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Membrane Fusion and the Cell Cycle: Cdc48p Participates in the Fusion of ER Membranes

Martin Latterich,* Kai-Uwe Fröhlich,† and Randy Schekman*
*Howard Hughes Medical Institute
and Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720
†Physiologisch-Chemisches Institut
Hoppe-Seyler-Strasse 4
72076 Tübingen
Federal Republic of Germany

Summary

The fusion of endoplasmic reticulum (ER) membranes in yeast is an essential process required for normal progression of the nuclear cell cycle, karyogamy, and the maintenance of an intact organellar compartment. We showed previously that this process requires a novel fusion machinery distinct from the classic membrane docking/fusion machinery containing Sec17p (α-SNAP) and Sec18p (NSF). Here we show that Cdc48p, a cell-cycle protein with homology to Sec18p, is required in ER fusion. A temperature-sensitive *cdc48* mutant is conditionally defective in ER fusion in vitro. Addition of purified Cdc48p restores the fusion of isolated *cdc48* mutant ER membranes. We propose that Cdc48p is part of an evolutionarily conserved fusion/docking machinery involved in multiple homotypic fusion events.

Introduction

The homotypic (self) fusion of intracellular membranes is an important means of regulating the assembly of organelles and maintaining their identity during the cell cycle in eukaryotes. Fragmentation of the nuclear envelope, the endoplasmic reticulum (ER), and the Golgi apparatus ensures that the organelles are partitioned during mitosis (Rothman and Warren, 1994). These membrane fragments assemble after mitosis in an organelle-specific manner. Membrane repair by homotypic fusion may also play an important role in organelle homeostasis. In the yeast Saccharomyces cerevisiae, nuclear and ER membranes remain intact throughout mitotic division. However, the fission of the nuclear envelope, a contiguous part of the ER network, requires at least one membrane fusion step to produce daughter nuclei. Furthermore, in the process of yeast cell mating, parental nuclei fuse at the nuclear envelope to form a diploid nucleus (Kurihara et al., 1994; Latterich and Schekman, 1994; Rose, 1991). Although vesiculation of secretory organelles does not occur during mitosis in yeast, it seems likely that many of the genetic requirements for nuclear fusion during mating, or nuclear fission during cytokinesis, are shared with corresponding homotypic fusion events during telophase in higher cells.

We studied selected genetic requirements for nuclear envelope fusion in vivo and developed a cell-free assay that reproduces some of these features (Kurihara et al., 1994; Latterich and Schekman, 1994). Tightly membrane-associated proteins required for nuclear membrane fusion after mating (karyogamy or *KAR* gene products) are involved in the in vivo fusion reaction. In contrast, proteins required for vesicular docking and fusion (Sec18p or N-ethylmaleimide-sensitive factor [NSF] and Sec17p or soluble NSF attachment protein α [α-SNAP]) are not required for homotypic fusion.

We reasoned that ER–membrane fusion must be required for nuclear fission in yeast. Thus, we expected a mutation inactivating a component of the homotypic fusion machinery would fail to segregate nuclei and therefore arrest late in the mitotic cell cycle. Among a group of late mitotic arrest mutants, we examined a *cdc48* mutant, which arrests as large budded cells with elongated nuclei that span the mother–daughter junction (Moir et al., 1982; Fröhlich et al., 1991). The original *cdc48-1* allele displayed a cold-sensitive medial nuclear division cell cycle arrest (Moir et al., 1982). Four cold-sensitive pseudorevertants that exhibited a temperature-sensitive phenotype were isolated in the same study. These new mutations are intragenic suppressors of the original cold-sensitive mutation. *CDC48* was cloned by complementation and found to be an essential gene. Of particular interest to us, the Cdc48p gene product shares significant sequence homology to Sec18p, the yeast NSF involved in vesicular docking and fusion in the secretory pathway (Fröhlich et al., 1991).

The *CDC48* gene product seems to be conserved throughout evolution. A homohexamer particle of unknown function sedimenting at 14.5S was found in Xenopus; its monomer, p97, shares 76% sequence identity with Cdc48p (Peters et al., 1990). A homolog, VCP (for valosin-containing protein, a misnomer because it is not the precursor to valosin), was identified in bovine, human, murine, and porcine sources (Egerton et al., 1992; Koller and Brownstein, 1987; Zhang et al., 1994). In pig, a 92 kDa VCP protein was characterized that is 70% identical to Cdc48p and also appears to form homohexamers. VCP was localized to the ER (Zhang et al., 1994) and also was shown in vitro to interact with clathrin heavy chain in bovine brain extracts (Pleasure et al., 1993). However, little is known about the function and significance of these interactions. Cdc48p is closely related to a subclass of ATPases, including Sec18p (vesicular docking/fusion; Eakle et al., 1988), Pas1p (peroxisome biogenesis; Erdmann et al., 1991), and Afg2p (unknown function; Thorsness et al., 1993), which have in common a duplicated nucleotide-binding motif and a duplicated adjacent region of close homology, termed the AAA motif. The functional significance of these homologies remains to be tested experimentally.

In this report, we present evidence that Cdc48p is necessary to allow ER membranes to fuse homotypically.

