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# Subcellular Localization of Cdc42p, a *Saccharomyces cerevisiae* GTP-binding Protein Involved in the Control of Cell Polarity

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The *Saccharomyces cerevisiae* Cdc42 protein, a member of the Ras superfamily of low-molecular-weight GTP-binding proteins, is involved in the control of cell polarity during the yeast cell cycle. This protein has a consensus sequence (CAAX) for geranylgeranyl modification and is likely to be associated, at least in part, with cell membranes. Using cell fractionation and immunolocalization techniques, we have investigated the subcellular localization of Cdc42p. Cdc42p was found in both soluble and particulate pools, and neither its abundance nor its distribution varied through the cell cycle. The particulate form of Cdc42p could be solubilized with detergents but not with NaCl or urea, suggesting that it is tightly associated with membranes. An increase in soluble Cdc42p was observed in a geranylgeranyltransferase mutant strain (*cdc43-2*$^{ts}$) grown at the restrictive temperature. In addition, Cdc42p from a *cdc42*$^{C188S}$ mutant strain (that has an alteration at the prenylation consensus site) was almost exclusively in the soluble fraction, suggesting that membrane localization is dependent on geranylgeranyl modification at Cys-188. Immunofluorescence and immunoelectron microscopy experiments demonstrated that Cdc42p localizes to the plasma membrane in the vicinity of secretory vesicles that were found at the site of bud emergence, at the tips and sides of enlarging buds, and within mating projections (shmoo tips) in $\alpha$-factor-arrested cells. These results indicate that Cdc42p is localized to the bud site early in the cell cycle and suggest that this localization is critical for the selection of the proper site for bud emergence and for polarized cell growth.

## INTRODUCTION

The *Saccharomyces cerevisiae* Cdc42 protein is a member of the Ras superfamily of low-molecular-weight GTP-binding proteins (Hall, 1990). It has been implicated in the control of morphogenetic events during the cell cycle, specifically in the generation of cellular polarity and the development of normal cell shape (Adams *et al.*, 1990; Johnson and Pringle, 1990; Ziman *et al.*, 1991; Johnson, 1993). Cellular polarity is manifested in at least two ways during the *S. cerevisiae* cell cycle: 1) selection of a nonrandom bud site at one of the two cell poles and 2) localized growth at the bud site resulting in the appearance and selective growth of the bud (Drubin, 1991). Analyses of the morphological phenotypes of temperature-sensitive and dominant-lethal *cdc42* alleles indicated that Cdc42p participates in the selection of the bud site and may play a role in polarized growth (Adams *et al.*, 1990; Ziman *et al.*, 1991; Johnson, 1993). Functional homologues of *CDC42* have been isolated from human cells (Munemitsu *et al.*, 1990; Shinjo *et al.*, 1990), *Schizosaccharomyces pombe* (Miller and Johnson, 1994), and *Caenorhabditis elegans* (Chen *et al.*, 1993), suggesting that Cdc42p is a fundamental component of the molecular machinery controlling cellular morphogenesis in all eukaryotes.

The C-termini of the *S. cerevisiae*, *S. pombe*, *C. elegans*, and human Cdc42 proteins consist of the sequence Cys-Xaa-Xaa-Leu (where Xaa is any amino acid); these termini are modified by the addition of a $C_{20}$ geranylgeranyl isoprenoid to the Cys residue (Maltese and Sher-

idan, 1990; Finegold *et al.*, 1991; Yamane *et al.*, 1991). The geranylgeranyl group is thought to anchor the protein to a cellular membrane (Maltese, 1990). In *S. cerevisiae*, geranylgeranylation depends on the Cdc43 protein (Johnson *et al.*, 1991; Finegold *et al.*, 1991; Ohya *et al.*, 1991), which is the β-subunit of the yeast type I protein geranylgeranyltransferase (Mayer *et al.*, 1992).

Several lines of evidence suggest that geranylgeranylation of Cdc42p (and, by inference, membrane association of the protein) is critical to its function. First, a Cys to Ser mutation at position 188 results in a nonfunctional protein (Ziman *et al.*, 1991). Second, the same C188S mutation can suppress the lethality associated with the otherwise dominant-lethal alleles $cdc42^{G12V}$, $cdc42^{Q61L}$, and $cdc42^{D118A}$ (Ziman *et al.*, 1991). Third, overproduction of the $Cdc42^{C188S}$ protein does not lead to abnormal positioning of budding sites, a phenotype characteristic of overproduction of the wild-type protein (Johnson and Pringle, 1990; Ziman *et al.*, 1991). This last result is notable in that it suggests that Cdc42p is unlike the Ras proteins in that delocalization because of a lack of isoprenylation cannot be reversed by overproduction (Deschenes and Broach, 1987).

The involvement of Cdc42p in the control of the bud emergence process suggests that it might localize to the site of bud emergence and to sites of new cellular growth at the tip of the emerging bud. We provide direct evidence for such localization using Cdc42p-specific antibodies in immunofluorescence and immunoelectron microscopy experiments. In addition, we used cell fractionation techniques to show that the total cellular complement of Cdc42p distributes to both soluble and particulate pools and that this distribution does not vary throughout the cell cycle. The particulate form of Cdc42p is only solubilized by membrane-disrupting reagents, suggesting that the particulate fraction of Cdc42p is tightly associated with a cellular membrane. Finally, we have found evidence supporting the idea that geranylgeranylation is essential for proper membrane localization.

## MATERIALS AND METHODS

### *Reagents, Media, and Strains*

Protein determinations were performed using the Bio-Rad protein assay kit (Richmond, CA), and immunoblots were developed using the Enhanced Chemiluminescence (ECL) system (Amersham, Arlington Heights, IL). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, protease inhibitors (phenylmethylsulfonyl fluoride [PMSF], N-tosyl-L-phenylalanine chloromethyl ketone, aprotinin, leupeptin, and pepstatin), glass beads (425–600 μm), and α-factor were obtained from Sigma Chemical (St. Louis, MO). AffiniPure goat anti-rabbit IgG and rabbit anti-goat IgG were obtained from Jackson Immuno Research Laboratories (West Grove, PA). Cdc42p-specific antibodies were isolated and purified as previously described (Ziman *et al.*, 1991). Yeast actin-specific antibodies were provided by Sue Lillie (University of Michigan). Affinity-purified anti-Pma1p antibodies were provided by Carolyn Slayman (Yale University). All other chemicals were obtained from standard commercial manufacturers.

