Subcellular Localization of a Protein Kinase Required for Cell Cycle Initiation in *Saccharomyces cerevisiae*: Evidence for an Association between the CDC28 Gene Product and the Insoluble Cytoplasmic Matrix

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Abstract. The product of the *Saccharomyces cerevisiae* gene CDC28, a protein kinase required for initiation of the cell division cycle, was localized within yeast cells. By using immunofluorescence methods, the CDC28 product was shown to be primarily cytoplasmic in distribution. The gene product was localized largely to the particulate fraction by differential centrifugation after mechanical disruption in aqueous buffers. The particulate association was not affected by the presence of nonionic detergent. To refine this localization further, a procedure was developed for the preparation of yeast cytoplasmic matrices which resemble the cytoskeletons of vertebrate cells on the basis of methodology, immunochemistry, and gross ultrastructure. A portion of the CDC28 product was found to be tightly associated with these detergent-insoluble cytoplasmic matrices by both immunofluorescence and immunoblotting procedures. Although, for technical reasons, precise quantitation was not possible, it is estimated that a minimum of 2–15% of the total CDC28 product pool is involved in the association with the insoluble matrix. Alcohol dehydrogenase, a soluble cytoplasmic protein, was found not to be associated with the cytoplasmic matrices at any detectable level, whereas, in contrast, ~10–40% of the total cellular actin, a bona fide cytoskeletal protein, was present in these structures. The proportion of CDC28 gene product associated with the particulate fraction, and perhaps the insoluble matrix, appears to be substantially decreased during the preparation of spheroplasts.

Temperature-sensitive mutants in the complementation group cdc28 are classical start mutants in that, at the permissive temperature, they show normal cell division behavior whereas, at elevated temperatures, they are incapable of initiating new cell cycles. Cells in the process of dividing progress through the cell cycle until they reach start (12, 30). The lesion appears to be cell cycle-specific inasmuch as macromolecular synthesis is unimpeded in arrested cells (16). The CDC28 gene was isolated from an *S. cerevisiae* recombinant plasmid library by transformational complementation of a cdc28 mutation (25). Sequence analysis of the cloned gene provided a predicted primary structure for the CDC28 product and revealed that the latter shared homology with a number of protein kinases (23). Using antisera prepared against the product of a chimeric gene containing part of the CDC28 coding region, we have demonstrated that the CDC28 product does in fact possess protein kinase activity (34). This finding suggests that this protein may participate in control of cell division as part of a signaling pathway involving protein phosphorylation events. At this time, the origins and ultimate targets of such a signal are unknown.

The purpose of the work reported here is to establish the
in intracellular location of the CDC28-encoded protein kinase in the hope of elucidating the molecular components and cellular organization of division-related signaling in yeast. First, we observed that the CDC28 gene product was localized primarily in the cytoplasm by immunofluorescence experiments and primarily to the particulate fraction by differential centrifugation followed by immunoblotting. Previous studies indicated that, in addition to cell cycle effects, cdc28 mutants showed aberrations in morphology and in organelar motility and transmission (9, 12, 30; our unpublished observations). These phenotypes are suggestive of functions associated with the cytoskeleton in higher cells (2, 13, 21, 38, 39, 42). We therefore sought to determine whether the CDC28 product was associated directly with the yeast cytoskeleton. Because the yeast cytoskeleton has not been extensively characterized, we proceeded by adapting methodology developed for cytoskeletal analysis in vertebrate cells (13) for use with S. cerevisiae. During the course of our studies we became aware of a similar approach being taken to isolate microtubule associated proteins from mitotic cells of S. cerevisiae (28). Although some similarities are clearly apparent, we cannot be certain that the structures we have prepared are precisely analogous to vertebrate cytoskeletons. We, therefore, refer to them as detergent-insoluble cytoplasmic matrices. The preparation of these structures and analysis of association of the CDC28 gene product with them is described.

**Materials and Methods**

**Strains and Media**

All strains used in this study were derivatives of BF264-15D (MATa, leu2, trpl, adet, his3) (34). A diploid derivative of BF264-15D, MMY13, and a diploid derivative of BF264-15D carrying the galactose-regulated allele of the CDC28 gene, MMY53 (described below), were constructed by rendering haploid cells homothallic by transient transformation with a plasmid containing the gene HO (15) and allowing the transformant cultures to diploidize. Individual cell clones were then screened for the ability to sporulate to confirm that diploidization had occurred.

Construction of a congeneric diploid strain, MMY53, where the CDC28 gene was placed under control of the regulatable GAL1 promoter, is described in Fig. 2 (a,b). The construction of the plasmid pFT1 was as follows. A BamHI restriction site was introduced by oligonucleotide-directed mutagenesis (44) 10 base pairs upstream from the translation initiation codon of the CDC28 gene on plasmid YRp7(CDC28&4) (33) to form plasmid YRp7(CDC28&4[BamHI]). A 4.4-kb XhoI-BglII fragment of this plasmid containing the CDC28 coding region and the sequence conferring autonomous replication in yeast (ARS) were removed, and the plasmid was reclosed by treatment with DNA polymerase followed by joining of the resulting blunt ends using DNA ligase. This plasmid (YRp7(CDC28&4[XhoI-

**Antibodies and Affinity Purification of CDC28 IgG**

Anti-yeast alcohol dehydrogenase (ADH) and preimmune serum from the same rabbit were a gift of Dr. Jill Ferguson (8). Anti-yeast actin antisera was a gift of Drs. Susan Brown (Research Institute of Scripps Clinic) and John Pringle (University of Michigan).