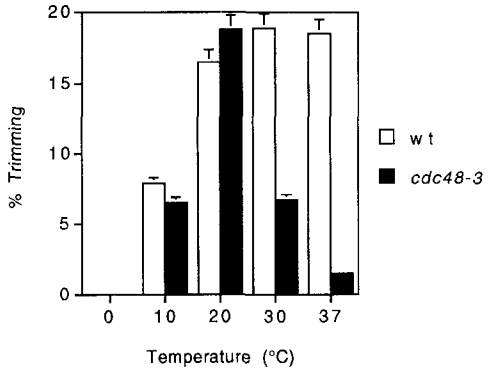

Figure 1. *cdc48-3* Membranes Are Defective for ER–Membrane Fusion In Vitro

Donor and acceptor membranes (1.5 mg of protein each) prepared either from wild-type strains (MLY1601 and MLY1600) or from *cdc48-3* mutant strains (MLY1641 and MLY1640) were combined in the presence of an ATP regeneration system in a total volume of 1 ml in B88 and held on ice. Aliquots (50 μl) were transferred into individual tubes and incubated for 60 min at various temperatures. The amount of glucose trimming, indicative of the successful fusion of membranes, was assessed as described before (Latterich and Schekman, 1994).

Results

cdc48-3 Membranes Are Defective for ER Fusion

Membranes were tested in the ER–membrane fusion reaction described before (Latterich and Schekman, 1994). In this assay, we mix crude membrane fractions from strains that either lack or contain an ER-limited glucosidase that is responsible for initiating the deglucosylation of newly synthesized glycoproteins. Yeast prepro-α-factor translocated into the lumen of glucosidase-deficient ER (*gls1*; donor membrane) is processed to the deglucosylated form when donor membranes are incubated with an acceptor, glucosidase-proficient (*GLS1*) ER membrane. We previously showed that oligosaccharide trimming is a direct measure of membrane fusion.

ER-enriched membranes were isolated from strains MLY1641 (*cdc48-3 gls1-1*) and MLY1640 (*cdc48-3*) that were grown at the permissive temperature of 24°C. These membranes were tested for fusion by incubating donor and acceptor membranes (75 μg of protein each) at different temperatures in the presence of an ATP regeneration system and in a final volume of 50 μl for 1 hr. In contrast to wild-type, *cdc48-3* membranes exhibited temperature-sensitive fusion. Mutant membranes remained fusion competent up to a temperature of approximately 20°C, but declined to near background levels at 37°C (Figure 1). The fusion defect was not due to membrane rupture. Glycosylated pro-α-factor translocated into *cdc48* mutant membranes remained trypsin inaccessible after a 1 hr incubation at 37°C. Our data is consistent with the hypothesis that the *CDC48* gene product directly participates in the membrane fusion process.

To understand further the role of *CDC48* in ER fusion, we investigated two other mutants, *cdc14* and *cdc15*, that arrest at a similar stage in the cell cycle (Culotti and Hartwell, 1971; Hartwell et al., 1970). Mutant strains were incubated at the restrictive temperature for 3 hr prior to membrane isolation to allow for expression of a cell-cycle defect. In spite of this preincubation, no defect was seen in fusion of membranes from either mutant or from control preparations from *sec18* and wild-type cells (Table 1). These data indicate that the role of Cdc48p in ER fusion is direct and not a general phenomenon observed during arrest of the cell cycle during mitosis. However, there may be cell cycle–regulated components of the ER fusion machinery that have not yet been identified by mutations.

Localization of Cdc48p

Cdc48p is predicted to be highly polar, with no significant length of hydrophobic peptide sufficient to embed the protein in a membrane (Fröhlich et al., 1991). Nevertheless, ER–membrane fusion is active without cytosolic protein, and membranes remain fusion competent after treatments that elute peripheral membrane proteins (Latterich and Schekman, 1994). Preliminary experiments designed to determine the subcellular localization of Cdc48p suggested that a fraction of the protein remained associated with a microsomal fraction (Fröhlich et al., 1991). To extend these observations, we used indirect immunofluorescence on wild-type yeast cells. Fixed and permeabilized cells were stained with affinity-purified polyclonal Cdc48p rabbit antibodies or a mouse monoclonal antibody raised against a C-terminal peptide of Kar2p, an ER lumenal protein. Cdc48p appeared to be associated with the nuclear envelope and peripheral ER in the majority of cells in an asynchronous cell population, reminiscent of the strong staining of nuclear and peripheral ER membranes by the Kar2p antibody (Figure 2). Additionally, a weaker staining in the cytoplasm and nucleoplasm was observed in the case of Cdc48p. Cells undergoing mitosis accumulated Cdc48p in the nucleoplasm (M. L., unpublished data), perhaps reflecting a requirement for Cdc48p in a process other than ER fusion. These data suggest that a fraction of Cdc48p is associated with the ER–membrane network, whereas other fractions are cytosolic and nuclear. We currently cannot rule out the possibility that Cdc48p is also associated with other organelles, such as the Golgi complex.

Table 1. ER–Membrane Fusion Competence of Mitotic Cell Cycle Mutants and Mutants in *CDC48* Relatives

Donor	Acceptor	Preshift Conditions	Trimming (%)
Wild-type	Wild-type	5 hr at 37°C	19.4 ± 1.2
cdc14-1	*cdc14-1*	3 hr at 37°C	19.2 ± 1.1
cdc15-2	*cdc15-2*	3 hr at 37°C	18.0 ± 0.8
cdc48-3	*cdc48-3*	5 hr at 37°C	1.4 ± 0.6
sec18-1	*sec18-1*	1 hr at 37°C	21.1 ± 0.6

Donor and acceptor membranes from both wild-type strains (MLY1601 and MLY1600) and from mutant strains, which all were preincubated as indicated, were combined in the presence of an ATP regeneration system (150 μg of membrane per 50 μl reaction) and incubated for 60 min at 37°C. The amount of glucose trimming, indicative of the successful fusion of membranes, was assessed as described before (Latterich and Schekman, 1994).