Conditions for the growth and maintenance of bacterial and yeast strains have been described (Sherman *et al.*, 1986; Sambrook *et al.*, 1989). The *S. cerevisiae* strains used were X2180-1A, *MAT*a (provided by J. Kurjan, University of Vermont); DC5, *MAT*a *his3 leu2 gal2* (Broach *et al.*, 1979); WTCB1, *MAT*a *ade2-1 his3-11,5 leu2-3, 112 trpl-1 ura3-1 can1-100 bar1::HIS3* (provided by T. Chen and J. Kurjan, University of Vermont); RAK21, *MAT*a *ade2-1 his3-11,5 leu2-3,112 trp1-1 ura3-1 can1-100 bar1::HIS3* (provided by R. Akada, University of Vermont); Y147, *MAT*a *cdc24-4 his3 leu2 ura3* (Bender and Pringle, 1991); DJTD2-16A, *MAT*a *cdc42-1 his4 leu2 trp1 ura3* (Johnson and Pringle, 1990); and DJD3-11A, *MAT*a *cdc43-2 leu2 trp1 GAL2*.

### *Cell Fractionation and Synchronization Experiments*

Cell fractionation experiments were performed using techniques described by Goud *et al.* 1988. Briefly, cells were grown at 30°C, 50 $OD_{600}$ units (~$1 \times 10^9$ cells) were collected, washed with water, resuspended in 0.5 ml of lysis buffer (0.8 M sorbitol, 1 mM EDTA, 10 mM 3-(*N*-morpholino) propanesulfonic acid pH 7.0) with protease inhibitors (0.5 mM PMSF, 1:1000 dilutions of 1 mg/ml stock of aprotinin in water, 1 mg/ml stock of N-tosyl-L-phenylalanine chloromethyl ketone in 95% ethanol, 1 mg/ml stock of leupeptin in water, and 1 mg/ml stock of pepstatin in methanol), and lysed on ice by vortexing with 400–500 μm acid-washed glass beads. Greater than 90% lysis was verified by light microscopy. Cells lysates were spun at 500 × *g* for 4 min at 4°C, and the pellets were resuspended in the same volume of lysis buffer as the supernatants. The 500 × *g*-supernatants were then spun at 10 000 × *g* for 10 min at 4°C, and the pellets were resuspended in the same volume of lysis buffer. Finally, the 10 000 × *g* supernatants were spun at 100 000 × *g* for 1 h at 4°C, and the pellets were resuspended in the same volume of lysis buffer. To assess the relative amount of Cdc42p in each fraction, equal volumes of each fraction were loaded onto a sodium dodecyl sulfate (SDS)-15% polyacrylamide gel (or 7.5% gel for Pma1p) for immunoblot analysis (see below).

In synchronization experiments, RAK21 cells were grown at 30°C to an $OD_{600}$ of ~0.6 and then incubated in the presence of 20 nM α-factor until cell cycle arrest was observed as defined by >90% unbudded cells (~90 min). After washing cells twice in cold 1% peptone, 1% succinic acid, 0.7% yeast nitrogen base, 0.5% yeast extract, 0.6% NaOH (YM-1), they were resuspended in prewarmed YM-1 with 2% glucose. The cells were incubated at 30°C, and 10 OD units were collected at 20-min intervals and washed once with water. The pellets were snap frozen in a dry ice-ethanol bath and stored at −70°C. At each time point, cell samples were sonicated (Branson Ultrasonics, Danbury, CT) and counted microscopically to determine the percentage of budded cells. For the fractionation of synchronized RAK21 cells, cell lysates were prepared as above. Lysates were spun at 500 × *g* for 4 min at 4°C to remove cell debris. Protein concentrations of the 500 × *g* supernatants were normalized, and equal amounts of protein extract were spun at 10 000 × *g* for 10 min at 4°C. The pellets were resuspended in the same volume as the supernatants. Equal volumes of each fraction were loaded onto a SDS-15% polyacrylamide gel for immunoblot analysis (see below).

For the fractionation and detection of Cdc42 mutant proteins, cells containing GAL promoter-driven *cdc42* mutant genes on plasmids (Ziman *et al.*, 1991) were grown under selective conditions in 2% raffinose to midlog phase; then 2% galactose was added to the media, and the cells were allowed to grow for 15 h before harvesting and lysis. For ts mutants, cells were grown at 23°C to midlog phase and then shifted to 37°C for the indicated times before harvesting and lysis.

### *Immunoblot Analyses*

Protein samples were diluted 1:1 in SDS-lysis buffer (Laemmli, 1970) containing 40% β-mercaptoethanol, heated at 100°C for 5 min, and separated on a SDS-polyacrylamide gel, and protein was transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH). Affinity-

purified anti-Cdc42p was used at 1:1000, and HRP-conjugated goat anti-rabbit IgG secondary antibody was used at 1:2000. Affinity-purified anti-actin antibodies were used at 1:9000. Affinity-purified anti-Pma1p antibodies were used at 1:1000. Antibody-antigen complexes were detected with the ECL system (Amersham).

