Golgi Localization and Functionally Important Domains in the NH₂ and COOH Terminus of the Yeast CLC Putative Chloride Channel Gef1p*

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GEF1 encodes the single CLC putative chloride channel in yeast. Its disruption leads to a defect in iron metabolism (Greene, J. R., Brown, N. H., DiDomenico, B. J., Kaplan, J., and Eide, D. (1993) *Mol. Gen. Genet.* 241, 542–553). Since disruption of *GEF2*, a subunit of the vacuolar H⁺-ATPase, leads to a similar phenotype, it was previously suggested that the chloride conductance provided by Gef1p is necessary for vacuolar acidification. We now show that *gef1* cells indeed grow less well at less acidic pH. However, no defect in vacuolar acidification is apparent from quinacrine staining, and Gef1p co-localizes with Mnt1p in the medial Golgi. Thus, Gef1p may be important in determining Golgi pH. Systematic alanine scanning of the amino and the carboxyl terminus revealed several regions essential for Gef1p localization and function. One sequence (FVTID) in the amino terminus conforms to a class of sorting signals containing aromatic amino acids. This was further supported by point mutations. Alanine scanning of the carboxyl terminus identified a stretch of roughly 25 amino acids which coincides with the second CBS domain, a conserved protein motif recently identified. Mutations in the first CBS domain also destroyed proper function and localization. The second CBS domain can be transplanted to the amino terminus without loss of function, but could not be replaced by the corresponding domain of the homologous mammalian channel ClC-2.

The CLC[1] proteins form an evolutionally old group of membrane proteins with homologues identified in bacteria, archaebacteria, yeast, plants, and animals (1, 2). Expression cloning of ClC-0, a voltage-gated chloride channel from *Torpedo* electric organ, yielded the first CLC cDNA (3). The completion of the *Saccharomyces cerevisiae* genome project shows that there is a single CLC gene in yeast.

Four members of the CLC family have been unambiguously shown to function as chloride channels. ClC-0 provides the high conductance of the non-innervated membrane of the *Torpedo* electric organ. In mammalian skeletal muscle, ClC-1 ensures a high resting chloride conductance. Mutations in its gene lead to myotonia because they render the muscle hyperexcitable (4). ClC-2 is an ubiquitously expressed chloride channel which is activated by cell swelling, hyperpolarization, or acidic pH (5, 6). The situation is less clear for ClC-5, a channel mutated in certain kidney stone diseases (7). It yields currents only in an unphysiological voltage range (8), which also applies to ClC-4.[2] To explain the proteinuria observed with ClC-5 mutations, it was proposed that ClC-5 operates in vesicles of the endocytotic pathway of proximal tubules (8, 9). Functional expression is controversial for ClC-3, ClC-Ka and -Kb, and no plasma membrane currents were detected when ClC-6 and ClC-7 were expressed in *Xenopus* oocytes (1, 10). One possible explanation is that some CLC proteins function as chloride channels in intracellular compartments.

Many intracellular organelles such as the Golgi, lysosomes, synaptic vesicles, and endosomes contain chloride channels, which have not yet been identified at the molecular level. There is some evidence that cystic fibrosis transmembrane conductance regulator, the cystic fibrosis chloride channel, may play a role in vesicle trafficking (11). Chloride channels of intracellular organelles are thought to facilitate intravesicular acidification by providing an electric shunt for the proton ATPase. An acidic lumenal pH is not only essential for enzymatic activities in lysosomes, but also for sorting in the endocytotic pathway. The pH gradient along the exocytotic pathway may be important for the retrieval of ER proteins (12) and the sorting of secretory proteins (13, 14). Furthermore, the retrieval of certain TGN proteins from the cell surface requires endosomal acidification (15).

In yeast, a genetic screen for an iron-suppressible *petite* phenotype identified a CLC gene dubbed *GEF1* (for glycerol/ethanol Fe-requiring, (16); to comply with the CLC nomenclature, we have also called this gene ScCLC (17)). Among four recently cloned plant CLCs from *Arabidopsis*, one could rescue the growth phenotype of *gef1* strains on low iron medium (17). Interestingly, *GEF2*, whose null mutation has a similar phenotype, encodes a subunit of the vacuolar H⁺-ATPase (16, 18). The strong homology of the *GEF1* gene to CLC chloride channel genes suggests that its gene product might provide an electrical shunt for the H⁺-ATPase, thereby facilitating the acidification of a common compartment. Greene *et al.* (16) hypothesized that this mechanism may be relevant to the vacuole which is involved in iron storage in yeast (19).

We now show that Gef1p resides in the Golgi. Cytosolic NH₂

[1] The abbreviations used are: CLC, generic name for a specific chloride channel gene family; ClC-X, member X of this chloride channel family; CBS domain, conserved protein domain found, *e.g.* in cystathione-β-synthase; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; LIM, low iron medium; WT, wild-type.

[2] T. Breiderhoff, T. Friedrich, and T. J. Jentsch, unpublished data.

and COOH termini of the protein were subjected to alanine scanning mutagenesis. It revealed a 5-amino acid motif in the NH_2 terminus that is essential for proper function. In the COOH terminus, mutating a larger domain conserved in all eukaryotic CLC homologues caused mislocalization of the mutant proteins which no longer complemented the *gef1* phenotype. Growth assays on titrated media revealed an increased sensitivity of *gef1* cells toward neutral pH. These results are compatible with a role for the putative chloride channel Gef1p in compartmental acidification of the Golgi apparatus.

MATERIALS AND METHODS

Strains and Media—Standard yeast media and genetic manipulations were as described (20). LIM50 low iron selection medium was prepared as described (21). pH-adjusted media for plates were titrated with free Tris base resulting in a Tris concentration between ≈1 mM for pH 5.5 and ≈6 mM for pH 6.5. Bromcresol purple (30 μg/ml) was added to these plates as a pH indicator. Methionine was generally omitted from the media to allow for maximal expression from plasmids pDR46 and p416MET25 (see below). The *GEF1* gene disruption was introduced into the strains K700α (*Matα, HMLα, HMBα, ho, ade2-1, trp1-1, can1-100, leu2-3, 2-112, his3-11, 3-15, ura3, ssd1*;(22)) and DF5a (*Mat a, lys2-801, leu2-3, 2-112, ura3-52, his3Δ200, trp1-1(am)* (23)) by transforming the strains with a linear DNA fragment containing 375 base pairs of the 5′ and 450 base pairs of the 3′ portion of the *GEF1* open reading frame with the HIS3 marker gene inserted between them. Gene disruption was verified by Southern blotting and polymerase chain reaction. Western blot analysis of the Gef1p GFP (green fluorescent protein) fusion protein and corresponding ALA mutants was performed on strain GPY385 transformed with the corresponding plasmids (*Mat a, leu2-3, 2-112, ura3-52, trp1-289, sst1-3, his4* or *his6 pep4::LEU2* (24)).