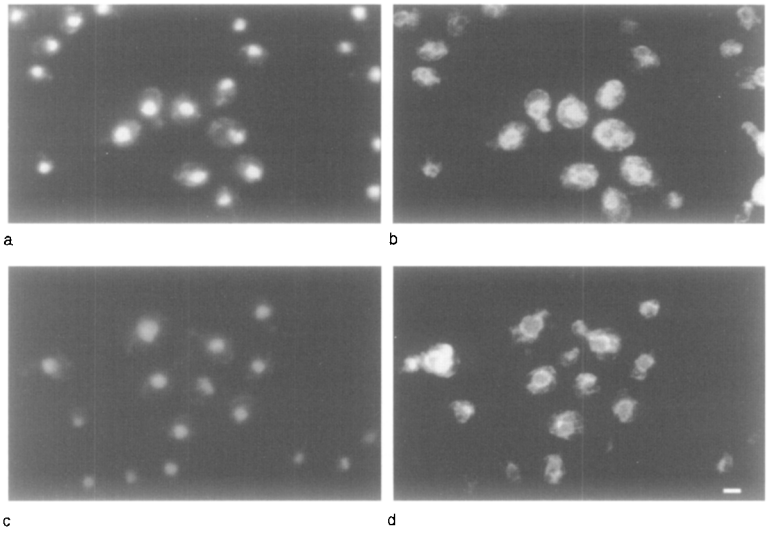

Figure 2. Immunolocalization of Cdc48p

Cdc48p protein was immunolocalized using affinity-purified antibodies against Cdc48p. Kar2p was localized using a monoclonal antibody raised against a Kar2p C-terminal peptide (see Experimental Procedures). Early log phase asynchronous cells of MLY1600 were observed under a Nikon fluorescence microscope with the diamidophenylindole (DAPI) channel to visualize nuclear DNA (panels a and c) or the fluorescein isothiocyanate (FITC) channel to detect Kar2p (panel b) or Cdc48p (panel d). The monoclonal antibody against Kar2p shows the characteristic perinuclear and peripheral ER staining pattern (b). Cdc48p is localized to the perinuclear and peripheral ER region, with some fluorescence seen in other areas of the cytoplasm and in the nucleoplasm (d). The scale bar represents 1 μm.

We used cell fractionation to localize Cdc48p to microsomal membranes used in the ER fusion assay. Wild-type and *cdc48-3* cells were converted to spheroplasts and gently lysed by Potter homogenization (Latterich and Schekman, 1994). After an initial 3,000 × g spin to sediment cellular debris, total microsomal membranes were sedimented by centrifugation for 20 min at 15,000 × g. Microsomal membranes enriched in ER were collected on a sucrose cushion as described before (Latterich and Schekman, 1994). A cytosolic fraction of proteins depleted of membranes was prepared as previously described (Rexach et al., 1994). Samples of membrane-depleted cytosol and microsomal membranes from both wild-type and *cdc48-3* cells were separated on SDS–polyacrylamide gels and quantitatively immunoblotted for Cdc48 protein (Rexach et al., 1994). Both wild-type and mutant cells contained cytosolic and membrane-associated pools of Cdc48 protein (Figure 3). Cdc48p remained tightly membrane associated even under conditions, such as 3 M urea, 2 M KCl, and mild trypsin treatment, that are sufficient to dissociate most peripheral membrane proteins, including Sec17p and Sec18p (Latterich and Schekman, 1994; Figure 3). This fractionation pattern was unchanged in a *cdc48-3* mutant strain, indicating that localization to microsomal membranes was not affected by the mutation. It appears that a quantity of Cdc48p sufficient to promote membrane fusion is associated with ER–nuclear envelope membranes, perhaps through a tight protein–protein interaction.

Cdc48p-Specific Antibodies Inhibit ER Fusion

Antibody fragments or complete antibodies have been used successfully to inhibit the function of proteins involved in vesicular transport and fusion (Beckers et al., 1989; Diaz et al., 1989; Griff et al., 1992; Latterich and Schekman, 1994; Rexach and Schekman, 1991). Because some of the Cdc48p protein is tightly membrane associated, we reasoned that Cdc48p antibodies should inhibit the fusion of isolated ER membranes. Indeed, polyclonal Cdc48p antibody inhibited the reaction approximately 50%, and this inhibition was competed by adding an equal amount of Cdc48p (Figure 4; see below). Neither preimmune serum from the same rabbit nor antibodies raised against the ER-localized translocation protein Sec61p inhibited fusion. These results provide another line of evidence that Cdc48p acts directly to promote ER fusion.

Cytosolic Cdc48p Rescues the Conditional Fusion Defect of *cdc48-3* Membranes

Because *cdc48-3* membranes are temperature sensitive for ER fusion in vitro and a fraction of Cdc48p is located in the cytosol, we added various cytosol fractions in an effort to restore the fusion of *cdc48-3* membranes at 37°C. Cytosol isolated from a wild-type yeast strain, as well as from a strain that overproduces Cdc48p 2-fold, partially repaired the fusion defect (Figure 5a). As expected, cytosol isolated from the *cdc48-3* mutant strain failed to restore fusion. Repair was proportional to the amount of Cdc48p in the cytosol fraction (Figure 5b). These results show that cytosolic Cdc48p can be recruited to membranes to act directly or indirectly in the fusion event.