### *Immunofluorescence and Immunoelectron Microscopy*

Basic procedures for immunofluorescence microscopy using formaldehyde-fixed cells have been previously described (Pringle *et al.*, 1989). Briefly, WTCB1 cells were grown to midlog phase in YM-1 plus 2% glucose, fixed with 3.6% formaldehyde in 1 M sorbitol, and spheroplasted using 1 mg/ml lyticase (Sigma). After fixation of spheroplasted cells to slides coated with polylysine, cells were incubated with 0.2% SDS for 5 min, followed by five washes with Buffer A (Pringle *et al.*, 1989; Lillie, personal communication). Cells were then incubated in Buffer A for 15 min before addition of primary antibodies. An antibody sandwich technique was then used; incubation with affinity-purified anti-Cdc42p (1:100) was followed by AffiniPure goat anti-rabbit (1:1000), rabbit anti-goat (1:1000), and FITC-labeled goat anti-rabbit (1:80) antibodies. Photomicrographs were taken using an Olympus BH-2 epifluorescence microscope (Lake Success, NY) with Hoffman Modulation Contrast optics (Hudson, MA).

Wild-type yeast cell sections used in the immunoelectron microscopy experiments were prepared and labeled as described in Preuss *et al.* (1992) and Mulholland *et al.* (unpublished data). Cells with an $\alpha$-factor-induced mating projection were prepared by adding $\alpha$-factor (Sigma) at 2.38 $\mu$M to a midlog culture ($1.87 \times 10^6$ cells/ml) of *MAT*a cells (DBY1034) (Sprague, 1991). When ~30% of the cell culture had formed mating projections as determined by microscopic examination (2.75 h after adding $\alpha$-factor), the culture was harvested and processed for immunoelectron microscopy as previously described (Mulholland *et al.*, unpublished data). Affinity purified anti-Cdc42p antibodies were used at a 1:10 dilution.

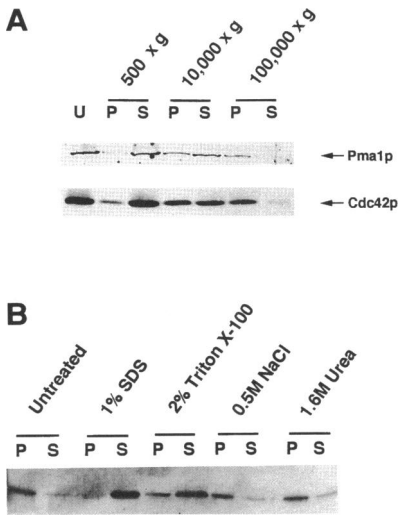


**Figure 1.** Immunoblot analysis of fractionated Cdc42p. (A) Protein extracts of X2180-1A cells were spun at 500 $\times$ *g* to produce pellet (P) and supernatant (S) fractions; the supernatant fraction was subsequently spun at 10 000 $\times$ *g* and 100 000 $\times$ *g*. Identical samples were run on either a 7.5% polyacrylamide gel (for Pma1p) or a 15% gel (for Cdc42p). U, unfractionated lysate. This result is representative of at least three independent experiments. (B) Solubilization of Cdc42p from particulate fraction. The 10 000 $\times$ *g* pellet was resuspended in lysis buffer, and aliquots were incubated with the indicated reagent for 30 min at 4°C. The samples were then spun at 10 000 $\times$ *g* to produce pellet (P) and supernatant (S) fractions. Equal volumes of protein extracts were run on a SDS-15% polyacrylamide gel and subjected to immunoblot analysis. This result is representative of two independent experiments.

## RESULTS

### *Subcellular Localization of Cdc42p by Cell Fractionation*

Cdc42p contains the sequence Cys-Thr-Ile-Leu at its C-terminus. The Cys residue (C188) is isoprenylated by the addition of a $C_{20}$ geranylgeranyl group (Finegold *et al.*, 1991). This modification is believed to play a crucial role in the anchoring of the protein to a subcellular membrane. To determine whether Cdc42p is associated with a subcellular membrane, cell fractionation of asynchronously growing cell cultures was performed. After centrifugation at 500 $\times$ *g*, Cdc42p was found in both soluble (~80%) and particulate (~20%) fractions (Figure 1A). When the 500 $\times$ *g* supernatant was fractionated at 10 000 $\times$ *g*, (conditions previously shown to pellet plasma membrane components as well as other dense materials; Goud *et al.*, 1988), Cdc42p was again found in both soluble and particulate fractions, although this time the amounts in each fraction were nearly the same. When the 10 000 $\times$ *g* supernatant was fractionated at 100 000 $\times$ *g*, which pellets 80–100 nm secretory vesicles (Goud *et al.*, 1988), once again the protein was found in both fractions; similar results were obtained previously with the human Cdc42Hs/G25K protein (Maltese and Sheridan, 1990). The yeast plasma membrane ATPase, Pma1p, exhibits a similar fractionation profile (Figure 1A), suggesting that Cdc42p may localize to the plasma membrane. These results are consistent with the partitioning of Cdc42p into at least two pools: soluble (supernatant at 100 000 $\times$ *g*) and particulate.

To determine the nature of the interaction between Cdc42p and the particulate (presumably membranous) structure(s), we treated aliquots of the 10 000 $\times$ *g* pellet with reagents that are known to disrupt membranes or release peripheral membrane proteins (Figure 1B). Treatment with 1% SDS completely solubilized the protein and treatment with 2% Triton X-100 solubilized most of the protein. Treatment with 0.5 M NaCl or 1.6 M urea had little effect. These results are consistent with tight association of Cdc42p with a cellular membrane(s).

To determine whether partitioning of Cdc42p between particulate and soluble fractions varies during the cell cycle, haploid *MAT*a cells were synchronized by arrest with $\alpha$-factor and fractionated at 10 000 $\times$ *g* at various times after release from cell-cycle arrest (Figure 2). Arrest by $\alpha$-factor resulted in >90% unbudded cells, and resumption of cell growth in a synchronous fashion was observed (Figure 2A). We found that the total level of Cdc42p, measured relative to the level of actin, did not vary significantly during the cell cycle (Figure 2B). Likewise, the partitioning of Cdc42p did