Plasmid Construction—All plasmids were derived from the wild-type *GEF1* gene fused to the GFP cDNA (mutant S65T) via a *Not*I restriction site engineered in the place of the *GEF1* stop codon. This construct was in pDR46, a yeast expression vector derived from pSEY8 (25). It carries the URA3 marker gene and the MET3 promoter fragment (26). Alanine scanning mutagenesis was performed by introducing windows of five alanines by polymerase chain reaction. Briefly, two fragments were amplified with primers containing the codons for the five alanines in an overlapping region and adjacent sequences that served to anchor the primer to the desired position in the *GEF1* sequence. The fragments were joined by recombinant polymerase chain reaction, digested with appropriate restriction endonucleases, and ligated into the backbone. Polymerase chain reaction-derived fragments and restriction sites used for ligation were sequenced. COOH-terminal domain swapping was based on a *Bgl*II restriction site naturally occurring in rat ClC-2. A *Bgl*II site was engineered in the same region of the *GEF1* gene thereby causing the mutation M652L. The mutated *GEF1* gene behaved like wild-type in all assays. Since there is a methionine in position 653 the *Bgl*II site could also be used to transplant this part of the protein to the NH_2 terminus by ligating it to the *Bam*HI site of the 5′ polylinker sequence of pDR46. In these constructs the same *Not*I site that was used for fusion of the GFP cDNA was used for linking the transplanted 3′ portion and the original 5′ portion of the *GEF1* gene. To this end the *Not*I site was engineered on the original initiator ATG (actually deleting the methionine) in a way that allowed in-frame fusion of the COOH and NH_2 terminus. Concatamers of different *GEF1* mutants were constructed using the same *Not*I restriction site.

Complementation Assays—Complementation of the *gef1* phenotype, the inability to grow on iron-limited plates, was assayed in at least three independent transformations for each construct. The wild-type *GEF1* GFP fusion construct was included in all experiments as a positive control. Equal aliquots from each transformation were spotted on synthetic complete selection plates lacking uracil and methionine as well as on LIM50 iron-limiting medium. Depending on the construct growth of all (wild type) or no (*e.g.* ALAN8, ALAC5, and ALAC7) transformants was observed on LIM50 medium.

GFP Fluorescence Assays—The evening before fluorescence microscopy a culture was inoculated from a stationary preculture in a dilution that resulted in early logarithmic growth the next day. Because formation of fluorescent GFP is more efficient at lower temperatures (27) cultures were transferred to 4 °C for a period of 1 to 4 h. In control experiments it was ensured that this did not change the observed staining patterns. Either live cells were attached to concanavalin A-coated microscope slides (28) and viewed directly, or cells were fixed, the cell wall was digested with zymolyase (see "Immunostaining of Yeast Cells"), and spheroblasts were attached to poly-L-lysine-coated slides. The two treatments result in slightly different appearance of the GFP staining pattern but differences between constructs (*e.g.* wild-type and significantly changed localizations) were always much larger. GFP fluorescence was observed and photographed using a Zeiss Axiophot 1000 microscope and a FITC filter set.

FM4-64 and Quinacrine Staining Assay—The styryl dye FM4-64 was used as described (28). Basically, we looked at steady-state labeling of the vacuole. FM4-64 fluorescence was observed using the rhodamine filter set of the Zeiss Axiophot 1000 epifluorescence microscope. The fluorescence of the dye was also visible as bright orange when using the FITC filter set allowing for a direct comparison of the FM4-64-stained and the GFP-stained structures. Quinacrine staining was performed as described (29). Cells were viewed and photographed using FITC optics.

Immunostaining of Yeast Cells—Cells were grown to early logarithmic phase, fixed at room temperature in freshly prepared fixative (29) for at least 8 h, washed in 0.1 M potassium phosphate, pH 6.5, and 1.2 M sorbitol in this buffer (30). The cell wall was digested with Zymolyase 100T (≈0.3 mg/ml; ICN) and spheroblasts were washed in 1.2 M sorbitol and permeabilized by a short incubation in 1% SDS in 1.2 M sorbitol (29). Subsequent staining with anti-FLAG M2 mouse monoclonal antibody (1 ng/μl, ABI), anti-Kar2 rabbit polyclonal antiserum (1:5,000, a gift of M. Rose), anti-c-Myc mouse monoclonal antibody 9E10 (10 ng/μl, Boehringer) and affinity-purified rabbit anti-Kex2 polyclonal antibody (1:100, a gift of Robert Fuller) followed Roberts *et al.* (29). The secondary antibody was a CY3- or FITC-conjugated anti-rabbit or anti-mouse IgG antibody raised in goat (1:1000, Jackson). Images of immunostained cells expressing GFP fusions of Gef1p and the mutants were recorded with a Bio-Rad confocal microscope.

Western Blotting—15 A_{600} units of yeast cells were harvested by centrifugation and resuspended in 100 μl of TEA (7.5 g/liter triethanolamine, 0.38 g/liter EDTA, pH 8.9, supplemented with "Complete" protease inhibitor (Boehringer Mannheim), and 1 mM diisopropyl fluorophosphate). Cells were lysed by 5×30 s vortexing cycles with the help of glass beads. Extract of approximately 1 A_{600} unit was loaded per lane and resolved on a 10% SDS gel. Gels were blotted on nitrocellulose. Horseradish peroxidase-coupled anti-mouse secondary antibody and Amersham ECL reagents were used for detection.

Colony Immunoblotting—Strains to be assayed for Kar2p secretion were streaked on a plate of synthetic complete medium lacking the appropriate amino acids. Streaks were overlaid with pre-wetted nitrocellulose and incubated at 30 °C overnight. The membrane was removed, washed in distilled water, and subsequently probed by standard Western blotting techniques with an anti-Kar2p rabbit polyclonal antiserum. Chemiluminescent detection used the ECL system (Amersham).

RESULTS

Gef1p Is Localized to the Golgi—To assess the subcellular localization of Gef1p, we expressed an epitope-tagged version of the protein in K700αΔPst (*gef1*) cells. Immunofluorescence revealed multiple dot-like structures in the cytosol that were rather variable in size and number (Fig. 1*A*). This pattern resembles the staining pattern observed for Golgi proteins (31). A similar pattern was observed with a construct in which the green fluorescent protein was fused to the carboxyl terminus of Gef1p (Fig. 1*B*). Different expression levels (obtained by adding different concentrations of methionine to the growth medium) gave results varying only in signal intensity (data not shown). Thus, the expression pattern is unlikely to be an artifact of overexpression. Both the FLAG-tagged and the GFP-tagged constructs complemented the growth defect of *gef1* strains on iron-limiting media as effectively as wild-type Gef1p, suggesting that they reach the compartment in which Gef1p is normally expressed (data not shown).

