Functional expression and cellular localization of the Na^+/H^+ exchanger Sod2 of the fission yeast *Schizosaccharomyces pombe*

Larry Fliegel, Christine Wiebe, Gordon Chua, and Paul G. Young

Abstract: In the fission yeast *Schizosaccharomyces pombe*, the Na^+/H^+ exchanger, Sod2, plays a major role in the removal of excess intracellular sodium, and its disruption results in a sodium-sensitive phenotype. We examined the subcellular distribution and dynamics of Sod2 expression in *S. pombe* using a sod2-GFP fusion protein under the control of an attenuated version of the inducible nmt promoter. Sod2 was localized throughout the plasma membrane, the nuclear envelope, and some internal membrane systems. In exponentially growing cells, in which sod2-GFP was expressed and then the promoter turned-off, previously synthesized sod2-GFP was stable for long periods and found localized to the plasma membrane in the medial regions of the cell. It was not present at the actively growing cell ends. This suggests that these regions of the cell contain old plasma membrane protein vs. newly synthesized plasma membrane without Sod2 at the growing ends. Sod2 localization was not affected by salt stress. The results suggest that Sod2 is both a plasma membrane protein and is present in intracellular membranes. It is likely tethered within discrete regions of the plasma membrane and is not free to diffuse throughout the bilayer.

Key words: Na^+/H^+ exchanger, *Schizosaccharomyces pombe*, cation binding, salt tolerance.

Résumé : Chez la levure fissipare, *Schizosaccharomyces pombe*, l'échangeur Na^+/H^+ Sod2 joue un rôle majeur dans l'élimination de l'excédent de sodium intracellulaire et sa désorganisation produit un phénotype sensible au sodium. Nous avons examiné la distribution subcellulaire et la dynamique de l'expression de Sod2 chez *S. pompe* en utilizant la protéine de fusion Sod2-GFP sous la régulation d'une version atténuée du promoteur nmt inductible. Nous avons localizé Sod2 dans toute la membrane plasmique ainsi que dans l'enveloppe nucléaire et quelques systèmes membranaires internes. Dans les cellules en croissance exponentielle, lorsque Sod2–GFP a été exprimée et que le promoteur a été supprimé, la protéine Sod2-GFP déjà synthétisée a été stable pendant de longues périodes et a été localizée dans la membrane plasmique des régions médiales de la cellule. Elle était absente au niveau des terminaisons des cellules en croissance. Ces observations laissent supposer que ces régions de la cellule pourraient contenir une protéine d'une ancienne membranaire plasmique par rapport à une membrane plasmique nouvellement synthétisée sans Sod2 au niveau des terminaisons en croissance. Le stress salin n'a pas influé sur la localization de Sod2. Les résultats donnent à penser que Sod2 est une protéine de la membrane plasmique et qu'elle est présente dans les membranes intracellulaires. Elle est probablement captive dans des régions discrètes de la membrane plasmique et ne peut diffuser librement dans la bicouche.

Mots clés : échangeur Na^+/H^+, *Schizosaccharomyces pombe*, liaison des cations, tolérance à la salinité.

[Traduit par la Rédaction]

Introduction

Sod2 is a gene product of the fission yeast *Schizosaccharomyces pombe.* It is a plasma membrane protein that exchanges sodium for protons and is responsible for sodium and lithium tolerance in this species. The *sod2* gene was cloned and characterized using selection for LiCl resistance (Jia et al. 1992). Disruption of the *sod2* gene results in an inability to extrude cytoplasmic Na^+ and take up external protons in exchange for internal sodium ions. Sod2 is a Na^+/H^+ exchanger, although the sequence of the hypothetical protein shows only a weak overall similarity with the mammalian and bacterial Na^+/H^+ exchangers. Previously, we showed that His 367, Asp241, and the Asp pair at amino acids 266, 267 are important for the activity of Sod2 (Dibrov et al. 1998), possibly being involved in a conserved motif important in Na^+/H^+ exchanger function (Dibrov and Fliegel 1998).

Sod2 functions to remove intracellular sodium from *S. pombe*. The energy of the proton gradient is used in Na^+/H^+ exchange that is believed to be electroneural (Jia et al. 1992). In an earlier report (Dibrov et al. 1997) we used immunocytochemistry of the overexpressed protein to show that sod2 is present in the plasma membrane of *S. pombe*.

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Some intracellular staining was noted, although we were unable to examine the subcellular localization of sod2 in detail using this system. In the present study, we constructed a Sod2-carboxyl terminal GFP fusion protein for use as a reporter of Sod2 expression, abundance, and localization within *S. pombe*. We used an attenuated version of the NMT promoter pREP-41 (Basi et al. 1993) so as not to overexpress the protein to a high level and to reduce possible errant localization of the protein. Our results suggest that the Na^+/H^+ exchanger sod2 is found on the plasma membrane, nuclear envelope, and some as yet unidentified intracellular membranes.