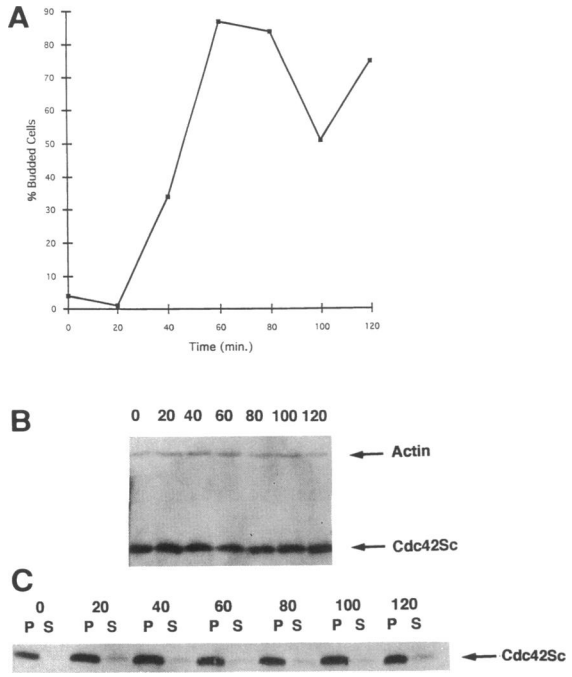


**Figure 2.** Immunoblot analysis of fractionated cell extracts from a synchronously growing culture. (A) RAK21 cells were synchronized and the percentage of budded cells was determined at 20-min intervals. (B) Unfractionated cell lysates from the same synchronization experiment were subjected to immunoblot analysis using affinity-purified anti-Cdc42p antibodies and anti-yeast actin antibodies. (C) Subcellular fractions from the same synchronization experiment were generated by differential centrifugation. P, 10 000 × *g* pellet; S, 10 000 × *g* supernatant. The results are representative of three different experiments.

not seem to vary (Figure 2C); however, in comparison to fractionation of asynchronous cells, we observed more Cdc42 protein in the particulate fraction relative to the soluble fraction, which may be because of effects of $\alpha$-factor arrest or lysate concentration. These results suggest that there is no cell-cycle specific regulation of the synthesis or localization of Cdc42 protein.

### *Fractionation of Cdc42p in Mutant Strains*

Mutation of the C-terminal Cys residue of Cdc42p presumably interferes with prenylation, resulting in a protein that no longer associates with membranes (Ziman *et al.*, 1991). To confirm this interpretation, we examined the partitioning of the C188S mutant protein (Figure 3A). Because this mutation results in a nonfunctional protein, the mutant allele was introduced into a wild-type strain on a high-copy number plasmid, and expression of the mutant protein was controlled with the *GAL* promoter. To ensure detection of only the overexpressed and not the endogenous Cdc42p, cells without plasmids were examined in parallel. Overexpression of wild-type Cdc42p resulted in a significant portion of the protein in the 10 000 × *g* pellet. In contrast, the C188S mutant protein was found entirely in the soluble fraction. These results confirm that alteration of the site of geranylgeranyl modification results in mislocalization of Cdc42p and lend support to the model that the particulate Cdc42p observed in wild-type strains is membrane associated.

In addition to *CDC42*, other genes have been implicated in control of cell polarity in *S. cerevisiae*, including *CDC43*, which encodes the $\beta$-subunit of the yeast type I protein geranylgeranyltransferase (Mayer *et al.*, 1992), and *CDC24*, which potentially catalyzes the exchange of Cdc42p-bound nucleotides (Hart *et al.*, 1991). The partitioning of Cdc42p was examined in strains with temperature-sensitive mutations in each of these genes (*cdc43-2* and *cdc24-4*), and in a strain with a *cdc42-1*$^{ts}$ mutation (Figure 3B). Neither the *cdc42-1* nor the *cdc24-4* mutations were observed to affect the partitioning of Cdc42p. However, as the *cdc43-2* strain was held at the restrictive temperature, the proportion of Cdc42p found in the soluble fraction increased, once again providing evidence that prenylation of Cdc42p is necessary for its association with the membranous pellet. Interestingly, the *cdc42-1* mutation results in dramatically reduced levels of total protein (Ziman *et al.*, 1991) and in two distinct Cdc42p bands (Figure 3B). Although the nature of these bands is unclear, they may reflect different states of protein modification.

### *Immunolocalization of Cdc42 Protein*

The preceding results indicate that a fraction of the cellular complement of Cdc42p is associated with mem-

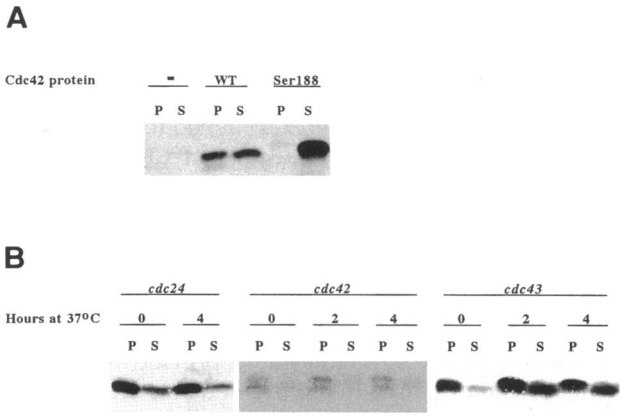


**Figure 3.** Fractionation of Cdc42p in mutant strains. (A) DC5 cells were transformed with p*GAL* plasmids containing *cdc42* mutant genes, and mutant proteins were expressed by growth on 2% galactose-containing medium (Ziman *et al.*, 1991). Cell extracts were prepared as described in Figure 2. −, cells without a plasmid; WT, cells overexpressing wild-type Cdc42p; Ser188, cells overexpressing the C188S mutation. The exposure times of these blots were adjusted to allow the detection of overexpressed Cdc42p but not endogenous Cdc42p. (B) mutant strains were grown to midlog phase at 23°C and shifted to 37°C; samples were collected at 2-h intervals. For the *cdc42-1* strain, more protein was loaded per lane because of lower levels of Cdc42p.

branes. We attempted to determine the location of this membrane-associated Cdc42p by immunofluorescence (Figure 4) and immunoelectron (Figures 5–7) microscopy using affinity-purified Cdc42p-specific antibodies. Using immunofluorescence microscopy, we found that Cdc42p was localized to a patch at the periphery of unbudded cells (Figure 4A), possibly marking the site at which a bud will form. In budded cells, the protein was found at the tips and sides of the enlarging buds, and, in *MAT*a cells arrested by α-factor, it was found at the tips of mating projections (Figure 4B).