Cytosolic complementation of the *cdc48* defect provided an assay for the purification of functional Cdc48p. Two previously published procedures describing the isolation of Cdc48p from a commercial source of baker's yeast (Fröhlich et al., 1991) were unsatisfactory when the source was a more defined lab strain (MLY1600; M. L. and R. S., unpublished data). We developed a procedure to obtain a nearly homogeneous preparation (see Experimental Procedures; Table 2; Figure 6a). Our procedure was optimized for resolution of Cdc48p from major contaminant protein species, not for yield. Further refinements in the purification procedure are underway to obtain larger quantities of pure protein. The last purification step, gel exclusion chromatography on Sephacryl S6, yielded a peak of protein that filtered at a position between size standards of 600 and 800 kDa. This apparent size is nearly the same

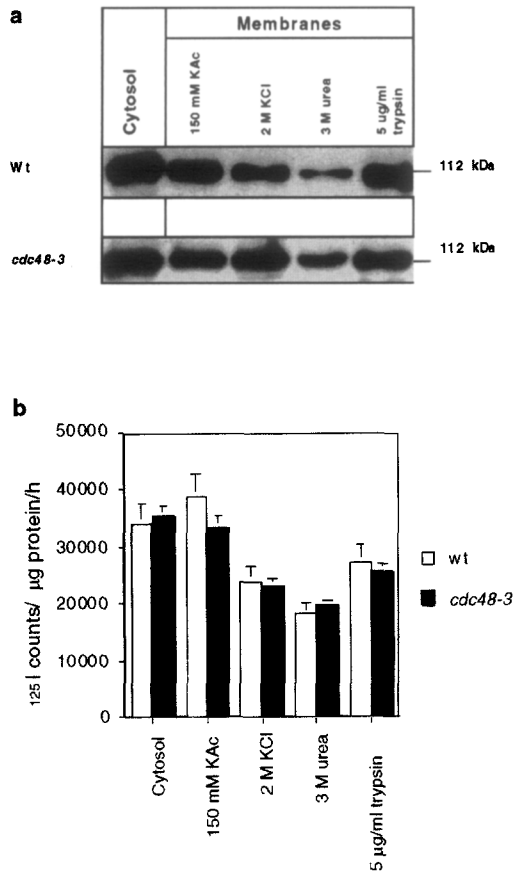

Figure 3. Cdc48p Fractionates in Cytosolic and Membrane-Associated Pools

(a) Membrane-depleted cytosol (50 µg) from wild-type (MLY1600) and *cdc48-3* (MLY1640) strains was loaded onto an 8% SDS–polyacrylamide gel. Membrane protein from microsomal fractions isolated from MLY1600 and MLY1640 was treated as follows: 100 µg of wild-type and mutant membranes were washed three times in 0.5 ml of B88 (150 mM KAc), twice with 0.5 ml of 2 M KCl, and once with B88, twice with 3 M urea and once with B88, or were incubated for 15 min on ice in the presence of 5 µg/ml trypsin, followed by 5 µg of 20 mg/ml trypsin inhibitor for 10 min on ice and two washes with B88. The washed pellets were dissolved in 100 µl of Laemmli's buffer, and 10 µl (10 µg of protein) was loaded onto the same SDS–polyacrylamide gel. Proteins were transferred onto nitrocellulose and immunoblotted for Cdc48p as described previously (Rexach et al., 1994). The relative Cdc48p concentration present in cytosol and on membranes is expressed as ^{125}I counts per µg of protein per hr of exposure and is corrected to the relative amount of Sec61p, an integral ER membrane protein, as a loading control (b).

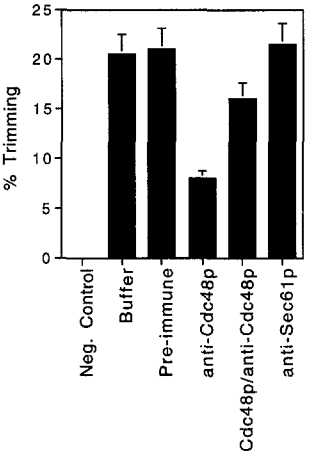

Figure 4. Anti-Cdc48p Antibodies Partially Inhibit ER–Membrane Fusion

Fusion activity of wild-type membranes (150 µg membranes per 50 µl reaction) was assessed on ice or at 30°C in the presence of B88, preimmune serum (5 µg per reaction), anti-Cdc48p antiserum (5 µg per reaction), anti-Cdc48p antiserum (5 µg per reaction) plus 5 µg of Cdc48p protein (>90% pure by SDS–polyacrylamide gel electrophoresis), and affinity-pure anti-Sec61p antibodies (5 µg per reaction). These reactions were preincubated on ice for 10 min before shifting to 30°C for 60 min.

as that deduced for the amphibian p97, which suggests that Cdc48p also exists as a homohexameric complex (Peters et al., 1990).

The highly enriched Cdc48p fraction restored ER fusion in a dose-dependent manner (Figure 6b). These results show that the active form of this molecule is an oligomer of Cdc48p subunits. Additional proteins, likely membrane associated, may be necessary to promote the action of Cdc48p.