Preparation of anti-CDC28 product antisera (anti-CDC28) by immunizing rabbits using an Escherichia coli-β-galactosidase-CDC28 product hybrid protein isolated from E. coli has been described previously (31). Antibodies with specificity for this fusion protein were affinity purified. The fusion protein was gel purified as described elsewhere (31) by separating crude bacterial lysate on preparative sodium dodecyl sulfate (SDS)-polyacrylamide gels and excising the appropriate bands after brief staining with Coomassie Brilliant Blue followed by brief destaining with water. Whole gel slices were then sealed in dialysis tubing filled with Tris-glycine running buffer without SDS. A number of these sealed gel slices were then discarded and the eluted protein was dialyzed against 0.1 M ammonium bicarbonate overnight. The solution was concentrated using a concentration apparatus (Amicon Corp., Danvers, MA) and finally lyophilized. Approximately 400 μg of protein was dissolved in 2 ml of 0.1 M NaHCO3, pH 8.3, 0.5 M NaCl by boiling for 2 min followed by centrifugation to remove insoluble debris. This was then reacted with 1 g of cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) as has been described by March et al. (24), yielding a 3.5-ml bed volume column. The column was washed with 10 vol of each of the following solutions in succession: 4.5 M MgCl2, 1 M NaCl, 0.1 M acetic acid, pH 4.8; 1 M NaCl, 0.1 M NaHCO3, pH 7.6; phosphate-buffered saline (PBS), pH 7.6. 3 ml of crude serum was then circulated through the column at 23°C for 2 h. The column was then washed with 10 vol of the following solutions in succession: PBS, pH 7.6; 1 M NaCl, 0.1 M acetic acid, pH 4.8; 1 M NaCl, 0.1 M NaHCO3, pH 7.6; PBS, pH 7.6. Bound antibodies were eluted by applying 4.5 M MgCl2. When the high-salt front appeared, two column volumes were collected and dialyzed against 0.1 M NaCl overnight at 4°C. The affinity-purified fraction was then dialyzed against PBS and finally concentrated to ~1 ml using an Amicon concentration apparatus. This gave an IgG solution of ~100 μg/ml as determined by comparison to a dilution series of normal rabbit IgG on SDS-polyacrylamide gels. Normal rabbit IgG was used at this concentration for control experiments.

Anti-CDC28 carboxy-terminal antisera (anti-CDC2825-297) was directed against a synthetic octadecapeptide (provided by Dr. Ralph Arlinghaus, University of Texas, Houston) homologous to the predicted carboxy-terminal amino acid sequence of the CDC28 gene product (23). This peptide was conjugated to keyhole limpet hemocyanin by glutaraldehyde crosslinking. Briefly, 5 mg of keyhole limpet hemocyanin was mixed with 5 mg of peptide in 0.25 M K2HPO4 buffer, pH 7.2, to a total volume of 0.7 ml. 4 μl of 25% glutaraldehyde was added stepwise with shaking and the mixture was allowed to sit on ice for 30 min. The reaction mixture was then dialyzed overnight against 50 mM sodium phosphate buffer, pH 6.0. The peptide was then conjugated (the equimolar ratio of 0.5 mg of peptide) was solubilized by bringing to 1% SDS and heating to 85°C for 5 min and then diluted 10-fold in PBS. This solution was emulsified with an equal volume of either complete or incomplete Freund's adjuvant for the initial or subsequent injections, respectively, and then used to immunize rabbits. Affinity purification of the anti-CDC2825-297 IgG was accomplished by the method described above except that the affinity matrix consisted of the carboxy-terminal peptide conjugated to cyanogen bromide-activated Sepharose 4B.

Both of the affinity-purified antisera (anti-CDC28, anti-CDC2825-297) react with a polypeptide of 34 kD on immunoblots (unpublished results). The discrepancy between the published molecular mass of the in vitro translation product observed by immunoprecipitation with the anti-CDC28 antisera (31) and that described here can be attributed solely to differences in the molecular mass standards used in these studies. The anti-CDC2825-297 antisera is a higher tier antisera and gives a substantially stronger signal on immunoblots.