The fluorescing dots did not overlap with the Nomarski-visible vacuole (Fig. 1, *A* and *B*). To confirm a non-vacuolar localization we stained the vacuole with FM4-64 (28). This styryl dye is endocytosed by yeast cells, and, after transiently labeling vesicles of the endocytotic pathway, it exclusively labels the vacuole. Qualitatively, *gef1* cells endocytose FM4-64 as well as wild-type cells (data not shown). These experiments showed that the Gef1p-GFP fusion protein is localized in compartments distinct from the vacuole (Fig. 1*C*). In order to assess
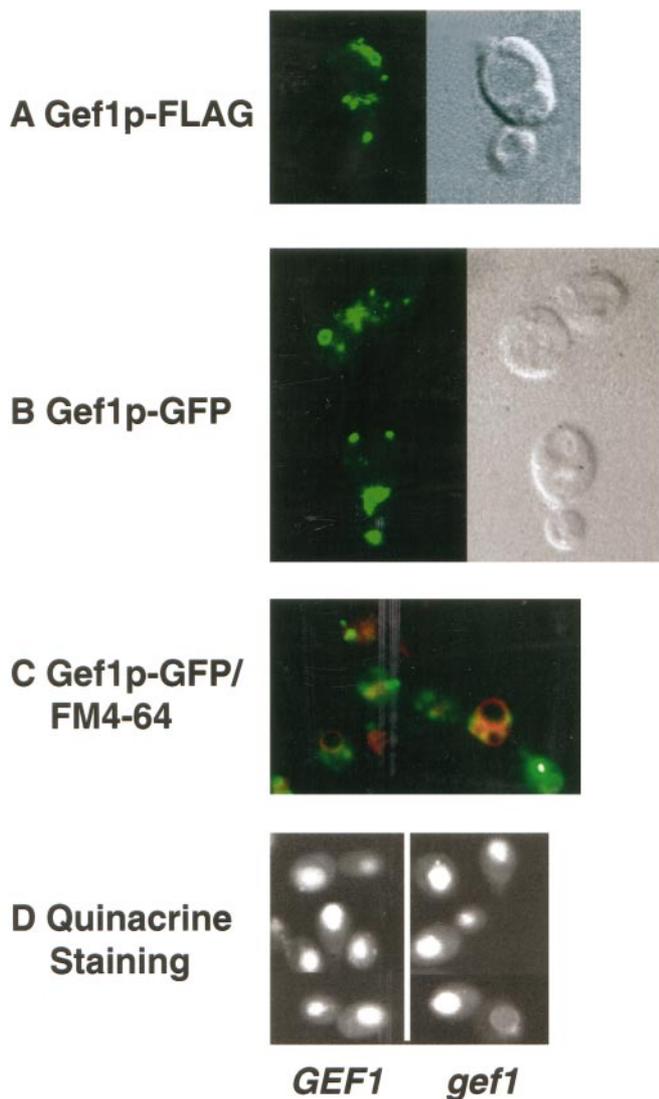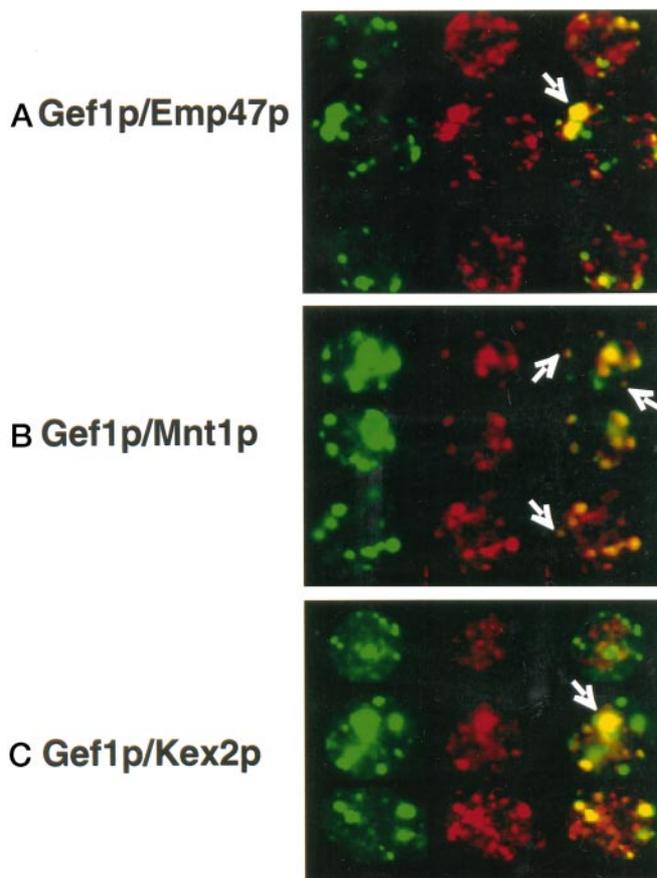
FIG. 1. Localization of Gef1p-FLAG and Gef1p-GFP. *A*, immunostaining of yeast cells expressing the FLAG epitope-tagged Gef1 protein. *Left*, immunofluorescence (FITC-coupled secondary antibody); *right*, Nomarski image. *B*, GFP fluorescence of Gef1p-GFP fusion protein. *Left*, GFP fluorescence; *right*, Nomarski image. *C*, cells expressing the Gef1p GFP fusion protein counterstained with the styryl dye FM4-64. Cells are shown after steady-state labeling has been achieved and the dye exclusively stains the vacuole. All images show cells from strain K700αΔPst (*gef1*) transformed with the corresponding plasmids. The vacuole appears in *red*, Gef1p in *green*. *D*, quinacrine staining of the vacuole in K700αWT (*left*) and K700αΔPst (*gef1*) cells (*right*).

FIG. 2. Co-staining of Gef1p GFP with three different marker proteins. For each marker three cells are shown. *Left*, GFP fluorescence (*green*); *middle*, CY3 fluorescence (*red*); *right*, overlay of GFP and CY3 fluorescences. *A*, co-staining with COOH-*myc*-tagged Emp47p expressed from a CEN plasmid under the control of its endogenous promoter. *Arrow* indicates co-staining in a strongly labeled structure exceeding the average size of labeled compartments. *B*, co-staining with COOH-*myc*-tagged Mnt1p expressed from a 2-μm plasmid under the control of its endogenous promoter. *Arrows* indicate that co-staining is also found in weakly labeled structures, suggesting that this is not due to overexpression. *C*, co-staining with Kex2p expressed from a CEN plasmid under the control of the *TDH3* promoter. Except for strongly labeled structures (*arrow*) there is no conspicuous co-staining. All images show cells from strain K700αΔPst (*gef1*) transformed with the corresponding plasmids.

a possible defect in vacuolar acidification, K700αΔPst (*gef1*) cells were stained with quinacrine (Fig. 1*D*). Quinacrine accumulated normally in the vacuole of *gef1* cells, excluding a gross defect of vacuolar acidification.

The localization of Gef1p was compared with that of three marker proteins representing the early, medial, and late portion of the yeast Golgi apparatus, respectively: Emp47p, Mnt1p, and Kex2p. Emp47p is a protein with homology to intracellular lectins. It recycles between the Golgi and the ER and is found in the Golgi complex at steady state (32). Mnt1p is a α1,2-mannosyl-transferase involved in *O*-glycosylation (33). The Kex2 protease resides in a late Golgi compartment, probably the equivalent of the mammalian TGN (34).

We expressed a *myc*-tagged version of Emp47p (35) under the control of the native promoter from a CEN plasmid. GFP-tagged Gef1p was co-expressed from a CEN plasmid under the control of the MET25 promoter (36). The two proteins were largely found in distinct structures (Fig. 2*A*). However, occasional overlap was observed, especially in strongly labeled structures exceeding the average size of stained structures (*white arrow* in Fig. 2*A*). Mnt1p was expressed under the control of its native promoter from a high copy number plasmid. Mnt1p and Gef1p co-localized to a high degree (Fig. 2*B*). Among the three marker proteins analyzed, Mnt1p matched the distribution of Gef1p most closely. Since Golgi-retention of Mnt1p is saturable (37), the degree of co-localization may be overestimated by looking at cells that gave a good signal. Thus, it is important that co-localization was not restricted to strongly labeled structures but was also seen with small, weakly labeled structures (*white arrows* in Fig. 2*B*). We expressed Kex2p from a CEN plasmid under the control of the *TDH3* promoter, which leads to a roughly 25-fold overproduction and a partial saturation of its TGN localization (38). There was a small degree of overlap between Kex2p and Gef1p, particularly in strongly labeled structures (*white arrow* in Fig. 2*C*). Thus, our data show that Gef1p is localized to the Golgi, with most of the protein probably residing in its medial portion.