Rescue of the *cdc48* Defect by Heterologous Cytosol

Cdc48p homologs have been found in other organisms in virtually all tissues examined (Egerton et al., 1992; Koller and Brownstein, 1987; Peters et al., 1990; Zhang et al., 1994). We isolated cytosol from Xenopus oocytes and rat brain to determine whether they were able to restore membrane fusion activity to *cdc48-3* yeast membranes. Soluble protein fractions (250 µg) obtained from S. cerevisiae (Rexach et al., 1994), Xenopus ovaries (Peters et al., 1990), and rat brain (M. Bennett, personal communication) were added to a standard fusion reaction containing 150 µg of *cdc48-3* membranes per reaction. Fusion reactions were incubated at 20°C and 37°C. Whereas buffer or an equivalent amount of bovine serum albumin allowed fusion only at the low temperature, Xenopus cytosol had the highest specific activity and stimulated fusion at both temperatures beyond wild-type levels (Figure 7). Rat brain cytosol restored fusion to almost wild-type levels, whereas yeast cytosol, surprisingly, had the lowest specific activity. These higher activities from Xenopus and rat may reflect a requirement for more active or more abundant homotypic ER fusion in these sources.

Discussion

Biochemical and genetic studies of vesicular traffic in mammals and yeast indicate that at least two universal components, NSF (Sec18p) and α-SNAP (Sec17p), are involved in the docking and fusion of vesicles in the secretory and endocytotic pathways (Block et al., 1988; Malhotra et al., 1988; Clary et al., 1990; Eakle et al., 1988; Griff et al., 1992; Wilson et al., 1989), excepting the transport

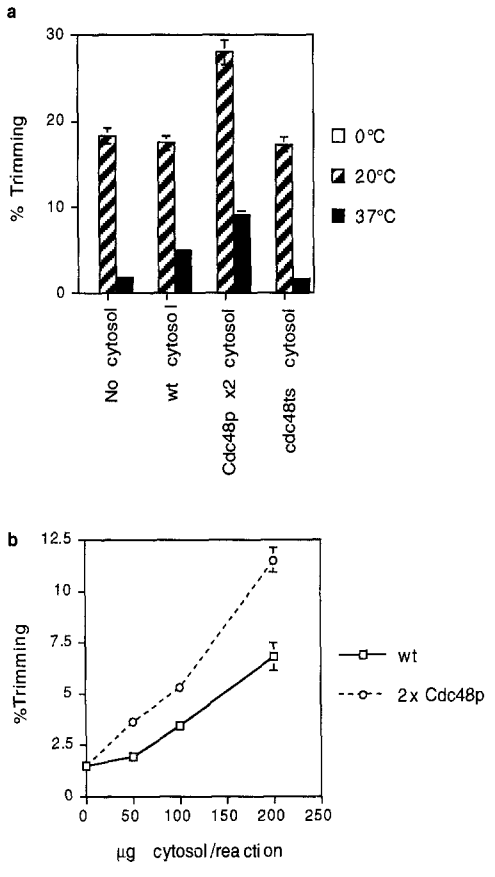

Figure 5. In Vitro Complementation of ER–Membrane Fusion by Yeast Cytosol

(a) Donor membranes and acceptor membranes isolated from MLY1641 (*cdc48-3 gls1-1*) and MLY1640 (*cdc48-3*) were incubated in a standard fusion reaction at the temperatures indicated in the presence of buffer, yeast cytosol (200 μg per reaction), cytosol isolated from MLY1600 (pKF441) after a 16 hr shift to 2% galactose-containing medium (200 μg per reaction), and cytosol isolated from MLY1640 (*cdc48-3*) (200 μg per reaction). MLY1600 (pKF441) incubated under the conditions described above accumulated twice as much Cdc48p as compared with MLY1600 by quantitative immunoblotting (data not shown).

(b) Donor membranes and acceptor membranes isolated from MLY1641 (*cdc48-3 gls1-1*) and MLY1640 (*cdc48-3*) were incubated in a standard fusion reaction at 37°C for 60 min at the cytosolic protein concentrations indicated using yeast cytosol and cytosol isolated from MLY1600 (pKF441) after a 16 hr shift to 2% galactose-containing medium (200 μg per reaction), conditions that overexpress Cdc48p 2-fold.

of secretory vesicles destined for the apical membrane in polarized epithelial cells (Ikonen et al., 1995). In contrast, we found that different proteins are involved in homotypic fusion of ER membranes (Kurihara et al., 1994; Latterich and Schekman, 1994).

This report describes a novel component involved in homotypic ER fusion that fortuitously emerged as a *SEC18* homolog involved at a late stage in mitotic nuclear division. Our attention was drawn to *CDC48* because the Cdc48 protein shares significant homology to the vesicular docking/fusion protein Sec18p and because temperature-sensitive *cdc48* mutant alleles arrest as large budded cells with undivided nuclei located in the neck between the mother and daughter cell. This phenotype is consistent with a defect in homotypic fusion of the inner nuclear membrane, which must precede complete fission to form daughter nuclei.

Membranes isolated from a temperature-sensitive *cdc48-3* strain grown at the permissive temperature are also temperature sensitive for ER fusion in vitro. The amino acid sequence indicates that Cdc48p is polar, and immunofluorescence and cell fractionation results demonstrate that a significant portion of the total Cdc48 protein is localized to the ER–membrane network, where it may act upon an integral membrane protein, perhaps a fusogen.