**Electrophoresis and Immunoblotting**

Samples for electrophoresis were prepared by suspending whole cells or cytoplasmic matrices in an equal volume of twice-concentrated gel sample.
buffer (20) and vortexing three times for 1 min with an equal volume of 0.45-mm glass beads (B. Braun Instruments, Burlingame, CA). The samples were then boiled for 3 min and the glass beads and cellular debris were removed by centrifugation. The proteins were resolved on SDS polyacrylamide gels (20) and either stained with Coomassie Brilliant Blue R or used for immunoblotting experiments. Immunoblotting experiments were performed as described (41). Nitrocellulose was blocked overnight with 5% nonfat dry milk (Carnation) and 0.45-mm glass beads (B. Braun Instruments, Burlingame, CA). The samples were then washed with PBS-BSA and incubated with the primary antibodies for 2 h at 23°C. The blots were then washed with PBS and incubated with the secondary antibody for 2 h at 23°C. Autoradiography was performed at -70°C with Kodak phosphatase-coupled goat anti-rabbit IgG (Promega Biotec, Madison, WI) and either stained with Coomassie Brilliant Blue R or used for densitometry using a ultrascann laser densitometer (LKB Instruments, Inc., Gaithersburg, MD).

Preparation of Cytoplasmic Matrices

Cells were grown to an approximate density of 2-4 x 10^7/ml and harvested by centrifugation. Cells were then resuspended in one-tenth the original culture volume of wall removal buffer: 0.2 M Tris-HCl, pH 9.4, 0.02 M EDTA, 1% 2-mercaptoethanol. After 10 min at 23°C, cells were collected by centrifugation and resuspended in an equivalent volume of YEPD medium stabilized with 1 M sorbitol and adjusted to 0.01 mg/ml chitinase (Sigma Chemical Co.), 0.02 mg/ml zymolyase (Kirk Brewery, Tokyo, Japan) and 1% glucose (Enzo Laboratories, New York). Incubation was at 30°C for 15-30 min. Wall removal was monitored as a function of susceptibility to lysis in 5% SDS. When cells became uniformly spheroplasted, they were collected by centrifugation at 23°C and washed once in stabilization buffer with 1 M sorbitol. Stabilization buffer was 0.15 M Pipes, pH 6.9, 0.1 mM EGTA, 0.5 mM MgCl_2, and 1 mM phenylmethylsulfonyl fluoride, containing pepstatin A and leupeptin at 1 µg/ml and aprotinin at 1:2,000 dilution. Preparation of cytoplasmic matrices was essentially as described by Heuser and Kirschner (13). Spheroplasts were resuspended in one tenth the original culture volume of stabilization buffer with 0.2% Triton X-100, pelleted immediately, and resuspended in the same buffer with 0.1 mg/ml bovine pancreatic DNase I for 60 min at 23°C. Cytoplasmic matrices were then washed once by resuspension in stabilization buffer with Triton X-100 followed by recentrifugation, and finally resuspended and washed twice in stabilization buffer without detergent.

Fluorescence Microscopy of Yeast Cells and Cytoplasmic Matrices

Approximately 10^7 log phase cells were collected by centrifugation and resuspended for 10 min in wall removal buffer (0.2 M Tris-HCl, pH 9.4, 0.02 M EDTA, 1% 2-mercaptoethanol), followed by centrifugation and resuspension in stabilization buffer brought to 3.7% formaldehyde. After fixation at 23°C for 30 min, cells were washed twice by centrifugation through stabilization buffer-1 M sorbitol and then resuspended in 10 ml of stabilization buffer-1 M sorbitol to which were added 150 µg of zymolyase, 50 mM Pipes, 35 µl of glutaraldehyde at 23°C and permeabilization was monitored by loss of refractility when cells were suspended in an equal volume of 10% SDS. Spheroplasts were washed twice in stabilization buffer-1 M sorbitol and then resuspended in methanol at -20°C and incubated at that temperature for 5 min. After pelleting, the spheroplasts were resuspended in acetic acid at -20°C and then pelleted immediately. After resuspension in PBS made 1 mg/ml with BSA (Sigma Chemical Co., Fraction V) (PBS-BSA), fixed spheroplasts were divided among four 1.5-ml microfuge tubes for reaction with antisera. Each tube was used for a staining experiment. Pellets were resuspended in 20 µl of 1:2 affinity-purified anti-CDC28 product IgG or 1:150 anti-ADH serum. All dilutions were with PBS-BSA. Reaction was for 1-2 h at 23°C. Five washes were performed by resuspension of the cells in 1 ml of PBS-BSA followed by centrifugation for 1 min using an Eppendorf microfuge (Brinkmann Instruments Co., Westbury, NY). Pellets were then resuspended in 20 µl of 1:16 affinity-purified fluorescein-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Naperville, IL). Incubation was for 1 h in the dark followed by five washes in PBS-BSA. Pellets were resuspended in 97% glycero/3% PBS containing the anti-blighting agent p-phenylenediamine as described by Adams and Pringle (1). Samples at this point could be stored at -20°C. Samples were then mounted and observed using a Zeiss Photomeasure II fitted with epifluorescence and a ×100 Planapo oil immersion objective. Tri-X Pan film was developed using Diaphine (Eastman Kodak Co.). Photomicrographs are shown at a magnification of ~1,500.