Materials and methods

Strains and media

Schizosaccharomyces pombe was maintained on yeast extract adenine (YEA) or low sodium minimal KMA (potassium, minimal media, adenine) medium using standard methods described earlier (Dibrov et al. 1998; Jia et al. 1992). KMA medium contained (per L): potassium hydrogen phthalate, 3 g; K_2HPO_4, 3 g; yeast nitrogen base without amino acids, 7 g; glucose, 20 g; and adenine, 200 mg. Leucine at 200 mg/L was added to maintain the *sod2::ura4 leu1-32* strain where appropriate and all media was buffered with 50 mmol/L Mes/Citrate and adjusted to pH 5.0 with KOH. Wherever appropriate, 2.5 or 10 mmol/L LiCl was added to the media. For growth curves, 2×10^6 cells from an overnight exponentially growing culture were used to inoculate 2.5 mL of fresh media liquid, and cultures were grown at 30 °C in a rotary shaker. At various times, aliquots of cells were harvested and the A_{600} determined. All growth curves were determined in duplicate a minimum of 3 times. *Schizosaccharomyces pombe* transformed with the pREP-41 plasmid (and derivatives) were routinely grown in medium containing 10 µmol/L thiamine. Thiamine was removed to induce expression from the nmt promoter. Addition of 10 µmol/L thiamine was used to suppress expression from the promoter.

Addition of the carboxyl terminal GFP tag to sod2

The GFP with a Ser65Thr mutation was added to the carboxyl terminal of sod2. The GFP protein was inserted into the Bam HI–Sma I sites of the plasmid pREP41 with a 9 amino acid Gly-Ala spacer preceding the initiator Met of GFP as described earlier (Taricani et al. 2001). GFP had an NdeI site removed by a silent mutation. To insert sod2 immediately ahead of the GFP, we used PCR with the high fidelity PWO DNA polymerase (Boehringer Mannheim). The forward primer 5′-GGGAATTCC ATA TGGGCTGGAGAC AACTTGA-3′ and the reverse primer 5′-CGC GGATCCGGA ACGTAATCTTCCTGT GAC TTA TC-3′ contained NdeI and Bam HI restriction sites, respectively (i.e., underlined). After digestion with Nde1 and BamH1, this allowed insertion in frame with a Gly-Ala linker immediately preceding GFP, resulting in the plasmid pREP41sod2GFP.

For expression of the sod2-GFP protein the sod2::ura4 strain (Jia et al. 1992) was transformed with the pREP41sod2GFP plasmid using the lithium acetate procedure (Okazaki et al. 1990) and grown in KMA medium.

A chromosomally integrated copy was constructed by selection of a stable integrant from a *sod2:ura4 ura4-D18* pREP41Sod2GFP strain. Random spore analysis (Jia et al. 1992) of the integrant showed no segregation, confirming the chromosomal integration of sod2. A strain containing an integrant under the nmt promoter at the *nmt1* locus was selected and used for further strain construction using standard methods.

Fluorescence Spectroscopy

Images were taken with a Leitz DMR fluorescence microscope (Leica Microsystems; Wetzlar, Germany) with a 100× objective equipped with a high performance CCD camera (Cooke Sensicam; Eugene, Ore.) and Slidebook image analysis software (Intelligent Imaging Systems; Eugene, Ore.). Excitation and emission wavelengths were 495 and 505 nm, respectively, and excitation and emission slit widths were 3 nm.

Results

We constructed a plasmid with the *S. pombe* Na^+/H^+ exchanger sod2 under control of the pREP41 nmt promoter. Initial experiments were performed to determine if the fusion of GFP to the carboxyl terminal of the sod2 protein resulted in a functional Na^+/H^+ exchanger. Sod2 confers salt resistance to *S. pombe,* and therefore cells transformed with the wild type or the GFP fusion were tested for resistance to LiCl in the growth medium as described earlier (Dibrov and Fliegel 1998). Figure 1A illustrates the growth properties of the *sod2::ura4 ura4-D18 leu1-32* knockout strain transformed with the plasmid pWH5sod2 containing a wild type copy of the *sod2* gene (Dibrov and Fliegel 1998). The cells grew well in up to 10 mmol/L LiCl, demonstrating resistance to this externally added cation. In contrast, the *sod2::ura4 ura4-D18 leu1-32* strain without the plasmid was retarded in growth in medium containing 5 or 10 mmol/L LiCl (Fig. 1B). When we tested the *sod2::ura4 ura4-D18 leu1-32* strain expressing sod2-GFP protein, we found that it was resistant to 5 and 10 mmol/L LiCl (Fig. 1C). Cells that expressed GFP alone from the pREP41 plasmid had no resistance to 5 and 10 mmol/L LiCl. These results showed that addition of GFP to the carboxyl terminal of sod2 resulted in a functional Na^+/H^+ exchanger protein that must be, at least partially, properly targeted to the plasma membrane. Similar results have been shown with other membrane proteins tagged with GFP (Kruckeberg et al. 1999).