NH₂- and COOH-terminal Truncations Render Gef1p Non-

FIG. 3. *A*, schematic diagram of Gef1p and NH₂- and COOH-terminal truncations. A general topological model for the CLC proteins is shown on *top of the panel*. The membrane is *hatched*. NH₂ and COOH termini reside in the cytosol. Domains D1 to D13 were deduced from the hydropathy plot of the first cloned member ClC-0 (3). Their relative position is modified according to current knowledge (65). The exact number of transmembrane domains between D9 and D12 is unclear. The two conserved CBS domains (43, 44) in the cytoplasmic COOH terminus are indicated. D13 coincides with CBS domain 2. *a*, schematic representation of Gef1p. The cytoplasmic NH₂ and COOH terminus are shown in *white*, whereas the transmembrane region is depicted in *black*. The two CBS domains in the COOH terminus are indicated. This and all other constructs are fused to the GFP at the COOH terminus. *b*, NH₂-terminal truncation mutant Gef1p-ΔN36. *c*, COOH-terminal truncation mutant Gef1p-ΔC198. Note that both CBS domains have been deleted. *B*, effect of NH₂- and COOH-terminal truncation of Gef1p in a complementation assay (*lower row*, transformants on synthetic complete medium lacking uracil; *upper row*, selection of transformants on LIM50 iron-limiting medium). *C*, GFP fluorescence of truncated Gef1 proteins. It differs markedly from the dot-like structures stained with WT Gef1p (see Fig. 1). Both assays were performed with strain K700αΔPst (*gef1*) transformed with the corresponding plasmids.
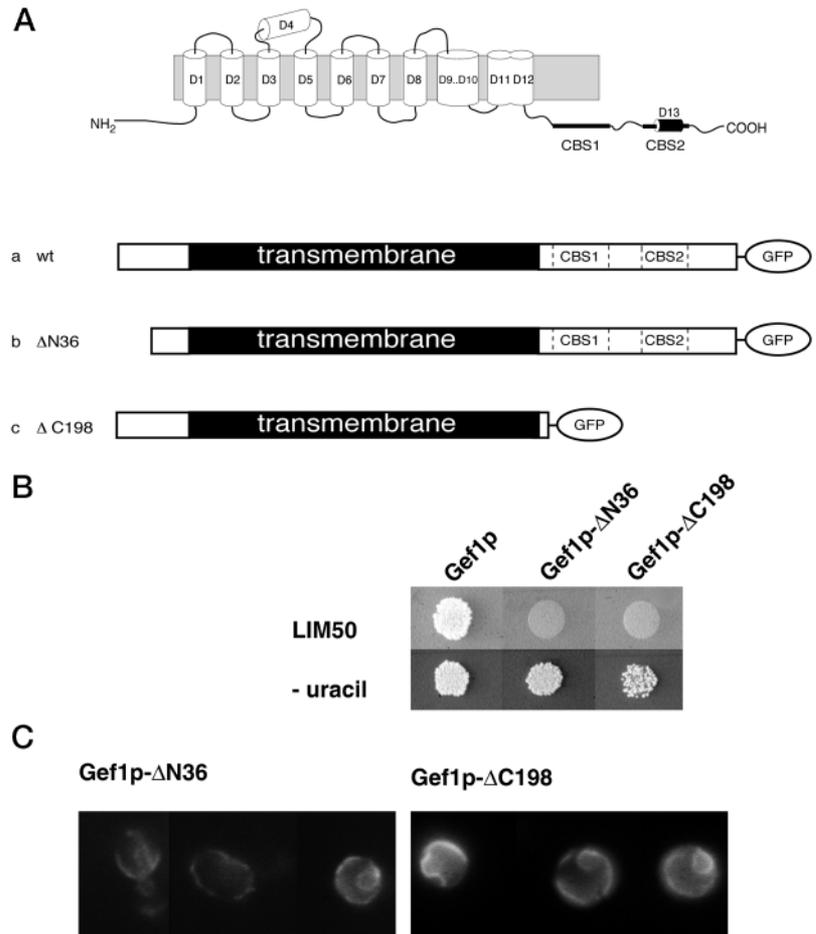
functional—In a search for functionally important domains in the cytoplasmic NH₂ and COOH termini of Gef1p, we truncated the first 36 or the last 198 amino acids of the protein and made COOH-terminal fusions to GFP (Fig. 3*A*, constructs *b* and *c*). These were expressed in *gef1* strain K700αΔPst. Neither of the truncated proteins could complement the growth defect of the cells on the iron-limiting LIM50 selective medium (Fig. 3*B*). The GFP fluorescence pattern observed for the truncated proteins lacked the dot-like structures obtained with WT Gef1p (Fig. 3*C*). Instead, a strong perinuclear fluorescence and staining close to the cell periphery were observed. We conclude that both the amino and the carboxyl terminus of Gef1p are necessary for the protein to fulfill its normal function. These regions may contain residues important for correct localization. Alternatively, the truncated proteins may be misfolded and retained in an early compartment by a quality control mechanism.

Alanine Scanning Identifies 5 Residues in the Gef1p NH₂ Terminus and 25 Residues in the Gef1p COOH Terminus That Are Essential for Proper Function—To narrow down the functionally important regions in the NH₂ and COOH termini of Gef1p, we systematically moved a window of 5 alanine residues through the regions of interest. For the NH₂ terminus, we scanned the whole region deleted in the experiment described above by constructing nine mutants in which 5 consecutive residues of the amino terminus were replaced by alanines (mutants ALAN1 to ALAN9, Fig. 4*A*). For the carboxyl terminus, we analyzed 17 such constructs (mutants ALAC1 to ALAC17).

From the 9 NH₂-terminal ALA mutants seven could not be distinguished from wild-type by either the complementation or the GFP fluorescence assay (Fig. 4, *A* and *C*). Mutant ALAN8 could not functionally substitute for the Gef1 protein and dis-

played an atypical GFP fluorescence. Mutant ALAN6 displayed weaker complementation than wild-type (transformants appeared 2 days later on LIM50 selective medium) and again showed an abnormal GFP fluorescence (Fig. 4*C*).

The motif destroyed in mutant ALAN8 ([36]FVTID) complies with a consensus sequence of sorting signals containing aromatic amino acids (see "Discussion"). We therefore mutated the two most conserved residues within these motifs either singly (F36A and I39G) or in combination. The single point mutations had no discernible effect on either complementation or localization when expressed in the *gef1* strain from the Met3 promotor at high levels (*i.e.* no methionine added). Decreasing the expression by increasing the methionine concentration to 25 or 250 μM, however, led to a decrease in complementation efficiency as compared with WT grown at the same concentrations of methionine (not shown). The double mutant, F36A/I39G, in contrast, led to the same loss-of-function phenotype as ALAN8, suggesting that FVTID may indeed function similarly as other sorting signals based on aromatic amino acids.