Cytoplasmic matrices prepared as described were resuspended in methanol at -20°C and incubated for 5 min. After pelleting and resuspension in PBS-BSA, they were stained with antibodies as described for whole cells. Antibody-stained cytoskeletons were then fixed by resuspension in stabilization buffer brought to 3.7% formaldehyde and incubation for 30 min at 23°C. Alternatively, cytoplasmic matrices without methanol extraction were stained for actin using rhodamine-phalloidin (Molecular Probes, Eugene, OR, 25 µg/ml in PBS for 1 h) or for DNA using 4,6-diamidino-2-phenylindole (DAPI) (Sigma; 10 µg/ml for 30 min). For staining with DAPI, matrices were prefixed in 3.7% formaldehyde for 15 min before addition of the dye. In all cases, several washes in PBS-BSA were performed before mounting. Mounting, observation, and photography were as described for whole, fixed cells except that phalloidin staining, n-propyl gallate at 0.2% was used in the mounting solution to retard photobleaching.

Electron Microscopy of Critical Point-dried Cytoplasmic Matrices

Preparation of cytoplasmic matrices for electron microscopy (EM) was by procedures adapted from those developed for visualization of the threedimensional structure of animal cell cytoskeletons (18, 43). Cytoplasmic matrices prepared as described above were fixed by resuspension in stabilization buffer containing 3% EM glutaraldehyde and then washed twice by centrifugation through stabilization buffer-1 M sorbitol and then resuspended in the same buffer containing 1% OsO_4 for 1 h followed by two washes in buffer without OsO_4 and one wash with water. The matrices were then placed on Formvar-coated grids which had been previously coated with 0.1% poly-l-lysine and allowed to settle by gravity. The grids were then dehydrated by submersion in water followed by a graded ethanol series (30%, 50%, 70%, 90%, and three changes of 100%) for 5 min each. It is critical that the cytoplasmic matrices not dry at any point during the procedure as this results in a collapse of the three-dimensional structure. Staining of the matrices was achieved by including 1% uranyl acetate in the 70% ethanol used in the graded ethanol series. The three 100% ethanol washes were followed by critical point drying in a Samdron-78 dryer (Tousimis Research Corp., Rockville, MD). The resulting critical-point-dried cytoplasmic matrices were photographed by transmission EM at the magnifications indicated in the figure legends.

Results

Localization of CDC28 Product in Whole Cells

We first sought to determine the location of the CDC28 product by immunofluorescence in whole, fixed yeast cells. A diploid strain was chosen for these experiments rather than a haploid strain in order to exploit the larger cell size for resolution of detail. Fig. 1, a and b shows fluorescence micrographs of diploid cells stained with anti-CDC28 product IgG and an equivalent concentration of normal rabbit IgG, respectively. A general cytoplasmic staining pattern was observed using anti-CDC28 product IgG (a) but not in the control panel (b). To insure that the IgG was reactive specifically with the CDC28 gene product and not another immunologically cross-reactive species, two additional control experiments were performed. A congenic diploid strain where the CDC28 gene had been placed under control of the inducible yeast GAL1 promoter was used. The methodology used to construct this strain is presented in Fig. 2 (see...
Figure 1. Immunofluorescence staining of fixed yeast cells. (a) Normal diploid cells (MMY13) stained with affinity-purified anti-CDC28 product IgG. (b) Same cells stained with equivalent concentration of normal rabbit IgG. (c) Diploid cells with CDC28 under GALI promoter control (MMY53) grown in galactose medium and stained with anti-CDC28 product IgG. (d) Same cells shifted to glucose medium for 36 h prior to staining with anti-CDC28 product IgG. (e) Normal diploid cells stained with anti-yeast alcohol dehydrogenase serum. (f) Same cells stained with preimmune serum. (g) MMY53 (galactose-regulated CDC28 allele) grown in galactose medium stained with anti-ADH serum. (h) MMY53 shifted to glucose medium for 36 h prior to staining with anti-ADH serum. For both the CDC28 product and ADH series, the exposure time was automatically set for normal diploid cells stained with immune sera. All other exposures in each series were manually normalized to this time. The second antibody in each case was fluorescein-conjugated (FITC) affinity-purified goat anti-rabbit IgG. Bars, 10 μm.

Materials and Methods). When these cells are grown with galactose as the sole carbon source, they contain ~100 times the wild-type level of CDC28 product based on both densitometry of immunoblots and biological activity (the ability to support cell division). These results are predicted by a comparison of the estimated efficiencies of the CDC28 and GALI promoters (31, 40). When cells are shifted to medium containing glucose as the sole carbon source, synthesis of CDC28 mRNA is terminated, and the preexisting protein is expected to be lost by turnover and dilution. The latter is confirmed because the cells cease dividing and become extremely morphologically aberrant after approximately seven generations of growth on glucose (data not shown). Anti-CDC28 product staining of cells in the overexpression and "depleted" states described above is compared to staining of normal wild-type cells in Fig. 1, c and d, respectively. As can be seen, galactose-grown cells stain much more intensely than the normal diploid whereas glucose-shifted cells, although at least 10 times larger than normal cells, show little staining at all. To substantiate that the differences observed were not a staining artifact attributable to diverse metabolic states of the cells under the various growth conditions, preparations of the same cells shown in Fig. 1, a-d were stained with anti-yeast ADH antiserum (Fig. 1, e, g, and h) and preimmune serum (Fig. 1). It is not anticipated that levels of ADH should change substantially and this is confirmed. In the case of glucose-shifted CDC28-depleted cultures most of the alcohol dehydrogenase staining appears to be localized near the ends of the rod-shaped cells. Although the reason for this is not known, it is conceivable that the rest of the cell in this terminal state consists mostly of endolytic vacuoles. The fact that the increase in anti-CDC28 product immunofluorescence signal is not proportional to the extent of increase in CDC28 gene expression observed in the presence of galactose can be explained by the semiquantitative nature of immunofluorescence. The immunofluorescence signal is not expected to increase in a linear fashion over a 100-fold range of antigen concentration. Based on these observations, we conclude that the CDC28 product is primarily cytoplasmic in its distribution.