We next examined the subcellular localization of the sod2-GFP fusion protein. When sod2-GFP was expressed from the nmt promoter it was present uniformly throughout the plasma membrane (Fig. 2A). We also found that sod2 was expressed in what appeared to be a perinuclear compartment. There was typically also a third region of expression, which were slender, irregular rod-like, and possibly membrane structures that led away from and contacted the nucleus. They typically went towards the apex of the cell. To confirm that the localization we observed was due to the sod2 part of the fusion protein, we examined the localization of the GFP protein by itself. The GFP protein was expressed behind the attenuated nmt promoter, but without fusion to any other protein. Figure 2B shows that GFP alone showed a

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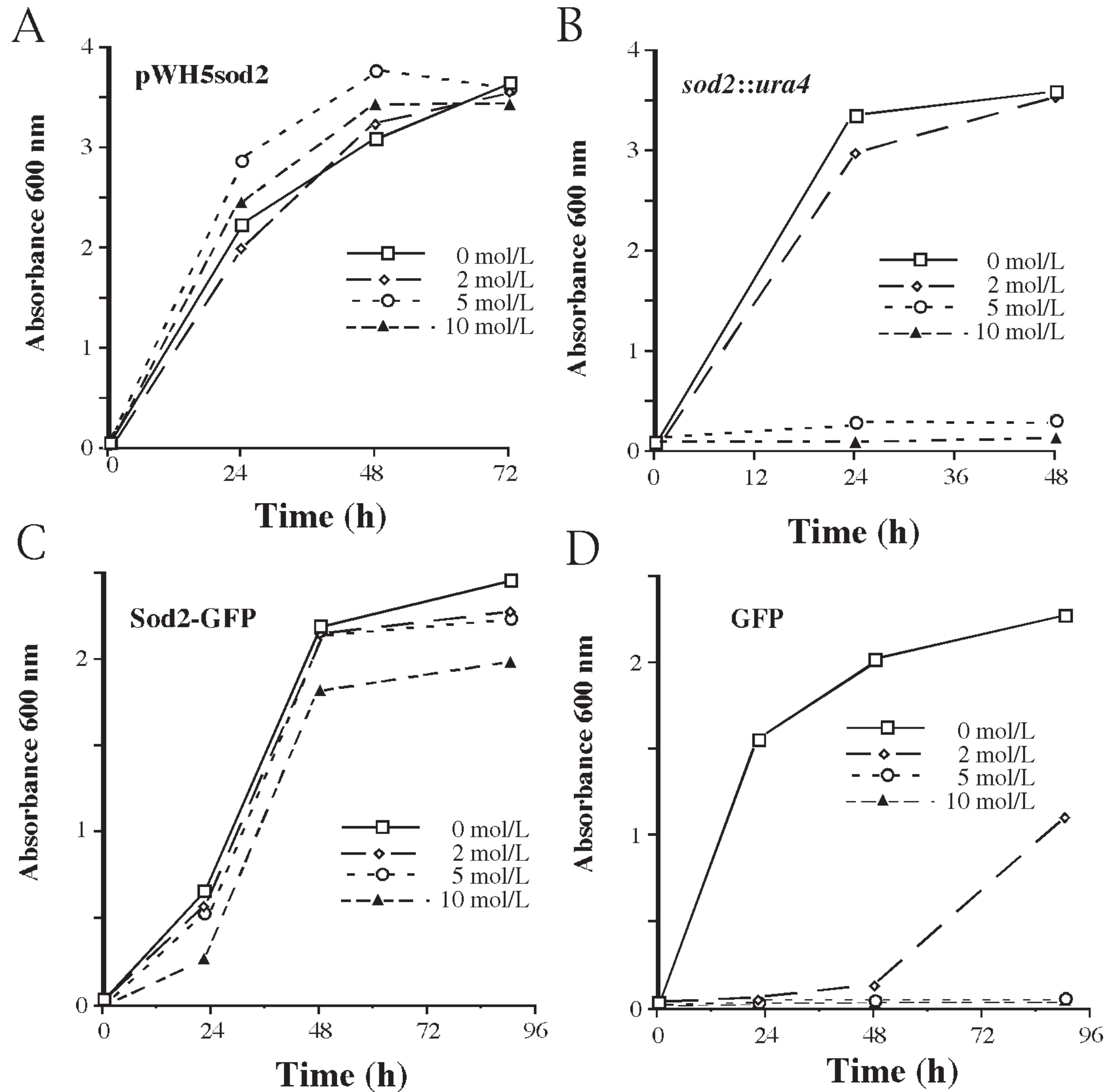
Fig. 1. Growth in LiCl containing media for *sod2::ura4* and *sod2::ura4* expressing sod2, sod2-GFP, or GFP. Sod2::ura4 ura4-D18 leu1-32 cells with or without plasmids expressing sod2 were inoculated into medium containing 0–10 mmol/L LiCl. (A) LiCl tolerance of the strain containing pWH5sod2 plasmid that expresses the wild type sod2. (B) LiCl tolerance of the disruption strain alone. (C) LiCl tolerance of the strain containing pREP41sod2GFP plasmid that expresses sod2 with GFP fused at the carboxyl terminal. (D) LiCl tolerance of the strain containing pREP41GFP plasmid that expresses GFP alone. Results are typical of at least 3 independent determinations.



diffuse fluorescence throughout the cell and was not apparent in either the plasma membrane, a nuclear envelope like structure, or the irregular rod-like structure. Figure 2C demonstrates that the fluorescence observed in Fig. 2A is due to induction of the nmt promoter. The addition of thiamine, which represses the promoter, resulted in the absence of the sod2-GFP signal that was present in Fig. 2A.