We took advantage of the *cdc48* temperature-sensitive defect to develop a biochemical complementation assay for the cytosolic form of the protein. Cytosolic repair of the membrane defect provided a simple and reliable assay to test the functional form of Cdc48p. The fact that in this assay Cdc48p alone rescues the fusion defect of *cdc48-3* membranes suggests that no other peripheral or integral membrane proteins are rate limiting in *cdc48* mutant membranes. In particular, no evidence for a Sec17p (α-SNAP) equivalent emerged from our fractionation. If homotypic fusion requires such a molecule, it may be sufficiently abundant on the surface of wild-type or *cdc48* mutant ER membranes. In addition to the effect of the *cdc48* mutation, Cdc48p antibodies inhibit the fusion activity of the wild-type protein. Based on these results, we believe that Cdc48p plays a direct role in homotypic fusion.

The fact that Cdc48p functions in a homotypic fusion reaction that is not dependent on Sec18p/NSF may imply that the mechanism of fusion is conserved and Cdc48p

Table 2. Purification of Cdc48p from Bead Beat Cell Extracts

Sample	Protein Concentration (mg/ml)	Volume (ml)	Cdc48p Concentration (μg/ml)	Enrichment (Fold)	Recovery (%)
Bead beat lysate	17	163	220		100
DEAE load	7.4	45	82	3.2	38.5
DEAE pool	0.48	64	79	32.8	36.5
HA pool A	1	2	176	35.2	2.5
MonoQ pool	3.5	0.5	690	39.4	2.5
Sephacryl S6 fraction 17	0.043	2	42	188	0.6

Cdc48p was purified as described in Experimental Procedures from 500 g of yeast cells (MLY1600). Protein concentrations were determined by the Bio-Rad Bradford assay. The Cdc48p concentration was assessed by quantitative Western blot, estimating the final protein concentration of the Cdc48p-containing fraction and relating the radioquantified data to this fraction. DEAE, diethylaminoethyl; HA, hydroxylapatite.

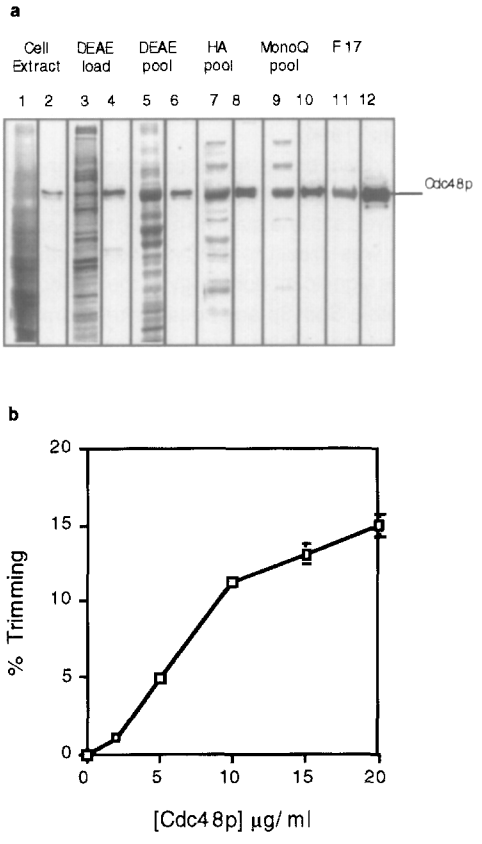

Figure 6. Cdc48p Restores Fusion of *cdc48-3* Membranes

(a) Purification of Cdc48p from total cell extract. Odd-numbered lanes are SDS–polyacrylamide gels stained by Coomassie blue; even-numbered lanes represent a Western blot of respective fractions with anti-Cdc48p antiserum. Total cell extract (20 μg of protein; lanes 1 and 2), 20 μg of dissolved $(NH_4)_2SO_4$ pellet (lanes 3 and 4), 10 μg of diethylaminoethyl (DEAE) pool (lanes 5 and 6), 2 μg of hydroxylapatite (HA) pool (lanes 7 and 8), 2 μg of Mono Q pool (lanes 9 and 10), and 1 μg of fraction 17 (lanes 11 and 12) were loaded. Fraction 17 eluting at the beginning of the Cdc48p peak fraction is the most pure fraction and is used subsequently as Cdc48p protein. From fraction 18 onward, other contaminant proteins elute together with the peak of Cdc48p (data not shown).

(b) Donor membranes and acceptor membranes isolated from MLY1641 (*cdc48-3 gls1-1*) and MLY1640 (*cdc48-3*) were incubated in standard fusion reactions at 37°C for 1 hr in the presence of the indicated amounts of Cdc48p derived from Sephacryl S6 fraction 17.

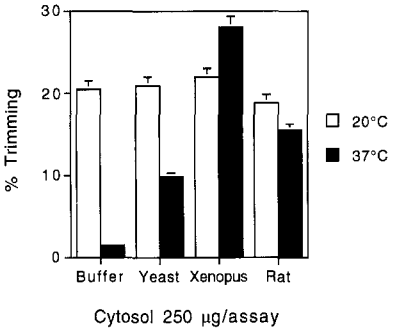

Figure 7. Evolutionary Conservation of ER–Membrane Fusion

Donor membranes and acceptor membranes isolated from MLY1641 (*cdc48-3 gls1-1*) and MLY1640 (*cdc48-3*) were incubated in a standard fusion reaction at 20°C (open bars) or at 37°C (closed bars) in the presence of buffer as control, yeast cytosol (250 μg per reaction), Xenopus oocyte cytosol (250 μg per reaction), and rat brain cytosol (250 μg per reaction).

may be a counterpart of Sec18p in ER fusion. However, the two proteins may have distinct functions. Sec18p is part of a complex including SNAPs and targeting molecules (SNAP receptors [SNAREs]) (Søgaard et al., 1994). Cdc48p-interacting membrane proteins have yet to be identified and analyzed. Cdc48p may be involved in the modulation of a fusion machinery in a cell cycle–dependent fashion, rather than directly in the fusion process.