In these experiments, the appearance of a Cdc42p-specific immunofluorescence signal required the addition of 0.2% SDS to the spheroplasted cells before the addition of primary antibodies. This presumably disrupts an interaction between Cdc42p and another cellular component, possibly the plasma membrane, but also tends to solubilize the cells, leading to a somewhat fuzzy appearance of immunofluorescence images. No localized immunofluorescence signal was observed with preimmune sera used at 1:100 or 1:800 dilutions, with cells that were not treated with 0.2% SDS, or with primary antibody that was incubated with the peptide immunogen. The Cdc42p-specific antibodies used in these experiments are directed against a Cdc42-specific C-terminal peptide (Ziman *et al.*, 1991) that is within the nonconserved hypervariable domain (unique to Cdc42p) and is predicted to be in close proximity to the plasma membrane.

Our immunofluorescence results clearly indicate that Cdc42p is concentrated near sites of cell growth. To extend these observations, we examined localization of Cdc42p using immunoelectron microscopy. As expected, we found scattered localization of Cdc42p throughout the cytoplasm. In addition, we found that Cdc42p was highly concentrated near the plasma membrane at the tips and sides of growing buds (Figures 5 and 6) and at the tips of mating projections (Figure 7). Particularly notable is the observation that the labeled region of the plasma membrane is in the immediate vicinity of secretory vesicles that accumulate, even in unbudded cells, at the point of bud emergence and maximum membrane growth. This association with the plasma membrane continues throughout bud growth, suggesting a role for Cdc42p at the site of fusion between vesicles and the plasma membrane. When thin sections were examined at high magnification, Cdc42p seemed to be localized to invaginations of the plasma membrane that are found in small- and medium-sized buds (Figures 5 and 6, inset). Although the function of these structures is unknown, they are often associated with actin cortical patches (Mulholland *et al.*, unpublished data). In some cells, Cdc42p was observed at the septum, but this observation was variable. The observed plasma membrane localization of Cdc42p, both at the presumed site of bud emergence and the tips of growing buds, is entirely consistent with its role in regulation of cell polarity.

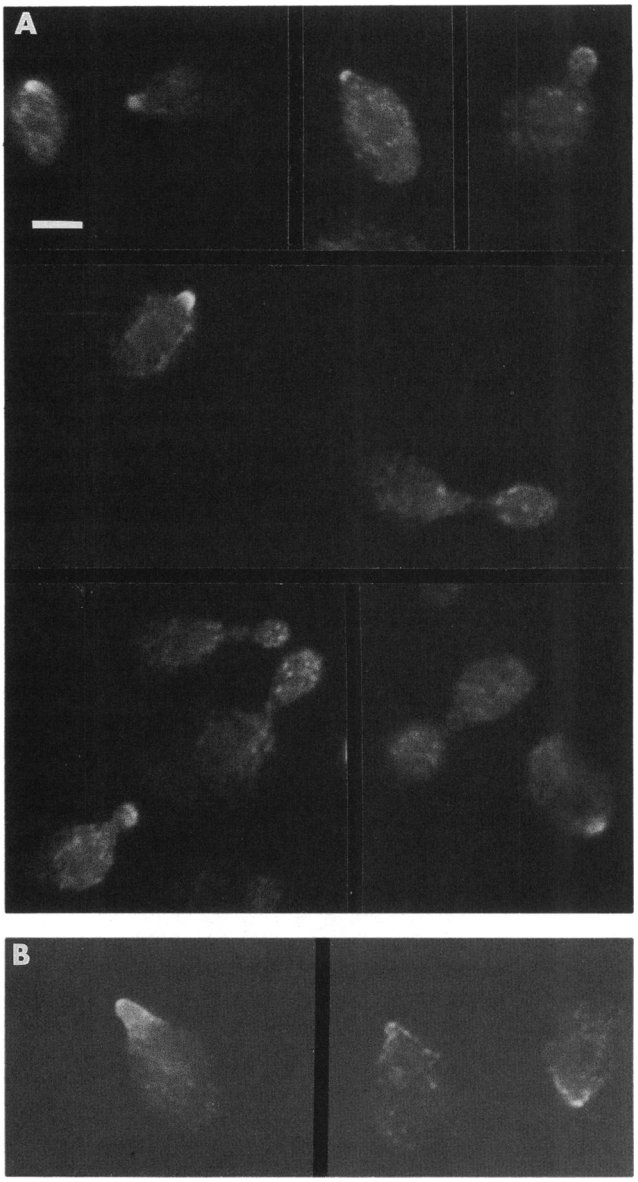


**Figure 4.** Immunofluorescent localization of Cdc42p in budding and α-factor-arrested cells. (A) Representative haploid cells in different stages of the cell cycle, as defined by bud size, are shown. Cdc42p localizes to the presumptive site of bud emergence as well as to the tips and sides of growing buds. (B) *MAT*a cells arrested with α-factor are shown. Cdc42p localizes to the ends and sides of shmoo tips. Bar, 5 μm.