To study the dynamics of sod2 turnover, we examined cells in which sod2-GFP was expressed under nmt promoter control and then added thiamine, which very rapidly suppresses activity from this promoter (Fig. 3). Prior to suppression, the characteristic sod2-GFP signal is present in the plasma membrane, nuclear envelope, and the irregular membrane-like systems extending from the nucleus. Panel B (Fig. 3) shows that in the same time period, 10 µmol/L thiamine prevents sod2 expression. Panels C–G show that in the presence of 10 µmol/L thiamine, expression of sod2-GFP in the cells declines. After 6 h, only faint levels of sod2-GFP are detected (Panel G). Panel C shows that with 1 h of repression, there is little change in sod2 localization. Though the intensity of expression decreases, sod2 is present in all 3 locations (i.e., the plasma membrane, perinuclear staining and irregular rod-like structures). Panel D shows that after 2 h of repression, the perinuclear staining is reduced, though there are traces of the irregular rod-like staining. Panels E through G show that sod2 localization changed, and between 3 to 6 h of repression it was found to localize mostly to discrete lateral regions of the plasma membrane. The staining was not uniform throughout the plasma membrane, but rather the lateral regions had more intense fluorescence,

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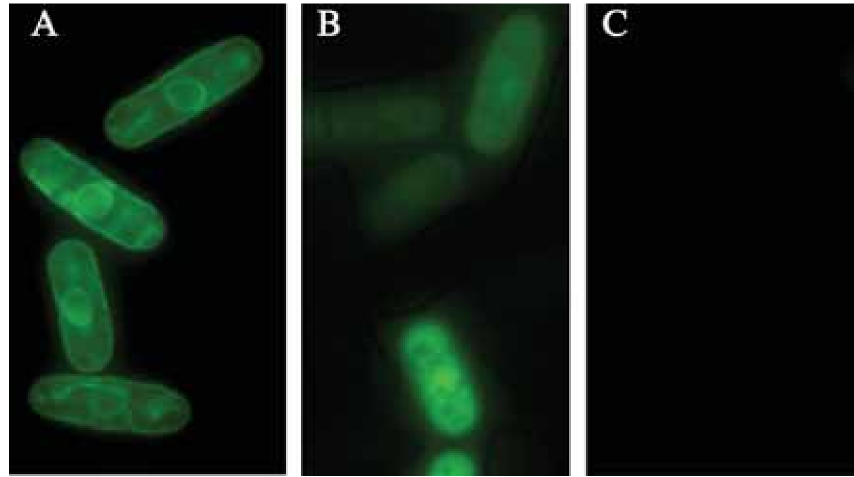
Fig. 2. Fluorescence microscopy of sod2-GFP in *S. pombe*. Overnight cultures of cells grown KMA (supplemented with 200 mg/L adenine) + 10 µmol/L thiamine to suppress expression. Cells were then harvested, washed 3 times with thiamine-free media, and introduced into 25 mL KMA with or without 10 µmol/L thiamine and allowed to grow for 15 h to an approximate final cell density of 0.5×10^7–1×10^7 cells/mL. (A) *sod2::ura4* containing pREP41sod2GFP plasmid (without thiamine). (B) sod2::ura4 strain containing pREP41sod2GFP plasmid (with thiamine). (C) *sod2::ura4* alone. All 3 images were exposed for an equivalent length of time.



whereas the ends of the cell showed much less fluorescent signal than the lateral regions. Perinuclear staining was absent and only E (3 h of repression) had some traces of irregular rod-like staining. Panel H is a control that demonstrates continued sod2-GFP expression after 21 h more in the absence of thiamine. It shows that the time-dependent changes observed in panels E–G require the presence of thiamine.

Since plasmid-borne constructs give considerable variability in expression in different cells because of variation in plasmid copy number, the chase experiment was repeated with the stably integrated sod2 (Fig. 4). To confirm that the apparent perinuclear staining was indeed surrounding the nucleus, cells were fixed and treated with DAPI to illustrate the nuclei. Panels E–H show the nuclear staining corresponding to Panels A–D respectively. Figure 4A shows the typical sod2-GFP localization with plasma membrane, nuclear, and rod-like staining. Comparison of Fig. 4A with 4E shows that the nuclear staining is indeed surrounding the DAPI stained nucleus. After 3 h (Fig. 4B), staining was reduced at the ends of the cell and some perinuclear staining was sometimes present. After 5 and 7 h, apical staining was greatly reduced, and most of the fluorescence was apparent in lateral regions of the plasma membrane.