From the 17 COOH-terminal ALA mutants 10 behaved like wild-type in every aspect tested. Five adjacent mutants could not functionally substitute for Gef1p (ALAC5 to ALAC9, Fig. 4, *A* and *B*) and displayed an atypical GFP fluorescence (see *e.g.* ALAC5, Fig. 4*C*). Very weak complementation, however, was observed with ALAC6, resulting in a few, very slow-growing colonies. Two additional mutants gave an intermediate phenotype. For ALAC3, complementation was weak and GFP fluorescence appeared again changed. Mutant ALAC10 displayed only abnormal fluorescence while showing complementation. Some of the mutant proteins were expressed to lower steady-state levels as judged by a lower level of GFP fluorescence and by Western blotting with an anti-GFP antiserum (data not
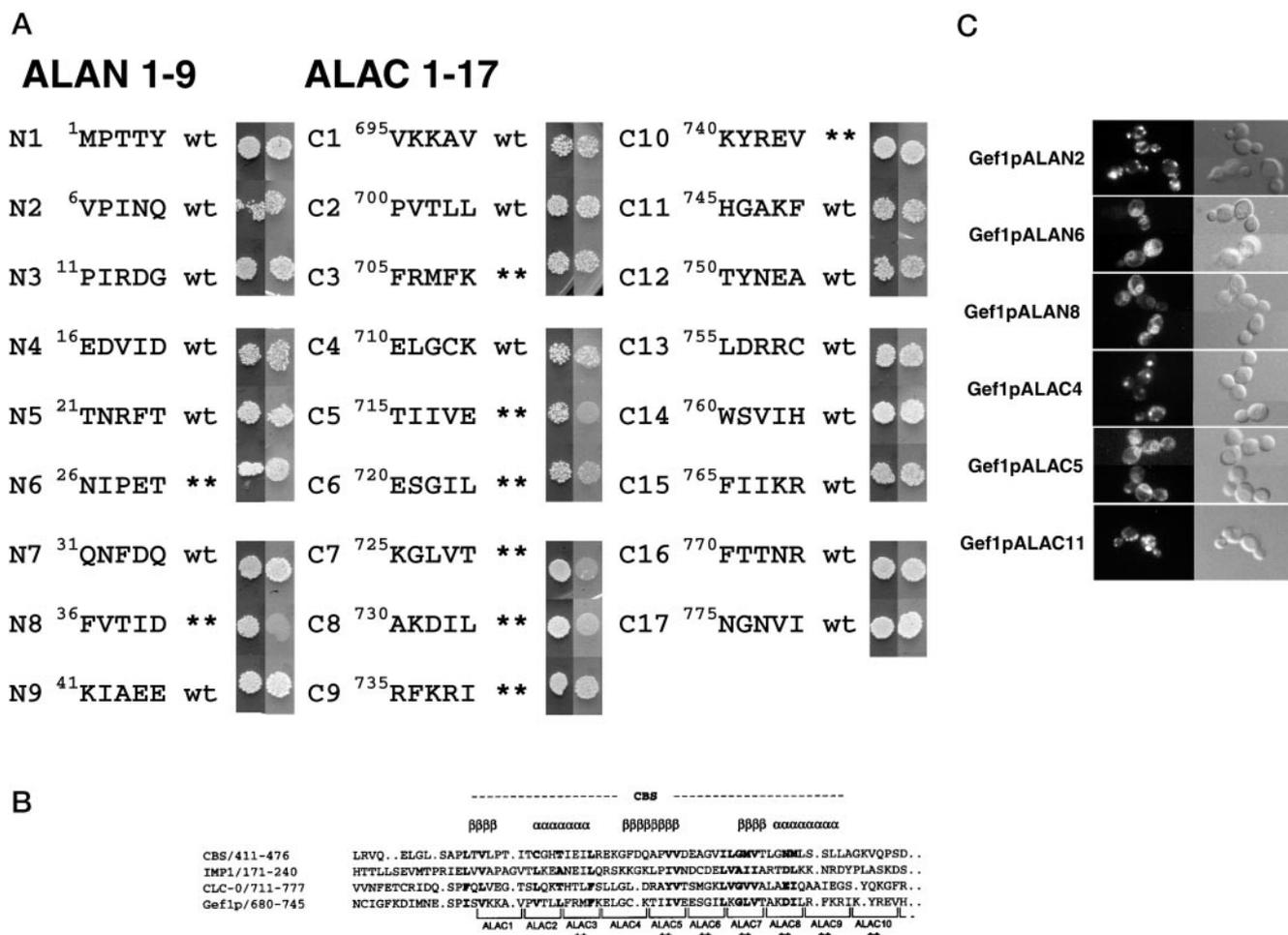
FIG. 4. **Alanine scan of Gef1p NH₂ and COOH terminus.** Five consecutive amino acids each, as indicated in single letter code and with the number of the first amino acid indicated by a superscript, were replaced by alanines. *A*, complementation assay with ALA mutants of Gef1p (*left rows*, transformants on synthetic complete medium lacking uracil; *right rows*, selection of transformants on LIM50 iron-limiting medium). *Asterisks* denote any property of a mutant that differed from wild-type, be it in the complementation or the GFP fluorescence assay. *B*, alignment of several CBS domains from human cystathionine β-synthase (*CBS*), human inosine-5′-monophosphate dehydrogenase (*IMP*), the *Torpedo* chloride channel ClC-0, and Gef1p. Conserved residues are shown in *bold*, the extent of the CBS domain and the predicted structure (β-sheet or α-helix) is indicated *above* (modified from Refs. 43 and 44). *Below*, positions of the amino acid sequences mutated in the COOH-terminal alanine scan, with *asterisks* indicating a loss of function. *C*, GFP fluorescence of selected Gef1p ALA mutants. *Left*, GFP fluorescence; *right*, Nomarski image. Both assays were performed with strain K700αΔPst (*gef1*) transformed with the corresponding plasmids.

shown), an observation not uncommon in mutagenesis studies (39, 40). There was no consistent correlation between expression levels and phenotypes.

Thus, our alanine scan showed that a 5-amino acid motif in the NH₂ terminus and a 25-amino acid domain in the COOH terminus could not be mutated without loss of function, which was always associated with an atypical GFP staining pattern.

Co-localization of Non-functional ALA Mutants with ER-resident Kar2p—In yeast, retention-defective mutant forms of resident Golgi proteins are often sorted to the vacuole (41). This is clearly not the case for our mutants. Their often perinuclear localization is rather compatible with a localization in the ER. We therefore examined co-localization with Kar2p, a lumenal ER chaperone of the Hsp70 family (42). K700αΔPst (*gef1*) cells expressing either Gef1p-GFP or two defective mutants, ALAN8 and ALAC5, were co-stained with an anti-Kar2 antiserum (Fig. 5). The wild-type protein was always found in structures distinct from those stained for Kar2p (Fig. 5A), while both mutants co-localized with this ER protein (Fig. 5, *B* and *C*). In addition to this typical perinuclear and submembraneous fluorescence, atypical structures were observed in cells expressing higher amounts of mutant Gef1 proteins, especially for ALAC5. This effect was the same in the corresponding *GEF1* strain
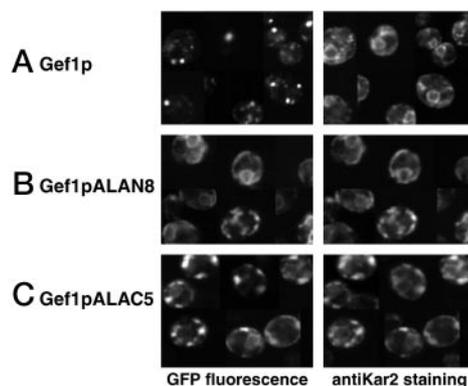
FIG. 5. **Co-localization of ALA mutants with an ER marker.** Co-staining of yeast cells expressing the wild-type Gef1p GFP fusion protein (*A*), mutant ALAN8 (*B*), and mutant ALAC5 (*C*) with an anti-Kar2 antiserum. *Left*, GFP fluorescence; *right*, CY3-fluorescence of immunostaining with anti-Kar2 antiserum.