It is tempting to speculate that Cdc48p-like proteins in higher organisms are involved in other homotypic fusion reactions, such as in the postmitotic reassembly of nuclear and Golgi membranes. Recently, others investigating the protein requirements for Golgi homotypic fusion reactions showed that p97, the Xenopus equivalent of Cdc48p, and ATP suffice to promote one step in this process (Acharya et al., 1995 [this issue of *Cell*]; Rabouille et al., 1995 [this issue of *Cell*]). It appears that NSF functions at the point of fusion between small Golgi-derived vesicles and p97 mediates the fusion of larger Golgi precursors or mitotic Golgi vesicles to form Golgi cisternae. No small vesicle intermediate is involved in the fusion of yeast ER membranes; therefore, Cdc48p may suffice for the bridging and fusion of larger membranes, such as the nuclear envelope.

Cytosol from both Xenopus and rat repairs the *cdc48-3* fusion defect. However, p97 isolated from both mitotic and interphase cytosol did not reverse the *cdc48-3* defect in vitro (M. L. and R. S., unpublished data). Similarly, a cDNA clone of the porcine VCP does not complement a deletion of the *CDC48* gene (K.-U. F., unpublished data). Perhaps a species-specific accessory factor, such as a SNAP, is required for p97/VCP function in yeast. A precedent for this is the observation that Sec18p interacts only with Sec17p, and not with bovine brain α-SNAP, when yeast cytosolic proteins are used to promote transport vesicle fusion in isolated mammalian Golgi membrane fractions (Clary et al., 1990). Alternatively, VCP may not be the functional homolog of Cdc48p in pig. Purification of the amphibian or mammalian factor(s) that restore fusion to yeast *cdc48* membranes and their subsequent analysis will address this issue.

What is the precise role of ER–membrane fusion and Cdc48p in mitotic nuclear division? We have no direct evidence that Cdc48p acts to bridge and fuse the inner nuclear membrane to initiate nuclear fission. Indeed, the wide distribution of Cdc48p along the ER–nuclear envelope network and in the nucleoplasm in mitotic cells suggests that the protein may act in several capacities. In addition to a direct role in nuclear fission, Cdc48p may promote nuclear envelope growth through homotypic fusion of unconnected ER tubules or vesicles. Growth of the envelope to a specific size may be necessary to set in train the events that lead to fission. In the absence of nuclear envelope growth, the mitotic cycle or perhaps just the nucleus may be held at a point prior to cytokinesis.

Cdc48p activity and localization may be cell-cycle regu-

lated. Such regulation, if it exists, must be posttranslational, because *CDC48* transcription and translation does not change during the cell cycle (K.-U. F., unpublished data). Phosphorylation of the mammalian counterpart of Cdc48p, VCP, has been documented in two instances. VCP becomes tyrosine phosphorylated during T cell and B cell antigen receptor activation (Egerton et al., 1992; Schulte et al., 1994). VCP is constitutively tyrosine phosphorylated in Rous sarcoma virus–transformed fibroblasts, but only transiently so in other cell lines (Schulte et al., 1994). Tyrosine phosphorylation of Cdc48p has been observed, but has not yet been correlated to its fusion activity or cell-cycle role (K.-U. F., unpublished data).

Why does the cell employ Cdc48p and other proteins for ER homotypic fusion, whereas most known fusion processes in the secretory pathway involving different organelles employ the same basic components, NSF and α-SNAP? Clearly NSF and α-SNAP do not provide the specificity in heterotypic vesicle docking/fusion. Instead, the specificity appears to be assured by specific Rab proteins and vesicle- and target-membrane SNARE molecules (v- and t-SNAREs) (Rothman and Warren, 1994). We find no requirement for GTP or a Rab protein relying on GTP hydrolysis in the ER fusion reaction. Various homotypic fusion events in a cell, such as among mitochondria, vacuoles, and peroxisomes, may be both specified and catalyzed by unique isoforms of Cdc48p. In this regard, two such homologs are known in yeast. *PAS1* encodes a member of the *SEC18* family that actually is more closely related to *CDC48* (Erdmann et al., 1991). *pas1* mutant cells are defective in peroxisome biogenesis, perhaps as a result of the failure to assemble a large functional organelle from small precursor vesicles. Another member of this family, *AFG2* (Thorsness et al., 1993), serves an essential but yet unknown function in mitotic growth. We predict that this isoform will promote the fusion of an essential organelle, such as the mitochondrion, as an obligate event in membrane maintenance or segregation during the cell cycle.

Experimental Procedures

Reagents and Strains

Reagents were as described before (Latterich and Schekman, 1994). Yeast strains used in this study are listed in Table 3. Yeast genetics and molecular biology methods have been described elsewhere (Rose et al., 1990; Sambrook et al., 1989), as has a method for quantitative immunoblots (Rexach et al., 1994).

Polyclonal Antibodies against Cdc48p

For antibody production, rabbits were injected subcutaneously with 200 μg of purified Cdc48p in complete Freund's adjuvant emulsion and boosted after 3 and 6 weeks with 100 μg of Cdc48p in incomplete Freund's adjuvant. Serum was collected starting 2 weeks after the last injection. IgG was purified from rabbit serum on HiTrap protein G columns (Pharmacia) as described by the manufacturer. Antiserum was affinity purified on nitrocellulose blots according to the protocol of Pringle et al. (1991), using 5 μg of purified Cdc48p as the affinity adsorbent.