Figure 5 shows the effect of osmotic stress on the localization of sod2-GFP after suppressing the nmt promoter by thiamine addition. The control (Panels A and B) show that sod2-GFP localization changed little, and after 5 h, sod2-GFP was found principally on the lateral regions of the plasma membrane. Panels C through F show that neither 300 nor 500 mmol/L NaCl affected the distribution or turnover of the sod2-GFP protein.

Discussion

Sod2 is a plasma membrane Na^+/H^+ exchanger of the yeast *S. pombe*. Though we have earlier studied its physiological function and the role of polar amino acids in sod2 activity (Dibrov et al. 1998; Jia et al. 1992), a detailed investigation of its localization still remained. The fact that sod2 can export intracellular Na^+ (Dibrov et al. 1998; Jia et al. 1992) demonstrates that it exists at the plasma membrane. In a preliminary report, we expressed hemagglutinin (HA)-tagged sod2 and used immunocytochemistry to examine its localization (Kruckeberg et al. 1999). Plasma membrane and intracellular sod2 were demonstrated, but precise localization was impossible using immunocytochemistry after the construction of sphaeroplasts. In addition, it was necessary to overexpress the protein to pick up any signal. This could lead to errant targeting.

In the present study, we linked sod2 to GFP. It was possible to examine the localization in intact live cells, as well as to readily follow the dynamics of its localization and turnover. Initially, we determined that addition of GFP to the C-terminus of sod2 did not impair its function. Sod2 was able to restore salt tolerance to the *sod2::ura4* knock out strain. When we examined the localization of sod2-GFP, we found that sod2-GFP exhibited plasmalemmal as well as perinuclear localization. In addition, it often appeared as an irregular rod-like structure associated with the nucleus and extending towards the apex of the cell. Whereas functional studies and our earlier studies suggested that sod2 was present in the plasma membrane, we had not yet shown these intracellular locations. Staining with DAPI confirmed that one of these locations was perinuclear. In mammals, the nuclear envelope has been shown to regulate Na^+ and K^+ gradients that exist between the nucleus and cytosol (Garner 2002). Recently, the mammalian NHE1 isoform of the Na^+/H^+ exchanger has been shown to be present on nuclear envelope membranes of cardiomyocytes, liver cells, and aortic vascular smooth muscle cells. The protein was suggested to contribute to nuclear pH and Na^+ regulation (Bkaily et al. 2004). To our knowledge, there are no reports of nuclear cytoplasmic Na^+ gradients or regulation of Na^+ fluxes in yeast. However, it is known that the pH of the nuclear compartment is identical to that of the cytoplasm (Karagiannis and Young 2001). It is conceivable that the sod2-GFP is in the