(data not shown). These structures also stained for Kar2p and might therefore represent abnormal ER-derived membranes. Thus, non-functional ALA mutants of Gef1p are retained in the
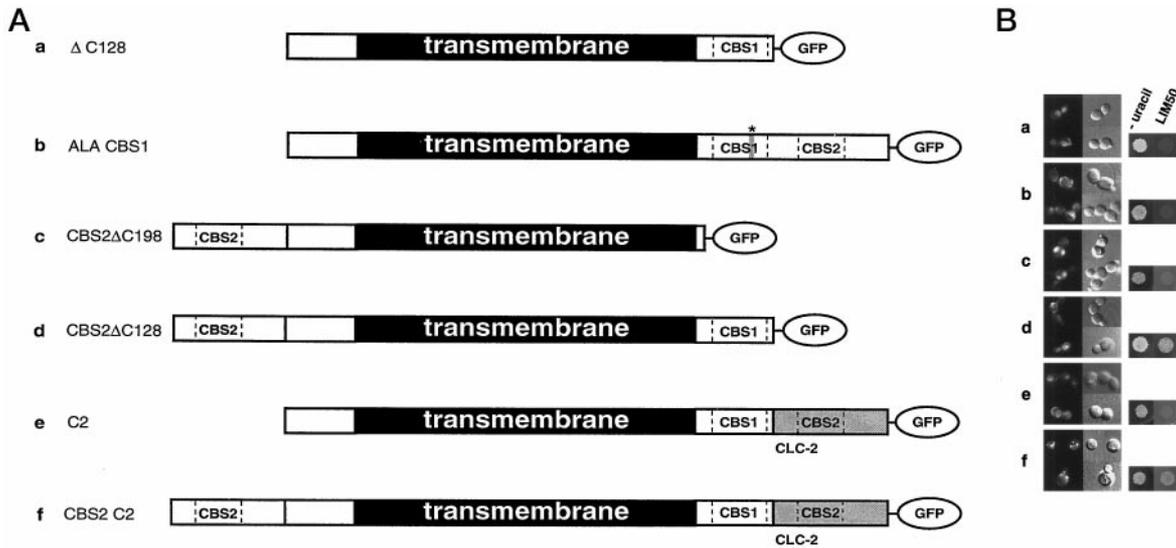
FIG. 6. **Importance of CBS domains for Gef1p function and localization.** *A*, schematic diagram of constructs; *B*, complementation assay (*right*) and GFP fluorescence (*left*). *a*, Gef1p truncated after the first CBS domain; *b*, Gef1p in which amino acids 640 to 644 in CBS1 have been replaced by alanines (indicated by the *asterisk*); *c*, a chimera in which CBS2 of Gef1p has been fused before the amino terminus of the COOH-terminal deletion mutant ΔC198 (see Fig. 3*A, c*); *d*, chimera in which CBS2 is fused before Gef1p truncated after CBS1 (*i.e.* fused to construct of *panel A (a)*); *e*, chimera in which the second part of Gef1p COOH terminus, including CBS2, has been replaced by the corresponding segment of the rat chloride channel ClC-2; *f*, chimera in which Gef1p CBS2 has been fused to the amino terminus of chimera (*panel A, e*). All constructs are COOH terminally fused to GFP.

ER, and expression of mutant proteins leads to abnormal Kar2p-containing structures.

The Carboxyl-terminal Essential Domain Coincides with CBS Domain 2 and Functions in a Position-independent Manner—Recently, Bateman (43) and Ponting (44) identified a new class of protein domains called CBS (for cystathione-β-synthase) domains. Two copies of this domain are found in the carboxyl termini of all eukaryotic CLC homologues, and deletion of both CBS domains in construct Gef1pΔC198 resulted in a loss of function as shown above. Interestingly, the region identified by our systematic alanine scanning of the COOH terminus nearly exactly co-incides with the second CBS domain (CBS2) (Fig. 4*B*). As expected from this scan, deletion of CBS2 (leaving CBS1 intact) also impaired Gef1p function (Fig. 6*a*). To examine whether the first CBS domain (CBS1) is also important, we replaced residues 640 to 644 (GYVLK, located centrally in CBS1) by alanines (Fig. 6*b*). Again, this mutant displayed perinuclear staining and could not restore growth under iron-limiting conditions. Thus, both CBS domains are needed for proper function.

When the last 128 amino acids of Gef1p (containing CBS2) were transplanted to the NH$_2$ terminus of the truncated protein that lacks both CBS domains at the COOH terminus, the resulting mutant was still non-functional but displayed a GFP-staining pattern that seemed no longer ER-like, but also differed from WT (Fig. 6*c*). We then fused CBS2 to the amino terminus of the Gef1p construct which was truncated after CBS1 (Fig. 6*d*). Surprisingly, this led to functional complementation of *gef1* cells on LIM50 plates. GFP fluorescence showed a mixture of dot-like staining resembling WT Gef1p and the ER-like fluorescence as typical for the truncated Gef1p protein.

In conclusion, both CBS domains are essential for Gef1p function. The function of the second CBS domain does not depend on its exact position within the protein.

The Gef1p COOH Terminus Cannot be Substituted by the ClC-2 COOH Terminus—To elucidate the role of the Gef1p COOH terminus further, we replaced the last 128 amino acids of Gef1p by the corresponding 127 residues of the rat ClC-2 protein (Fig. 6*e*). Chimeric ClC-2 proteins in which the COOH terminus was replaced by that of ClC-1 still yield currents

when expressed in *Xenopus* oocytes, although their characteristics are changed (6). In contrast, the chimeric protein Gef1p/C2 could not complement the growth defect of the *gef1* strain on iron-limiting LIM50 plates, and the GFP staining pattern resembled that observed for truncated Gef1 proteins and non-functional ALA mutants. Thus, the COOH-terminal region contains essential residues that are not conserved in the corresponding sequence of ClC-2. We next fused the Gef1p CBS2 domain to the amino terminus of the chimera Gef1p/C2 (Fig. 6*f*). This construct complemented the growth of *gef1* cells on LIM50 plates, and the associated GFP fluorescence resembled the punctate staining of WT Gef1p. ALAC mutations which destroyed proper Gef1p function when inserted into CBS2 at the COOH terminus (Fig. 4) also destroyed the function of the chimera when inserted into the CBS2 domain transplanted to the amino terminus (data not shown). This demonstrates that the effect of the transplantation is due to a specific effect of the CBS domain.