## DISCUSSION

Cdc42p belongs to the Ras superfamily of low-molecular-weight GTPases that act as molecular switches to control a variety of cellular processes (Hall, 1990; Bourne

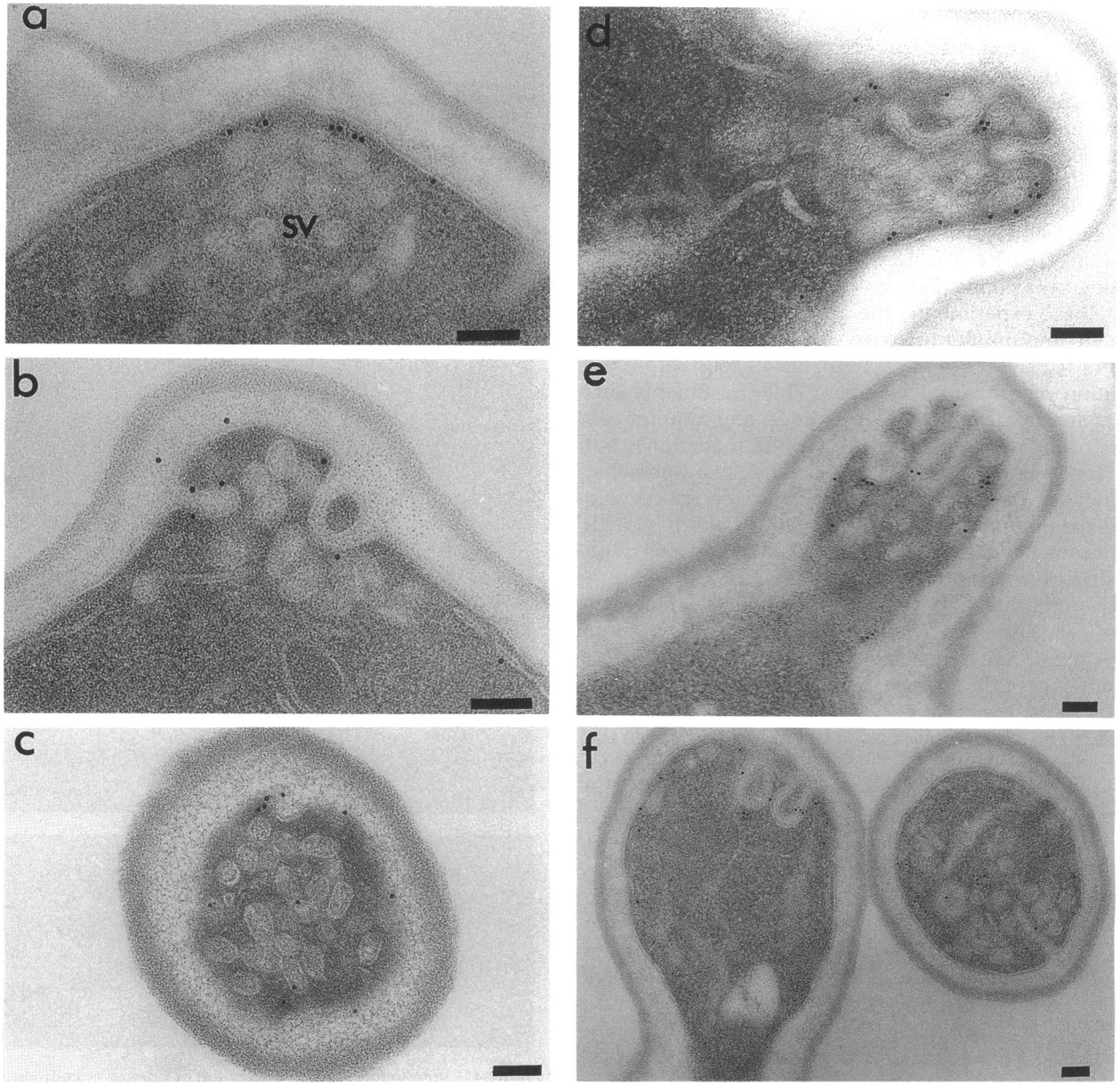


**Figure 5.** Immunogold localization of Cdc42p. Representative haploid cells at several stages of the cell cycle, as defined by bud size and organelle content, are shown. Cdc42p is localized predominately to the plasma membrane of the tip and sides of the growing bud, and this localization is strongest at apparent invaginations of the plasma membrane. Occassionally, secretory vesicles within the small bud also appear to be labeled (c). Small buds, a and b; cross-section through tip of small bud, c; small to medium-sized bud, d; medium to large buds, e and f. SV, secretory vesicles. Bars, 0.1 $\mu$m.

*et al.*, 1991). These proteins are generally active in the GTP-bound state and inactive in the GDP-bound state. Though the nucleotide-bound state of these proteins is critical to their functions, the proper membrane localization of these proteins, which often depends on the attachment of a farnesyl or geranylgeranyl group to their C-termini followed by proteolysis and carboxymethylation, also plays a vital role (Maltese, 1990). Members of this superfamily bind to the plasma membrane and transmit signals from the extracellular environment or bind to intracellular membranes, such as the Golgi membrane or 80–100 nm secretory vesicles, and function in the movement of membrane-bound organelles within the cell (Bourne *et al.*, 1991).

Bud emergence occurs at a specific time during the yeast cell cycle and at a specific location within the plasma membrane. Analysis of the *cdc42*$^{C188S}$ mutant phenotype (Ziman *et al.*, 1991) previously suggested that