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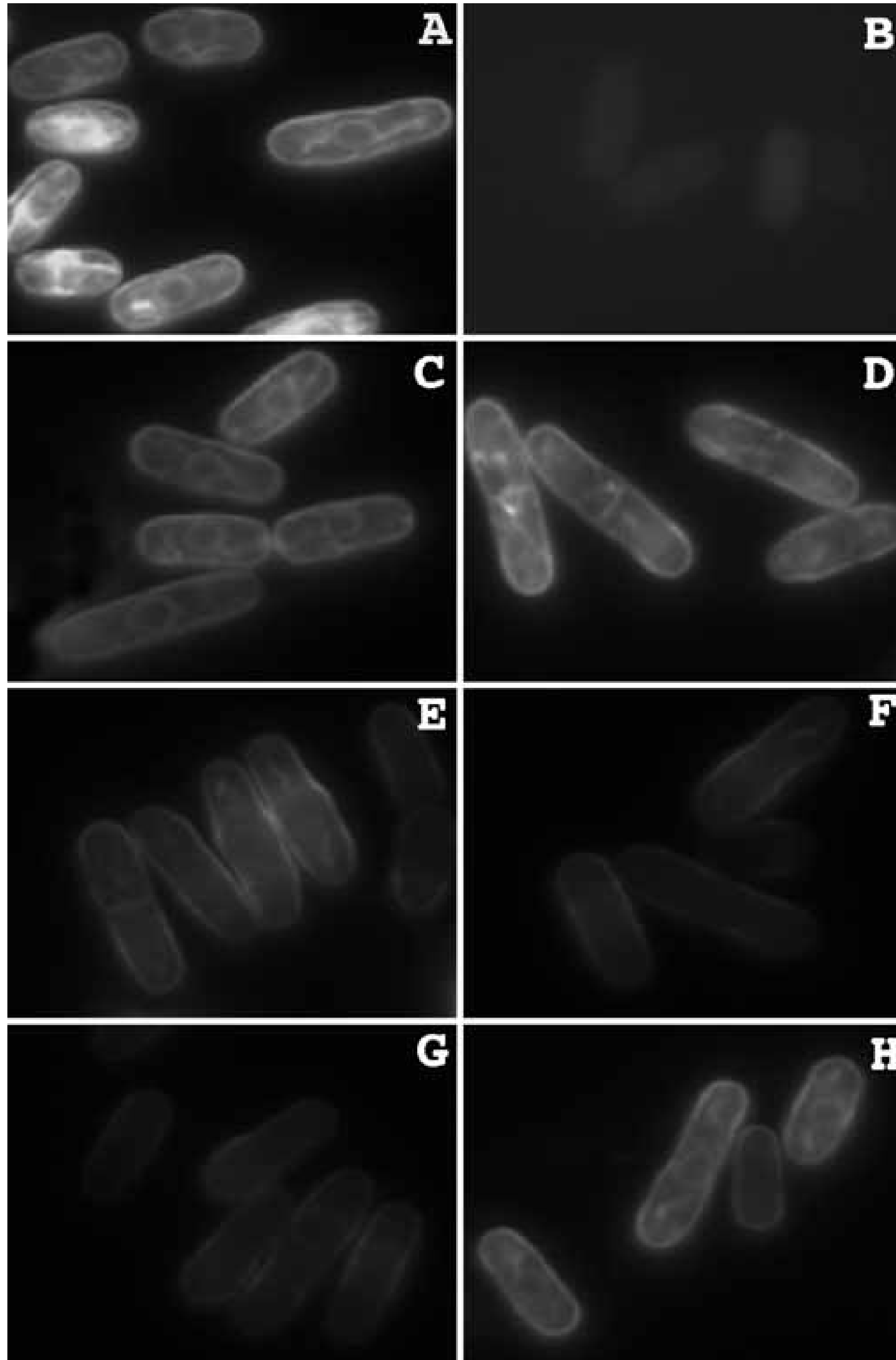
Fig. 3. Effect of nmt promoter suppression sod2 localization and stability. Overnight cultures of cells grown KMA (supplemented with 200 mg/L adenine) + 10 µmol/L thiamine to suppress expression. Cells were then harvested, washed 3 times with thiamine-free media, and introduced into 25 mL KMA with or without 10 µmol/L thiamine and allowed to grow for 15 h to an approximate final cell density of 0.5×10^7–1×10^7 cells/mL. (A) Cells expressing sod2-GFP for 15 h (in the absence of thiamine). (B) Cells treated as for A except in the presence of 10 µmol/L thiamine. After expression for 15 h in thiamine-free medium, sod2-GFP gene expression was prevented by addition of thiamine for (C) 1, (D) 2, (E) 3, (F) 5, or (G) 6 h. (H) Expression of sod2-GFP after 21 h in the absence of thiamine.



outer membrane of this double membrane, or simply present because of the continuity with the ER. There is conceivably a pH and Na^+ concentration difference between the ER and the cytosol. In the hepatocyte nuclei, a Na^+,K^+-ATPase appears to remove Na^+ from the nucleoplasm, possibly to regulate DNA structure and interactions between binding

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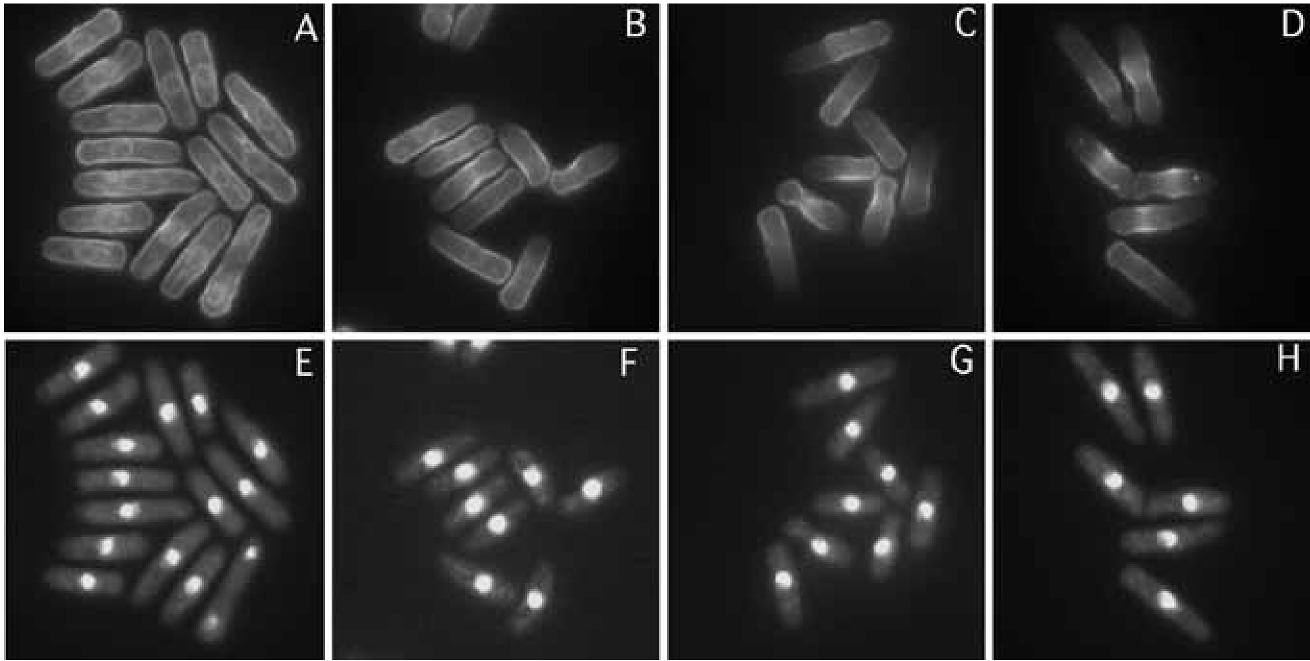
Fig. 4. Expresssion and stability of sod2-GFP in a strain containing a stable *nmt41:sod2:GFP* integrant. Thiamine (10 μmol/L) was added to log phase cells expressing sod2-GFP to turn off expression from the nmt promoter. The distribution of sod2-GFP was examined at (A) 0, (B) 3, (C) 5, and (D) 7 h after addition of thiamine. Fluorescence was faint and camera exposure times varied: 8 s for (A) and (B), and 10 and 15 s for (C) and (D), respectively. Cells were harvested and fixed with 100% methanol. Lower panels (E-H) illustrate DAPI staining.