Concatamers of Wild-type and Mutant Gef1 Proteins Are Functional—ER retention of the non-functional GEF1 mutants could result from a misfolding of the protein rather than reflect specific functions of the mutated residues. We linked mutant and wild-type proteins in a concatameric fashion in order to address this question. We reasoned that the recognition of a misfolded protein region by some quality control mechanism in the ER should not be influenced by linking it with WT Gef1p. Thus, ER retention due to misfolding should also operate on the concatamer, resulting in a non-functional protein.

However, concatamers linking mutant ALAC6 to WT Gef1p efficiently complemented the knockout phenotype irrespective of the order in which the proteins were fused (data not shown). Examining their intracellular localization by GFP fluorescence revealed that cells with almost wild-type appearance were mixed with cells that exhibited untypical staining. These findings argue against ER retention of the mutant proteins being due to misfolding.

CLC Knockout Strains Display Reduced Tolerance Toward Neutral pH—If Gef1p is involved in pH regulation, one might expect a growth phenotype on media adjusted to different pH values. This hypothesis was tested for two different genetic
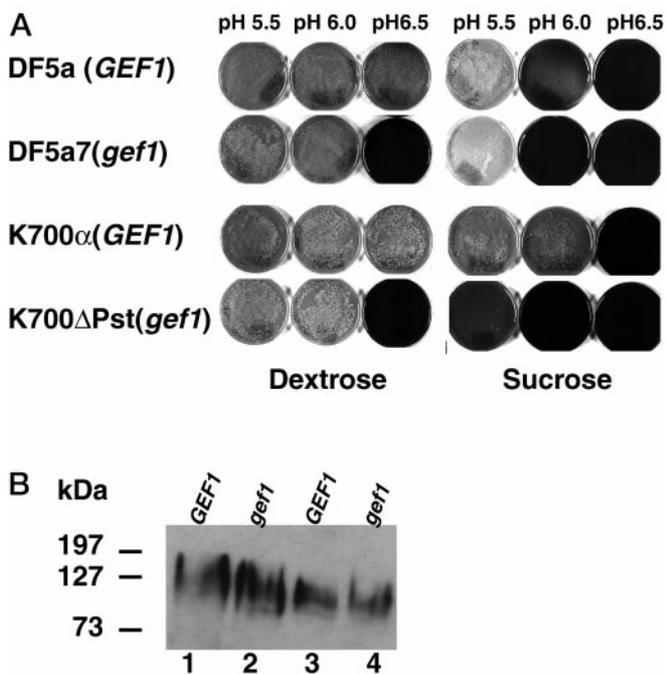
FIG. 7. **pH sensitivity of *gef1* cells.** *A*, growth behavior of two different yeast strains and the corresponding *gef1* strains on media titrated to different pH values and containing dextrose or sucrose as the carbon source. 30 μg/ml bromcresol purple was added to the plates as an pH indicator that turns yellow upon acidification caused by growth of the yeast cells. Thus, dark plates indicate a lack of growth. *B*, glycosylation of invertase in two different yeast strains and the corresponding *gef1* strains. COOH-*myc*-tagged invertase was detected in total protein extracts blotted onto nitrocellulose (9E10 mouse monoclonal antibody, Boehringer Mannheim, 2 μg/ml).

backgrounds, K700α and DF5a. Growth was assayed on synthetic complete medium titrated to pH 5.5, 6.0, and 6.5, respectively (Fig. 7*A*). Two different carbon sources, dextrose and sucrose, were used. On dextrose, both wild-type strains were able to grow on plates titrated to all three pH values, whereas *gef1* strains could not grow on plates titrated to pH 6.5. On sucrose both wild-type strains were unable to grow on plates titrated to pH 6.5, and the corresponding *gef1* strains stopped growing at pH 6. Growth on sucrose was probably more sensitive toward neutral pH since invertase, the key enzyme in sucrose metabolization, requires acidic pH for optimal function (45). These results show that *gef1* strains are less tolerant toward neutral pH than the corresponding wild-type strains. The same result was found when growth of the same strains was assayed on full medium buffered with 100 mM Tris-HCl to pH 7.5. No growth of the *gef1* strains was observed whereas the corresponding wild-type strain grew slowly.

Thus, Gef1p may have a role in acidification, a hypothesis supported by the fact that *GEF2* encodes a subunit of the vacuolar H$^+$-ATPase (16). However, the fact that quinacrine accumulates normally in *gef1* cells suggests that these cells deviate from the *vma* phenotype. The inability to grow on rich media supplemented with 100 mM CaCl$_2$ is another hallmark of this phenotype (49). *gef1* cells do not show this growth defect (data not shown), implying that the *gef1* phenotype only partially overlaps with the *vma* phenotype.

Since our studies indicated that Gef1p localizes to the Golgi, we tested for two effects a change of pH in that organelle might have. The binding of mammalian KDEL-tagged proteins to the KDEL receptor is more efficient at acidic pH (12), and pH differences between the ER and the Golgi are important in maintaining the specific distribution of proteins along that pathway. Kar2p is a resident ER protein which is retrieved from the Golgi by binding to the yeast HDEL receptor. Deleting its HDEL signal results in Kar2p secretion (46). Using colony immunoblots, we asked whether Kar2p might be secreted in yeast cells lacking a functional *GEF1* gene, and used two strains with different genetic backgrounds (DF5a and K700α). A strain expressing a HDEL-deleted Kar2p was used as positive control. The *gef1* strains did not secrete Kar2p, nor did the expression of mutants ALAN8, ALAC3, C6, and C7 in the K700α background lead to the secretion of Kar2p (data not shown).

Glycosylation of proteins might also depend on Golgi pH. We therefore asked if glycosylation of invertase was affected in *gef1* strains and expressed COOH-Myc-tagged invertase which was then detected by Western analysis of total protein extracts (Fig. 7*B*). Due to heterogeneous glycosylation, invertase migrated as a smear in the high molecular weight range for both wild-type and *gef1* strains. Thus, we could not detect a gross effect of Gef1p on glycosylation.

DISCUSSION

Many intracellular compartments of eukaryotic cells are acidified. An acidic intravesicular pH, and the pH gradient along endo- and exocytotic pathways, is not only important for certain enzymatic activities, but also for receptor-ligand interactions, protein sorting, and vesicle trafficking (for review, see *e.g.* Ref. 47). Acidification depends on a vacuolar-type H$^+$-ATPase. This pump is electrogenic and therefore creates a transmembrane potential that in turn inhibits pumping. A parallel conductive pathway can relieve this energetic constraint on proton pumping. In higher eukaryotes, this conductance is provided by chloride channels, the molecular identity of which is still unclear.

Greene *et al.* (16) reported that disruption of either *GEF1*, a member of the CLC family of chloride channels (1, 2), or of *GEF2* (*VMA3*), a subunit of the V-type ATPase, led to the same phenotype. This indirectly suggested that both proteins functionally interacted in the acidification of an intracellular organelle. Since the vacuolar H$^+$-ATPase is required for the acidification of the vacuole, which is a site of iron-storage in yeast (19), it seemed reasonable to hypothesize that Gef1p resides in that organelle. However, in line with previous unpublished observations (16), our results suggest that vacuolar pH is normal in *gef1* cells and that Gef1p is not (predominantly) localized to the vacuole. Moreover, iron storage in yeast was reported to not depend on vacuolar acidification (48).