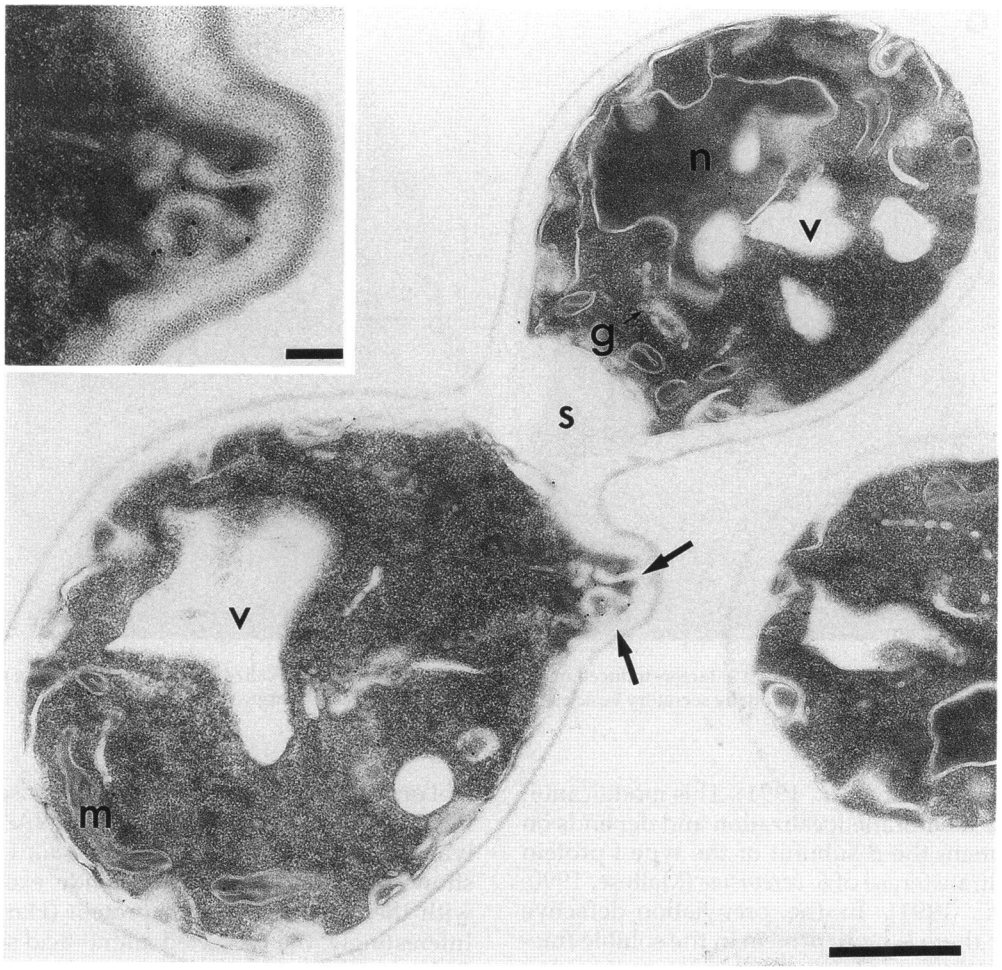


**Figure 6.** Immunogold localization of Cdc42p at the site of bud emergence. A representative mother-daughter pair that has undergone septation but not cell separation is shown. A new bud has formed on the mother cell, and Cdc42p is primarily localized to this emerging bud (arrows). A high magnification view of the emerging bud (inset) shows Cdc42p localizes to apparent invaginations of the plasma membrane. g, Golgi; m, mitochondrion; n, nucleus; s, septum; v, vacuole. Bar, 0.5 $\mu$m; inset bar, 0.1 $\mu$m.

proper subcellular localization of Cdc42p during the cell cycle is vital to its function. The results described in this report strongly support this model; Cdc42p is selectively associated with membranes in a position entirely consistent with a role in bud emergence and polarized growth.

### *Subcellular Localization of Cdc42p*

Cdc42p is found in both soluble and particulate fractions, as is the human Cdc42Hs/G25K protein (Maltese and Sheridan, 1990). It can only be solubilized from the particulate fraction by membrane-disrupting reagents, suggesting that it is tightly associated with a cellular membrane(s). The immunofluorescence and immunoelectron microscopy studies support this model and show that most of the membrane-associated Cdc42p is near the plasma membrane at the sites of bud emergence and maximal bud growth. Its localization at the plasma membrane is typically accompanied by the accumulation of secretory vesicles.

We consistently found that some of the cellular complement of Cdc42p is soluble and that some of the Cdc42p-specific antibodies are localized to the cytoplasm. Although the nature of this soluble material is unclear, it might represent newly synthesized protein. Because bud emergence occurs at a specific time within the cell cycle, it is significant that the distribution of Cdc42p between soluble and particulate fractions does not dramatically vary during the cell cycle. Regulation of the activity of Cdc42p through the cell cycle could be accomplished by controlling its binding to GTP, rather than by regulating its localization to membranes.

The altered fractionation profile of the $Cdc42^{C188S}$ protein probably results from the absence of geranylgeranyl modification at the C-terminal Cys residue (Ya-

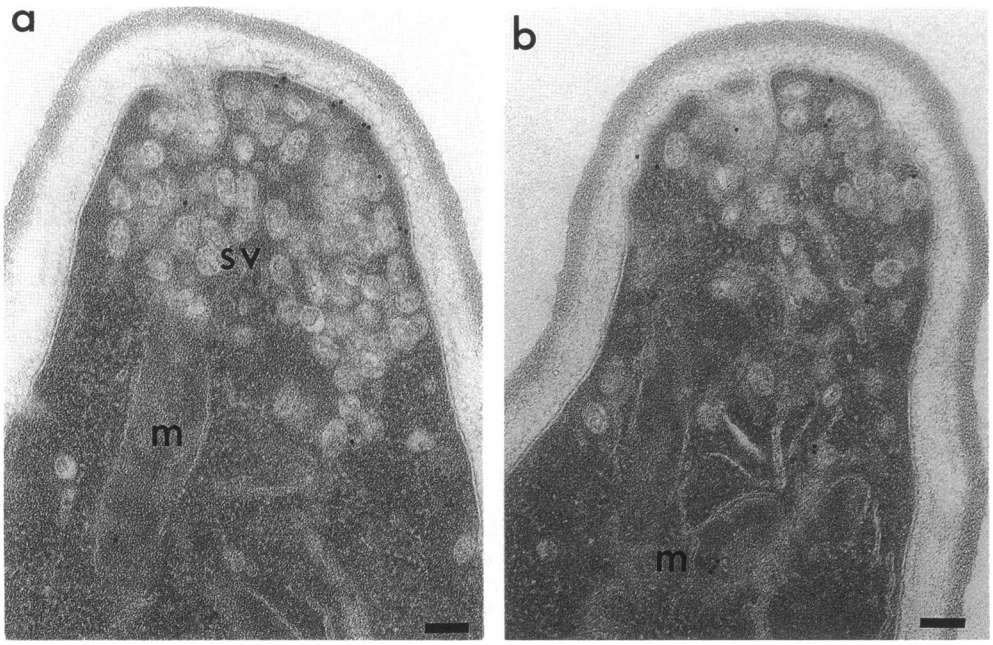


**Figure 7.** Immunolocalization of Cdc42p in $\alpha$-factor–induced mating projections. Cdc42p is localized predominately to the plasma membrane at the tip of the mating projection. Occassionally secretory vesicles that are near the cell surface appear to localize Cdc42p (b). m, mitochondrion; sv, secretory vesicles. Bar, 0.1 $\mu$m.