proteins (Garner 2002). Whereas it is possible that sod2 was inappropriately targeted to the nuclear envelope because of overexpression, we believe that this is unlikely because of the relatively low level of expression of the protein from the nmt promoter. This promoter was attenuated approximately 7-fold relative to the wild type nmt promoter (Basi et al. 1993). GFP alone did not localize to the same regions of the cell and was widely distributed throughout the cytosol. This suggests that the localization of sod2 was not induced by the GFP tag. However, at this stage we cannot rule out that the tag contributes to problem with folding of some portion of the expressed sod2-GFP. Attempts to generate good antibodies against the sod2 protein have to date failed, so we were unable to directly localize sod2.

The identity of the irregular rod-like structures extending from the nuclear region is not known at this time. They sometimes appeared to be connected to the nuclear envelope, but this was not always the case. Comparison with other studies on the localization of ER proteins in fission yeast shows that the structure does not match the distribution of ER under normal conditions. (Facanha et al. 2002). We examined the localization of a series of *S. pombe* fusion proteins with GFP and compared them with the localization of the sod2-GFP fusion (Ding et al. 2000). There were no proteins with similar localization, with the possible exceptions of a putative yeast ammonium transporter (Accession No. AB027805) and a drug/metabolite transporter (Accession number AB027863). The putative ammonium transporter in particular had a perinuclear type of staining with rod-like structures originating from the nucleus and going towards the apex of the cell. Though both of these proteins are predicted to be integral membrane proteins, there is little information available on them aside from their sequence and localization. Neither possessed any regions of similarity with sod2. Future experiments will attempt to determine the identity of the unknown compartment to which sod2 localizes. It is quite possible that it is a portion of ER, and the fact that sod2-GFP chases through this compartment when the promoter is suppressed supports this suggestion.

An interesting aspect of sod2 localization in the plasma membrane was that following suppression of the promoter, previously synthesized sod2-GFP remained localized to discrete regions of the plasma membrane during subsequent cell growth and division. This localization was predominantly limited to the lateral regions of the plasma membrane. This has several implications. Clearly, sod2's lateral mobility within the membrane is restricted. It appears to be flowing from the apex to the lateral region of the cells but not the reverse direction. It is interesting that the lateral membranes of the cell are known to be sterol rich as shown by filipin staining, and thus may have substantial structure associated with it. However, the sterol rich region also normally extends to the growing cell tips, suggesting that sod2 localization does not strictly follow sterol distribution (Takeda et al. 2004). Sod2 has a small hydrophilic 35 amino acid extension from the C-terminus of the protein. When we attached a hemagglutinin tag to this region, it was necessary to permeabilize cells to get an immunofluorescent signal, suggesting that this extension was cytoplasmic (Dibrov et al. 1997). If the protein is tethered to some intracellular anchor-like proteins, it seems likely that it would be through this region. Analysis of the short cytoplasmic tail suggests that several phosphoryl-