On the other hand, our finding that *gef1* cells are more sensitive toward neutral pH is compatible with a role of the yeast CLC in intracompartmental acidification. We have shown that Gef1p is present in the yeast Golgi apparatus, and the significant overlap with Mnt1p suggests that the medial Golgi is the predominant site of expression. It is known that the Golgi lumen is acidified, and that an increasingly acidic pH along the pathway from the ER to the TGN is important for protein localization and sorting. However, we could not detect an effect on the retention of Kar2p, an ER protein which is retained by an HDEL signal. Interestingly, Manolson *et al.* (49) found a novel isoform of Vph1p, the 100-kDa subunit of the V-ATPase. In contrast to Vph1p, this isoform (Stv1p) did not localize to the central vacuole. It contains two F-*X*-F-*X*-D motifs that are necessary for the efficient retention of dipeptidyl aminopeptidase A in the yeast Golgi, and may therefore be localized to that compartment (50).

The Golgi localization of Gef1p does not offer an obvious explanation for the poor growth of *gef1* cells on low iron media. Radisky *et al.* (51) have recently characterized a gene, *VPS41*, that is required for vacuolar trafficking and high-affinity iron transport. In the *vps41* mutant, incorporation of copper into the

multi-copper oxidase Fet3p is impaired. Fet3p is an enzyme necessary for high-affinity iron uptake. Since glycosylation of Fet3p is normal in this mutant, the defect seems to lie beyond the TGN, probably in the prevacuolar endosome. Conceivably, the *gef1* phenotype may also be due to a trafficking defect of some essential component of yeast iron metabolism. We could not detect an obvious effect of *GEF1* disruption on the glycosylation of invertase, but this does not rule out a change in Golgi pH which may affect the sorting of some proteins. Interestingly, inhibition of the vacuolar proton pump in mammalian cells can induce retrograde transport from the TGN to the Golgi stack (52). However, the mechanism by which disruption of a putative Golgi chloride channel leads to the defect in iron metabolism of *gef1* cells remains to be elucidated.

In a search for functionally important domains, we performed systematic alanine scanning experiments at the amino and carboxyl terminus. This revealed that a 5-amino acid motif in the amino terminus and a larger domain in the COOH terminus are essential for the proper function and localization of the protein. Some mutant proteins were affected in the staining pattern of the Gef1p-GFP fusion protein but remained functional. However, we cannot exclude that low level expression, which may suffice for functional complementation, still occurred at the normal site within the cell. All non-functional mutants displayed an abnormal GFP fluorescence pattern. The scan identified no mutant that seemed to be correctly localized and non-functional. However, in analogy to the many mutations analyzed in mammalian CLC channels, we expect that such phenotypes could be obtained by mutating residues in the transmembrane region.

The NH_2-terminal essential motif FVTID complies with a consensus of sorting signals containing aromatic amino acids that are involved in localizing proteins to the Golgi and in cycling mechanisms between the Golgi and the plasma membrane. These include the localization determinants of the cation-independent mannose 6-phosphate receptor in mammals (YSKV (53)), the yeast Kex1 and Kex2 Golgi-resident proteases (YTSI and YEFDII, respectively (41, 38)), and the internalization signal in the NH_2 terminus of the mammalian glucose transporter GLUT4 (FQQI (54)). The consensus for these signals is $\phi–\pi$, where ϕ stands for an aromatic and π for large aliphatic side chains (55). Therefore, we additionally mutated these residues either singly or in combination (F36A and I39G). Although expression levels had to be decreased to see an effect of the single point mutants and only the double mutant fully destroyed Gef1p function, these results further support the notion that it constitutes such a motif. Whether it truly functions as a determinant for Gef1p localization remains to be shown. It is mostly proteins with only one transmembrane domain where similar motifs were shown to be necessary and sufficient for localization. The issue may be more complicated for multispanning proteins like GLUT4 where at least two localization determinants were identified (54, 56, 57). Interestingly this amino-terminal motif is highly conserved in the mammalian ClC-3, -4, and -5 homologues (FHTID in ClC-3 and −4, FNTID in ClC-5 as compared with FVTID in Gef1p).

For two TGN proteins, TGN38 and furin, endosomal acidification is needed for retrieval into the Golgi compartment (15). Acidification-dependent localization to the TGN depends on the cytosolic tail of these two proteins (58, 59) and is mediated by a tyrosine-containing motif. If a similar pH-dependent sorting process would operate on a chloride channel involved in intracompartmental acidification, it would reside in the compartment to be acidified until the correct pH is reached and would then be sorted to the Golgi. However, if the phenylalanine-containing motif can truly mediate localization of Gef1p to the Golgi, this invokes a different role for this type of motif than described for many other proteins.

The ER localization of many mutants may reflect a retention of misfolded protein by a quality control mechanism, but our experiments with concatamers and the effect of point mutations at the NH_2 terminus argue against this possibility. Alternatively, forward sorting of Gef1p out of the ER may have been disrupted. For the yeast amino acid permeases a protein required for ER exit is known (60). A sorting signal promoting the ER exit of the vesicular stomatitis virus glycoprotein has been characterized in mammalian cells (61). Thus, it is conceivable that some of the mutants are no longer recognized as cargo destined for ER exit.

Our systematic alanine scanning of the COOH terminus showed that a broad region of about 25 amino acids is also necessary for proper function. This region coincides with the second CBS domain of Gef1p. These domains, dubbed CBS for cystathionine-β-synthase, have recently been identified in a number of otherwise unrelated proteins and occur in organisms as diverse as archaebacteria and man (43, 44). The CBS domain is thought to fold into a compact structure of three β-strands with two short α-helices occurring after strands 1 and 3 (Fig. 4*B*). The function of these domains is presently unknown. There are two CBS domains in the COOH termini of all eukaryotic CLC proteins. Truncation of the muscle channel ClC-1 before the second CBS domain led to a loss of function, and co-expression of the truncated channel protein together with the COOH terminus containing CBS2 again yielded functional channels (62). With ClC-0, a similarly truncated channel could also be rescued by injecting the COOH-terminal peptide encompassing CBS2 into the oocytes (63). This effect could be blocked by brefeldin A, suggesting (in line with this work) an effect of CBS2 on trafficking. In the baculovirus system, however, an in-frame deletion of CBS2 in ClC-1 (leaving the extreme COOH terminus intact) still gave currents, showing that CBS2 is not absolutely necessary for ClC-1 function upon overexpression (64).

We have functionally delineated the borders of the second CBS domain, which coincide well with the borders suggested by Bateman (43) and Ponting (44). CBS1 is also essential, and CBS2 functions independently of its localization within the protein. This suggests that it acts as an independent structure, probably by binding to another protein domain. Whether this domain is on the channel itself, or on a different protein involved, *e.g.* in determining the localization of Gef1p, are problems whose importance clearly extends beyond CLC channels.

Note Added in Proof—After this work was accepted, Gaxiola *et al*. (Gaxiola, R. A., Yuan, D. S., Klausner, R. D., and Fink, G. R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4046–4050) reported that Gef1p and the copper-ATPase Ccc2p co-localize and showed that Gef1p is necessary for copper loading of Fet3p, explaining the iron requirement of the *gef1* strain.

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