To determine whether the CDC28 gene product is in a particulate or a soluble form, differential centrifugation of whole-cell lysates was performed. Cells were mechanically disrupted by vortexing with glass beads in 50 mM Tris, pH 7.0. Cell lysis was determined to be >99% by microscopic examination. Particulate fractions were prepared by sedimentation at 5,000, 20,000, or 100,000 g. These fractions, as well as the soluble supernatant fraction, were examined by immunoblotting for the presence of the CDC28 gene product (Fig. 3, top panel, lanes 1–4, respectively). The
presence of alcohol dehydrogenase was also monitored as a control for efficient lysis and washing (bottom panel, lanes 1–4, respectively). Greater than 70% of the gene product sediments in the 5,000- and 20,000-g fractions, with <20% in either the 100,000-g pellet or the soluble supernatant. In contrast, the pellet fractions are devoid of detectable soluble protein as judged by the absence of ADH. This confirms that these fractions do not contain an appreciable number of unlysed cells or trapped soluble protein. Furthermore, the CDC28 gene product associated with the 5,000- and 20,000-g pellets is not released when the pellet is washed with 1.0% Triton X-100 (not shown), suggesting that it is not associated with membrane. In fact, when the same fractionation scheme is performed using a buffer commonly used for the preparation of vertebrate cytoskeletons (discussed below), containing 0.2% Triton X-100 for the disruption of cellular and organelar membranes, a similar result is observed (Fig. 3, lanes 5–8). The only qualitative difference in these results is the absence of the CDC28 product in the 100,000-g fraction (lane 7) and the possibility of a slight amount of contamination with soluble protein in the 20,000-g fraction (lane 6). These same results are observed when Triton-X100 is absent from the buffer (data not shown). We conclude that under each of these conditions the CDC28 gene product is associated with particulate, nonmembranous structures. In addition, because most of the ribosomes sediment in the 100,000-g fraction (36), it is doubtful that a significant portion of this particulate CDC28 gene product is ribosome-associated.

**Preparation of Detergent-insoluble Cytoplasmic Matrices**

Considering the general cytoplasmic staining observed by immunofluorescence experiments along with localization to
Figure 4. (A) Analysis of proteins in cytoplasmic matrices. Cells were spheroplasted and extracted with detergent as described. Cytoplasmic matrix, whole spheroplast, and extraction supernatant fractions were homogenized by vortexing with glass beads and boiled in SDS-polyacrylamide gel sample buffer prior to electrophoresis through a 4%/8.5% discontinuous SDS-polyacrylamide gel. The gel was then stained with Coomassie Brilliant Blue R, destained, and photographed. Lanes 2, 3, and 4 correspond to cell number equivalents (approximately 1 x 10^7 cells/sample) of spheroplast, extraction supernatant, and matrix fractions, respectively. Lane 1 corresponds to matrix fraction proteins from 10 times as many cells as the other lanes. (B) Analysis of cytoplasmic matrix components by immunoblotting. Sample preparation, electrophoresis, and immunoblotting were as described. Whole cell and cytoplasmic matrix samples (2 x 10^7 and 6 x 10^7 cells/lane, respectively) were stained with anti-yeast actin (lanes 1 and 2, respectively) or anti-yeast ADH (lanes 3 and 4, respectively). The ADH signal was intentionally overexposed to emphasize the absence of ADH in the cytoplasmic matrices. Molecular weight standards are phosphorylase b, BSA, actin, carbonic anhydrase, and soybean trypsin inhibitor. Values given are x10^-3.

Accordingly, spheroplasts were prepared and extracted with 0.2% Triton X-100 according to a modification of the procedure of Heuser and Kirschner (13) followed by treatment with DNase I as described in Materials and Methods. Such structures rapidly lose most of their protein content (Fig. 4). Fig. 4 A summarizes the gross biochemical results of detergent extraction of spheroplasts. Proteins from whole yeast cells, Triton X-100 extraction supernatant, and extracted cytoplasmic matrices were analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 2 and 3, equal cell equivalents of whole-cell lysate and detergent supernatant, respectively, indicate that all of the most abundant cellular proteins are accounted for quantitatively in the extraction supernatant. Lane 4 contains an equal cell equivalent of detergent-insoluble cytoplasmic matrices and serves to demonstrate that most of the cellular protein has been removed in the process. Lane 1 displays an approximate protein equivalent of the matrix sample. It is obvious that the cytoplasmic matrix is composed of a small number of major polypeptides and a larger number of minor species. Furthermore, several of the proteins enriched in the matrix fraction (lane 1) are visibly depleted in the supernatant fraction (lane 3). The polypeptide composition of the matrix bears no resemblance to that seen for whole cells and close scrutiny of individual bands suggests that most of the principal matrix species are, at best, minor species in the cell, but are highly enriched in this structure. The identities of the majority of polypeptides present in the cytoplasmic matrix fraction are

