

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

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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.

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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 *Agef1* 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 *NotI* 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 *NotI* 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.

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2.4. Immunoblotting

Yeast extracts were prepared from 1.8 OD₆₀₀ 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 \approx 30 kDa and a C-terminal fragment of \approx 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 pre-

dominantly localized to the membranes containing strong Pep12p immunoreactivity, a marker for the pre-vacuole. A larger band of \approx 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 CIC-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 CIC-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

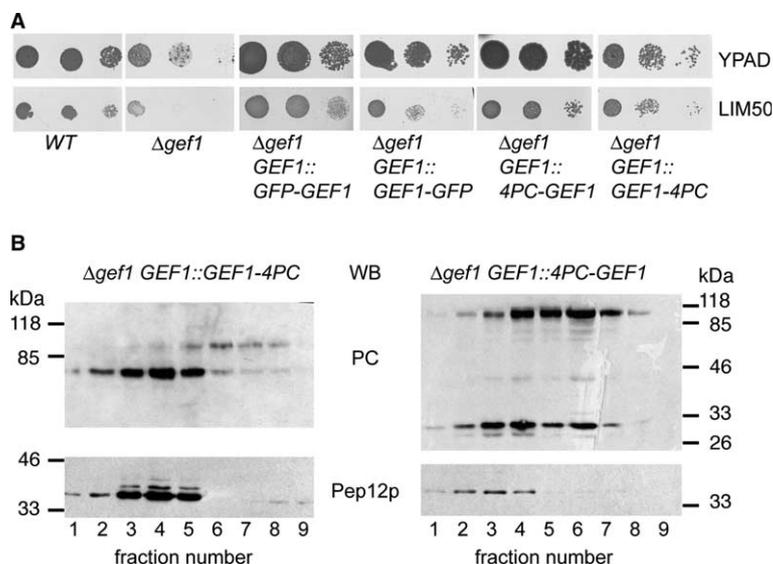


Fig. 1. Gef1p is proteolytically processed. (A) Four tagged versions of Gef1p complement the growth defect on iron-limiting medium when expressed under control of the *GEF1* promoter. All strains were created using integrating plasmids containing the *GEF1* 5' and 3' untranslated regions. Serial dilutions 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 *gef1* 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.

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