the protein to the prevacuole does not depend on cleavage. The GFP fluorescence pattern of the overexpressed mutant protein was indistinguishable from the wildtype pattern (Fig. 3C).

To gain further insight into the possible functional significance of the proteolytic processing of Gef1p by Kex2p in the late Golgi, we expressed proteins engineered to mimic the

N- and C-terminal processing products. To this end, a stop codon was introduced to the *GEF1* open reading frame at codon 138 to mimic the situation after Kex2p cleavage has taken place (*GEF1*ΔC137). A second protease, Kex1p, is known to further process Kex2p cleavage products by removing the two basic residues at the C-terminus of the N-terminal fragment [17].

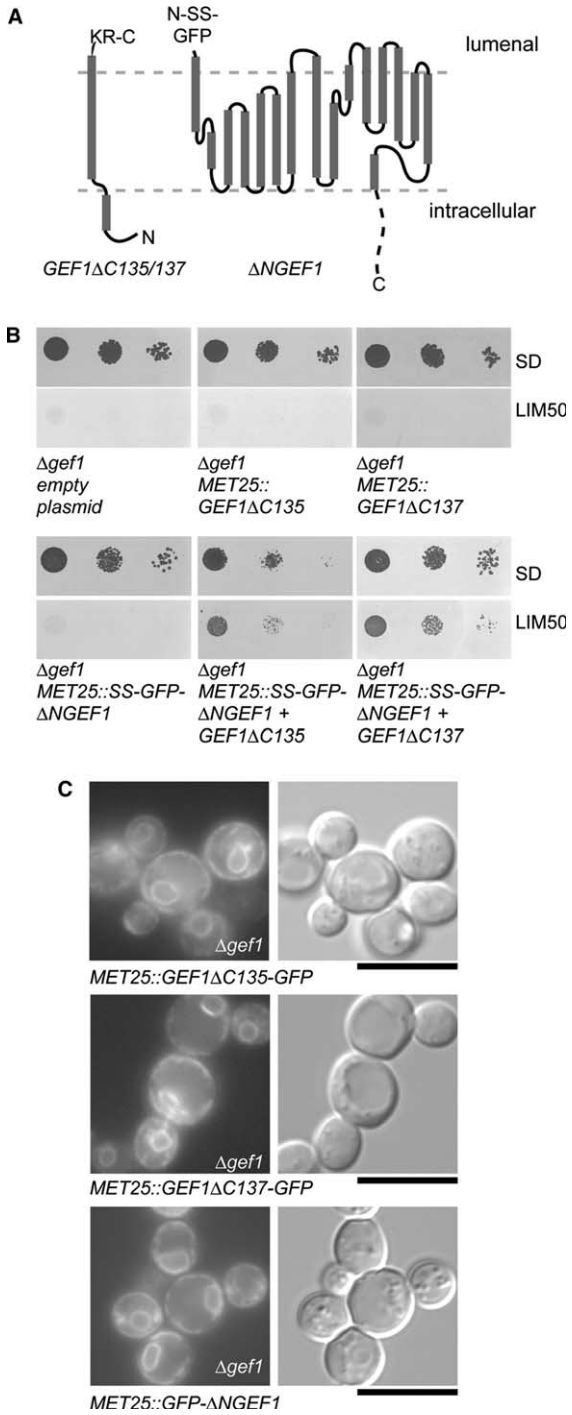

Fig. 4. The individual cleavage products are non-functional but can co-assemble to yield functional channels. (A) Schematic representation of the constructs. (B) Complementation assay as described in Fig. 3A (C) GFP fluorescence patterns of cells transformed with plasmids encoding Gef1p–GFP fusion constructs as indicated. Nomarski images are shown on the right. Size bar is 10 μm.

Thus, we engineered a second variant of Gef1p with a stop codon introduced instead of codon 136 to mimick a possible Kex1p processing product (*GEF1*Δ*C*135; Fig. 4A). In order to create a protein similar to the C-terminal fragment of Gef1p generated by Kex2p, we created a fusion protein of the carboxypeptidase Y signal sequence and GFP [18] joined to residue 138 of Gef1p (Δ*NGEF1*). All three Gef1p fragments were tested for complementation of the high-affinity iron uptake deficiency (Fig. 4B). Neither N-terminal nor the C-terminal fragment was able to complement the growth defect when expressed alone. In contrast, complementation was observed when we co-expressed the C-terminal fragment with either N-terminal fragment indicating that these proteins assemble to form functional channels. When the GFP fluorescence pattern of the two N-terminal fragments fused to GFP as well as the C-terminal fragment was analyzed most of the protein seemed to reside in the ER (Fig. 4C) as evident from the strong perinuclear and cortical staining. This pattern did not change upon co-expression of the two fragments (not shown) suggesting that the functional population of channels reaching the late secretory pathway (where Gef1p is thought to affect copper loading to Fet3p and thus high-affinity iron uptake) may be very small.

4. Discussion

Our data demonstrate that the yeast CLC chloride channel Gef1p is proteolytically processed by the Kex2p protease at position R137. Consistent with the localization of Kex2p to late Golgi compartments, the unprocessed form of Gef1p was separated from the processed form on sucrose gradients whilst the N- and C-terminal products of the cleavage reaction co-migrated with each other and with a marker of the prevacuolar compartment. Activation of proteins by proteolytic processing events late in the secretory pathway is a common regulatory mechanism. We find that Gef1p activity can be restored by co-expression of fragments resembling the products of the cleavage reaction. This is consistent with the idea that the split protein is, indeed, active. Active proteins have been shown to reassemble from engineered fragments of soluble as well as membrane proteins and even CLC channels [19,20]. On the other hand, the non-cleavable form was functional as well as assayed by complementation of the Δ*gef1* growth phenotype on iron-limited medium. Thus, cleavage by Kex2p does not represent a simple on or off switch for Gef1p channel activity. We are left with the possibility that cleavage of Gef1p is an erratic by-product of evolution without any functional significance or the hypothesis that this mechanism may be in place to achieve a more subtle regulation of the localization or function of Gef1p. The development of assays with better resolution might make it possible to distinguish between these alternatives.

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