the particulate fraction in cell lysates, we chose to examine the yeast cytoplasmic matrix as a potential site of association of the CDC28 gene product. In vertebrate cells, the cytoplasmic matrix or cytoskeleton is defined as the residual structure after detergent extraction of cells (4, 13, 22, 43). Adaptation of the methods used with vertebrate cells for use with yeast required several important modifications. First, because intact yeast cells are resistant to detergent extractions as a result of a dense protective cell wall, enzymatic pretreatment is required. We found that after a short treatment with a mixture of several hydrolytic enzymes, the cells, presumably now spheroplasts, became accessible to extraction with detergent. This was the case even though, based on both light and electron microscopy, the cells were still mostly encased in a matrix of, now presumably porous, cell wall material. Next, because yeast cells do not attach to a substratum, as do most vertebrate cells used in cytoskeletal studies, a principal modification involved the use of centrifugation to perform detergent washes and other extractions. Here, the remaining cell wall matrix proved to be useful in that it protected the material inside from the mechanical shock associated with repeated centrifugation, although it appeared to present no barrier to the extraction of detergent-soluble proteins. This was crucial to the procedure as demonstrated by the fact that, when the same extraction procedures described below are applied to true protoplasts (devoid of all cell wall material), the remaining material becomes condensed and intractable to further manipulation or analysis.
unknown. The majority of tubulin is lost during the preparation of these structures (de Barros Lopes, M., C. Wittenberg, and S. Reed, unpublished observations). Fluorescence microscopy using the DNA-specific dye DAPI indicates that a significant portion, if not all of, the nuclear DNA remains in the structures prior to treatment with DNase I (Fig. 5 a). Treatment with DNase I, a procedure routinely incorporated into our preparation of insoluble cytoplasmic matrices, removes the DNA (Fig. 5 b), thus averting the concern that chromatin in the structures might contribute to nonspecific adhesion of soluble proteins.

The presence of actin in these cytoplasmic matrices is demonstrated both by fluorescence microscopy using rhodamine-tagged phalloidin (Fig. 5 c) and by immunoblotting (discussed below). An organized network of detergent-resistant actin bundles is observed. Cortical actin spots, seen when whole, fixed cells are stained (1, 19, 26, 32), are not seen in cytoplasmic matrices. The reason for this selective extraction is not known. The presence of actin is demonstrated on a biochemical level by immunoblots of whole-cell and cytoplasmic matrix polypeptides reacted with anti-yeast actin antiserum. Such an experiment is shown in Fig. 4 B (lanes 1 and 2). A strongly reacting band is observed in both whole-cell and cytoplasmic matrix preparations which comigrates with vertebrate actin as previously demonstrated for authentic yeast actin by Greer and Scheckman (10). This result corroborates those observed with rhodamine-labeled phalloidin. DNase I treatment has no significant effect on the
Figure 6. Immunofluorescence analysis of yeast cytoplasmic matrices. (a) Cytoplasmic matrix preparation stained with affinity-purified anti-CDC28 product IgG. (b) Cytoplasmic matrix preparation stained with normal rabbit IgG. (c) Cytoplasmic matrix preparation stained with anti-yeast ADH serum. (d) Cytoplasmic matrix preparation stained with anti-ADH preimmune serum. Second antibody in all cases is fluorescein-conjugated (FITC) affinity-purified goat anti-rabbit IgG. Bars, 10 μm.

The structure of these cytoplasmic matrices is revealed most clearly by preparing whole mounts after critical point drying and observing by EM (Fig. 5, d and e). This procedure is considered to conserve the three-dimensional structure of vertebrate cytoskeletons and to allow for clear visualization of spatial relationships (43). A cytoplasmic matrix visualized in this manner is shown in Fig. 5, d and e. One observes a complex network of filamentous material revealed by the complete removal of the cell wall surrounding the new cell bud. Cell wall material obscures the majority of the cell depicted in Fig. 5 d. Most of the cells in these preparations display only occasional gaps revealing the internal structure. Further ultrastructural and biochemical analysis of this structure will be required to determine the organization and composition of these matrices.
 Localization of CDC28 Product in Cytoplasmic Matrices