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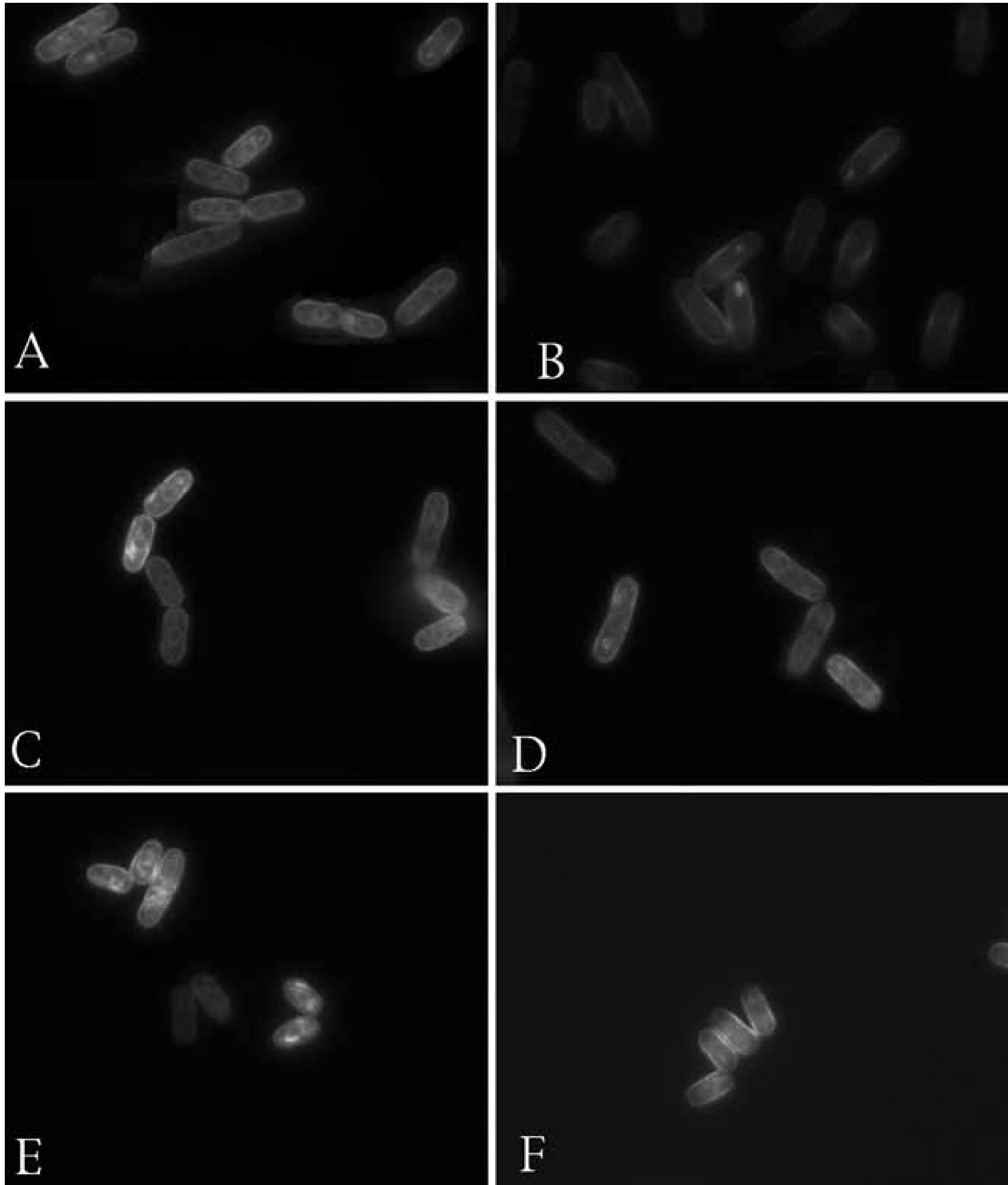
Fig. 5. Effect of salt stress on sod2-GFP localization after thiamine add back. Thiamine was added to log phase *sod2::ura4* pREP41sod2-GFP cells as described in Fig. 4. Simultaneously, 300 mmol/L (C and D) or 500 mmol/L (E and F) NaCl was added as a stressor to the cells. The distribution of sod2-GFP was observed over time. Thiamine returned to the medium for (A) 1 and (B) 5 h in the absence of NaCl. Thiamine and 300 mmol/L NaCl were added for (C) 30 and (D) 60 min. Thiamine and 500 mmol/L NaCl were added for (E) 1 and (F) 5 h. Exposure times varied.



ation sites could be in this region plus a myristoylation site, although it is not known whether the protein is modified. Binding sites for other anchoring proteins were not identified. Future studies could examine protein-protein interactions of this region, possibly by 2-hybrid analysis. Because sod2 is responsible for Na^+ export and salt tolerance, we rea-

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soned that the stability of the protein and its localization might be affected by salt stress. However, our results suggested that there was little effect of salt stress on sod2 localization. With either 300 or 500 mmol/L salt challenge, the subcellular distribution of sod2 was not altered. Previous studies had shown that it is constitutively transcribed (Jia et al. 1992) and the current results suggest that the protein's translation and localization are not regulated by sodium.

Overall our study has clearly demonstrated that sod2 is present in the plasma membrane and 2 intracellular compartments at least during synthesis. We have shown that the protein is not free to diffuse laterally in the membrane, since attenuation of the promoter results in a discrete accumulation in the lateral regions of the cell, and an absence of the protein in the apical growing tips where presumably new membrane is extruded. Sod2 may serve as a structural marker for old vs. newly synthesized plasma membrane.

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