Indirect Immunofluorescence Microscopy

Yeast cells were fixed for immunofluorescence using antibodies against Cdc48p (1:1000) and Kar2p (C-terminal mouse monoclonal antibody; 23 residues of C-terminus) (1:500) as previously described (Latterich and Schekman, 1994).

Isolation of ER Membranes and Yeast Cytosol and ER Fusion Assay

The isolation of ER membranes and cytosol and the ER fusion assay have been described (Latterich and Schekman, 1994).

Purification of Cdc48p

MLY1600 cells (500 g) were suspended in 200 ml of buffer H (20 mM triethanolamine hydrochloride [pH 7.4], 1.5 mM EDTA, 1 mM $MgCl_2$, and 5 mM β-mercaptoethanol) containing 1 mM o-phenanthroline and 2 mM PMSF. An equal volume of glass beads (0.5 mm diameter) was added, and cells were disrupted by vigorous shaking seven times for 1 min each in a bead beater. The suspension was centrifuged at 3,000 × g for 10 min to remove cell debris and glass beads and subsequently at 18,000 × g for 30 min to sediment membranes and vesicular components. Proteins were precipitated with ammonium sulfate at 40% saturation, dissolved to an approximate protein concentration of 10 mg/ml, and dialyzed twice against 5 l of buffer H for 4 hr and 8 hr, respectively (Fröhlich et al., 1995). The solution was applied to a 3 cm × 18 cm DEAE Sephacel (Pharmacia) column, washed with 1 vol of buffer H, and eluted with a 500 ml linear gradient of 0–600 mM NaCl in buffer H. Fractions of 7 ml were collected (Fröhlich et al.,

Table 3. Strains and Plasmids Used in This Study

Strain	Genotype	Reference
DBY473	MATα *his4-619 gal mal*	Moir et al., 1982
DBY2030	MATa *ade2-101 lys2-801 ura3-52 cdc48-1*	Fröhlich et al., 1991
DBY4057	MATα *his4-619 gal mal cdc48-2*	Moir et al., 1982
DBY4060	MATα *his4-619 gal mal cdc48-3*	Moir et al., 1982
MLY1600	MATα *ura3-52 leu2-3,112 Δpep4::URA3*	Latterich and Schekman, 1994
MLY1601	MATa *ura3-52 leu2-3,112 Δpep4::URA3 gls1-1*	Latterich and Schekman, 1994
MLY1602	MATa *ura3-52 Δpep4::URA3 sec18-1*	Latterich and Schekman, 1994
MLY1604	MATa *ura3-52 leu2-3,112 Δpep4::URA3 sec18-1 gls1-1*	Latterich and Schekman, 1994
MLY1636	MATa *leu2-3,112 Δpep4::URA3 cdc48-1*	This study
MLY1639	MATa *leu2-3,112 Δpep4::URA3 gls1-1 cdc48-3*	This study
MLY1640	MATa *leu2-3,112 Δpep4::URA3 cdc48-3*	This study
MLY1641	MATα *his4-619 Δpep4::URA3 gls1-1 cdc48-3*	This study
MLY1642	MATa *ura3-52 leu2-3,112 Δpep4::URA3 gls1-1 cdc14-1*	This study
MLY1643	MATa *ura3-52 leu2-3,112 Δpep4::URA3 cdc14-1*	This study
MLY1644	MATa *ura3-52 leu2-3,112 Δpep4::URA3 gls1-1 cdc15-2*	This study
MLY1645	MATa *ura3-52 leu2-3,112 Δpep4::URA3 cdc15-2*	This study
MLY1646	MATa *ura3-52 leu2-3,112 Δpep4::URA3 gls1-1*	This study
MLY1647	MATα *ura3-52 leu2-3,112 Δpep4::URA3 pKF441 (GAL1/10::CDC48, LEU2)*	This study

1995). Fractions containing Cdc48p were pooled, adjusted to 50 mM potassium phosphate with a 1 M potassium phosphate (pH 7.4) stock solution, and applied to a 2 cm × 10 cm column of hydroxylapatite, which was prepared fresh each time using the method of Bernardi (1971). The column was washed with 2 vol of 40 mM potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 5 mM β-mercaptoethanol and eluted with a 120 ml gradient of 50–200 mM potassium phosphate (pH 7.4) in 1 mM $MgCl_2$, 5 mM β-mercaptoethanol. Cdc48p-containing peak fractions that eluted from hydroxylapatite and were free of major high molecular weight contaminants were pooled and concentrated by ammonium sulfate precipitation to 90% saturation. The precipitate was dissolved into 2 ml of buffer H100 (20 mM triethanolamine [pH 7.5], 100 mM NaCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol) and loaded onto a 1 ml Mono Q column (Pharmacia). The column was washed with 2 ml of buffer H100, protein was eluted with a 20 ml of 100–600 mM NaCl linear gradient in buffer H (20 mM triethanolamine [pH 7.5], 1 mM $MgCl_2$, 5 mM β-mercaptoethanol), and 0.5 ml fractions were collected. Cdc48p-containing fractions were pooled and applied to a 125 ml Sephacryl S6 gel filtration column (Pharmacia) equilibrated in B88, 100 μM ATP, 5 mM β-mercaptoethanol. Fractions of 2 ml were collected, and those containing Cdc48p were pooled and concentrated at least 10-fold using Centricon 30 (Amicon) membranes.

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