To determine whether the CDC28 gene product is associated with detergent extracted cytoplasmic matrices, these structures were prepared, reacted with affinity-purified anti-CDC28 product IgG and subjected to indirect immunofluorescence microscopy. Cytoplasmic matrices were also reacted with normal rabbit IgG and anti-ADH serum. The concentrations and reaction times for all immunologic reagents were identical to those used in experiments with whole fixed cells. Fig. 6, a and b, respectively, shows cytoplasmic matrices stained with anti-CDC28 product IgG and the normal IgG control. The matrices display a staining pattern similar to that seen for whole cells. For reasons to be discussed below, we do not feel that quantitative conclusions are justified from this experiment although the level of staining appears to be roughly comparable. Cytoplasmic matrices reacted with anti-ADH and corresponding preimmune sera show no staining (Fig. 6, c and d, respectively). This lack of staining is consistent with the observation by immunoblotting experiments were performed using anti-CDC28 product IgG. This antiserum was of higher titer than the anti-CDC28 sera and could be used in immunoblotting experiments at a lower concentration without loss of signal. This is an advantage that nonspecific background is decreased at lower antisera concentrations. Cytoplasmic matrices were prepared as described. Immunoblots were prepared from SDS-polyacrylamide gels of whole-cell and cytoplasmic matrix polypeptides using antisera directed against the CDC28 gene product (anti-CDC28 product IgG, lanes 1 and 2, respectively) or with the same antibody blocked by preincubation with 0.5 μg/ml of the peptide antigen (lanes 3 and 4, respectively). The position of the CDC28 gene product on the gel is denoted (pCDC28). Molecular weight standards were the same as those given in Fig. 4. Values given are x10^3.

![Figure 7. Immunoblotting analysis of CDC28 product in cytoplasmic matrices. Sample preparation, electrophoresis, and immunoblotting were as described. Whole-cell and cytoplasmic matrix samples (2 x 10^7 and 6 x 10^7 cells/lane, respectively) were stained with affinity purified anti-CDC28 product IgG (lanes 1 and 2, respectively) or with the same antibody blocked by preincubation with 0.5 μg/ml of the peptide antigen (lanes 3 and 4, respectively). The position of the CDC28 gene product on the gel is denoted (pCDC28). Molecular weight standards were the same as those given in Fig. 4. Values given are x10^3.](image-url)

Quantitation of the proportion of the CDC28 gene product in the cytoplasmic matrix is complicated by a number of factors, the most substantial of which are the difficulties in determining the yield of matrices and in controlling proteolysis. There is variability between preparations of cytoplasmic matrices in the yield of CDC28 gene product as well as the ratio of actin to CDC28 gene product. With consideration for these limitations, the upper and lower limits for the proportion of CDC28 gene product associated with the cytoplasmic matrix when prepared in the manner described are 15% and 2%, respectively. A portion of the remaining CDC28 gene product can be detected in the dialyzed supernatant of the first Triton X-100 extraction by immunoblotting (data not shown). Actin association with the cytoplasmic matrix ranges from ~10% to 40% of the whole cell level. Presumably, a portion of the actin associated with the "cortical dots" (1, 19) as well as any soluble actin is lost during the preparation of the cytoplasmic matrices. Neither the CDC28 gene product nor actin is affected by the DNase I digestion used to prepare the insoluble cytoplasmic matrices (data not shown).

It is notable that when the insoluble fraction is isolated by centrifugation of lysates from yeast spheroplasts mechanically disrupted in buffer with or without detergent, approximately the same proportion of the CDC28 gene product is recovered as in cytoplasmic matrices (data not shown). The portion remaining in the supernatant is not sedimented by centrifugation at 100,000 g for 90 min and can, therefore, be considered truly soluble. This is in strong contrast to the relatively large proportion of the gene product found in the particulate fraction when whole non-pretreated cells are similarly disrupted (Fig. 3). This argues that the association of the CDC28 gene product with the particulate fraction is
strongly influenced by some aspect of the spheroplasting process. The finding that approximately the same proportion of the gene product is associated with the particulate fraction in the presence or absence of detergent further supports the argument that this association is not due to a surface artifact.

**Discussion**

These results indicate that the majority of the *CDC28* protein kinase of *S. cerevisiae* resides in the cytoplasmic compartment and that at least a portion of it is tightly associated with the insoluble cytoplasmic matrix. Cytoplasmic localization was demonstrated by indirect-immunofluorescence studies. Due to the small size of yeast cells and nuclei, in particular, we cannot eliminate the possibility that this protein is present in the nucleus as well. Such resolution must await immunocytochemical analysis at the electron microscopic level.

The localization of the *CDC28* gene product within the cytoplasmic compartment has been examined using several approaches. First, the distribution of the gene product within particulate and soluble fractions of mechanically disrupted whole cells was determined by immunoblotting. The material in the particulate fraction is unlikely to be membrane associated in that it is not extractable by concentration of Triton X-100 as high as 2%.

To refine the localization of this particulate fraction further within the cytoplasm, a detergent-resistant fraction of yeast spheroplasts was prepared. We refer to this fraction as the insoluble cytoplasmic matrix. Based on immunofluorescence, it would appear that the majority of the *CDC28* product is matrix bound. However, it is conceivable that matrices, which are of low density and are not aldehyde fixed in our procedure, are more accessible and reactive with antibodies than are prefixed, whole yeast cells. As a result, quantitative assessments are probably not justified. On the other hand, based on immunoblotting experiments, it appears that only a fraction (2–15%) of the *CDC28* product is in the detergent-resistant fraction of spheroplasts.