mane *et al.*, 1991; Ziman *et al.*, 1991). This modification is necessary for membrane localization and depends on the Cdc43 protein, the $\beta$-subunit of the type I protein geranylgeranyltransferase of *S. cerevisiae* (Maltese, 1990; Finegold *et al.*, 1991). In the prenylation-defective *cdc43*$^{ts}$ mutant, there is more protein in the soluble fraction than in *cdc24*$^{ts}$ or *cdc42*$^{ts}$ strains, suggesting that prenylation is necessary for membrane association of Cdc42. The gross fractionation pattern of Cdc42p is not altered in *cdc42*$^{ts}$ or *cdc24*$^{ts}$ strains.

The mechanism that controls the subcellular localization of Cdc42p has not been determined. Potentially, it could be regulated by protein-protein interactions between Cdc42p and other components of the pathway, such as proteins that determine the guanine nucleotide bound state of the protein. The nature of the bound guanine nucleotide has been implicated in the membrane localization of other GTP-binding proteins. In the case of *smg* p25A and *rhoA*, the GDP-bound form of the protein can associate with a specific guanine dissociation inhibitor or stimulator, leading to dissociation from membranes (Araki *et al.*, 1990; Hori *et al.*, 1991). Therefore, membrane localization may depend on which nucleotide is bound to Cdc42p and on the association of the nucleotide-bound Cdc42p with a putative guanine nucleotide exchange factor.

### *Interaction of Cdc42p with Other Factors Involved in Bud Emergence*

Cdc42p-specific exchange factors have not been biochemically identified in yeast to date, but the human Dbl oncoprotein, which was originally isolated through DNA transfection studies using DNA from a B-cell lymphoma (Eva *et al.*, 1988; Ron *et al.*, 1991), has been shown to have guanine-nucleotide exchange activity with the human Cdc42Hs protein (Hart *et al.*, 1991). Interestingly, the predicted amino-acid sequence of the Dbl oncoprotein displays limited sequence similarity to the yeast Cdc24 protein (Miyamoto *et al.*, 1987, 1991; Ron *et al.*, 1991), suggesting that Cdc24p may play a similar role with Cdc42p. Several genetic experiments suggest that Cdc24p and Cdc42p interact in vivo. First, *cdc24*$^{ts}$ mutants (Sloat and Pringle, 1978; Sloat *et al.*, 1981) have identical phenotypes to the *cdc42-1*$^{ts}$ mutant (Adams *et al.*, 1990). Second, *cdc42*$^{ts}$ *cdc24*$^{ts}$ double mutants are synthetic lethal mutants (Ziman and Johnson, 1994). Third, a *cdc24*$^{ts}$ mutation can be suppressed by overproduction of Cdc42p (Bender and Pringle, 1989; Munemitsu *et al.*, 1990), suggesting either that Cdc24p and Cdc42p interact or that Cdc42p acts downstream of Cdc24p. Finally, overproduction of Cdc24p can suppress *cdc42*$^{D118A}$, a dominant-negative mutation that results in a loss of cell polarity (Ziman *et al.*, 1991; Ziman and Johnson, 1994). The fractionation profile of Cdc42p does not seem to vary in *cdc24-4*$^{ts}$ arrested cells (Figure 3), suggesting that Cdc24p does not play a major role in the localization of Cdc42 protein. However, we cannot rule out subtle alterations in the cellular localization of Cdc42p in these cells. If Cdc24p interacts with Cdc42p as a guanine nucleotide exchange factor, it may be differentially localized to the plasma membrane

leading to differential activation of Cdc42p; a hypothesis we will test in the future.

### *Other Proteins that Localize to the Site of Bud Emergence*

Our results from immunofluorescence and immunoelectron microscopy experiments indicate that Cdc42p is localized to the plasma membrane at the presumed site of bud emergence before a nascent bud can be seen in the light microscope, as well as to the tips and sides of growing buds. The latter observation was an unexpected result because genetic experiments had only implicated Cdc42p in the organization of the bud site preceding bud emergence and not in the control of polarized cell growth during the cell cycle (Adams *et al.*, 1990). Mutational analysis of *S. pombe cdc42* mutants, however, has also implicated Cdc42p in the control of polarized growth (Miller and Johnson, 1994), suggesting that Cdc42p may play a conserved role in controlling polarized growth in all eukaryotes. Even though Cdc42p is localized to sites of polarized growth throughout the cell cycle, it is not known if it is in an active GTP-bound state throughout the cycle.

Several other yeast proteins have also been localized to the site of bud emergence and regions of polarized growth. Actin is found in cortical spots at the sites of new cellular growth as well as in cables that are directed into the enlarging bud; however, its overall staining pattern is significantly different from Cdc42p (Adams and Pringle, 1984; Novick and Botstein, 1985). The staining pattern of Cdc42p is similar to those of Spa2p (Snyder, 1989) and calmodulin (Brockerhoff and Davis, 1992), two other yeast proteins that localize to the sites of new cell growth. Spa2p, actin, and calmodulin localize to the site of bud emergence before visualization of a nascent bud in the light microscope, but calmodulin appears at this site after the appearance of actin (Brockerhoff and Davis, 1992). Both calmodulin and actin localize to the region of cell septation at the end of the cell cycle, and, although some Cdc42p was occasionally found at the cell septum, its abundance was relatively less than in buds. These results suggest that Cdc42p may be required both at the time of bud emergence as well as during the growth of the bud and may play a role in directing new cellular constituents to their proper location within the enlarging bud. The determination of the temporal and spatial localization of Cdc42p relative to actin, Spa2p, and calmodulin through the cell cycle may help unravel the relationships between these proteins.

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