We cannot be certain that our recovery of cytoplasmic matrices is quantitative and to what extent they have been damaged. Rhodamine-phalloidin staining and immunoblotting of protein extracts of whole cells versus cytoskeletons suggests that a considerable portion of actin is lost during our extraction procedure (as much as 90%). It is possible that extractable and nonextractable actin represent biochemically distinct populations. This hypothesis is supported by our observation that so-called cortical actin spots seen in stained whole cells (1, 19) are not seen in stained cytoskeletons whereas filamentous actin appears to be conserved (Fig. 5 c). It is also likely, however, that there has been a systematic loss of cytoskeletal proteins during the extraction procedure. With respect to this point, we have observed that the variability in the extent of association between the *CDC28* gene product and the detergent-resistant matrix correlates well with the extent to which those matrices have become proteolyzed during their preparation. Proteolysis varies between preparations as evidenced by a relatively low yield of high molecular weight polypeptides in some preparations based on analysis by SDS-polyacrylamide gel electrophoresis. Although precautions have been taken, the sensitivity of cytoplasmic matrices to proteolysis and the abundance of proteases in yeast pose a formidable barrier to precise quantitation (29).

Finally, the *CDC28* product may be largely matrix-associated in a manner which is affected by the spheroplasting process. The presence of >70% of the gene product in the particulate fraction when whole cells are disrupted under buffer and detergent conditions similar to those used for the preparation of insoluble cytoplasmic matrices and the differences observed between spheroplasted and non-spheroplasted cells (discussed further below) suggest that a larger portion of the gene product may, in fact, be associated with the matrix in living cells. The nature of this effect is unclear at present. The loss observed during the preparation of cytoplasmic matrices cannot be explained simply by association with cell wall debris because the spheroplasts retain a significant proportion of their cell wall (Fig. 5 d). Considering these complications, it is clear that absolute quantitation of matrix association of the *CDC28* product must await further investigation.

Treatment of detergent-extracted spheroplasts with DNase I has not been observed to affect the proportion of actin or *CDC28* gene product with the insoluble material. The absence of an effect on actin content and structure, at least so far as can be discerned by rhodamine-phalloidin staining, is surprising in light of the ability of actin to specifically bind to DNase I (10) and the observation that DNase I can induce depolymerization of F-actin under some conditions (14). Clearly, actin, in the state present in these matrices, either does not interact with DNase I or, at least, is not affected by such interaction. Similar observations have been made concerning DNase I treatment of vertebrate cytoskeletal preparations (7).

It is not clear at the moment whether association of the *CDC28* product with the cytoplasmic matrix has any quantitative meaning or biological significance. A cytoskeleton-related function for the *CDC28* protein kinase is consistent with a number of experimental observations. G1 arrest conferred by *cdc28* mutations is accompanied by a number of gross morphologic changes. Most notably, cell growth is asymmetric in the absence of budding, causing a loss of the normal spheroid shape and a transition to large amorphous cells often called "shmoos" (9, 30). Such extreme changes of form must involve cytoskeletal interactions at some level. This is confirmed by observing the structure of actin in *cdc28* mutant cells. Shortly after a shift of temperature sensitive *cdc28* mutants to the restrictive temperature, we have observed the disintegration of actin fibers and the concomitant appearance of apparently disorganized actin foci (32). We, of course, do not know whether this effect of *cdc28* dysfunction on actin structure is direct, but it serves to underscore an inherent relationship of the *cdc28* phenotype to cytoskeletal components. Similar alterations in actin structure are observed in temperature-sensitive actin mutants at the restrictive temperature (26). Another phenotype conferred by *cdc28* mutants is a defect in nuclear fusion (karyogamy) during conjugation (9). Initial reports described a unilateral kar defect (9), meaning that only one parent need be mutant, to observe the effect on conjugation for *cdc28* mutants. We have recently found, however, that in our strains, both conjugants need to be *cdc28* mutants (bilateral kar defect; unpublished observations). Mutant zygotes stained using DAPI in order to visual-
ize nuclei indicate a defect in nuclear migration (unpublished observations). It is expected that organelle motility involves cytoskeletal function (42).

We report the preparation of detergent-resistant cytoplasmic matrices from yeast spheroplasts by a procedure adapted from a regime developed for the preparation of cytoskeletons from vertebrate cells (13). A preliminary ultrastructural and biochemical analysis of the resulting structures is presented. If the complete extraction of alcohol dehydrogenase is an accurate criterion, these structures are devoid of soluble proteins. The procedure results in a complex three-dimensional structure which conserves the structure of the actin cage revealed by rhodamine-phalloidin staining of whole cells. As is the case with vertebrate cells, only a limited subset of proteins is retained during the course of the extraction procedure. Evidence is presented that one of these is actin. Filamentous structures of several different forms are recognizable in electron micrographs. One of these is likely to be actin filaments. Another form appears to have a tubular structure similar to known vertebrate cytoskeletal elements such as microtubules and intermediate filaments. At this time, there is no strong evidence for the existence in yeast of intermediate filaments, a major constituent of vertebrate cytoskeletons (21). Our structures do not contain significant amounts of tubulin, as is expected because extractions were not carried out under conditions necessary to stabilize microtubules. Similar procedures which preserve microtuble structure have been adapted for use with S. cerevisiae (28). The procedure we have developed makes use of the residual cell wall to protect the cytoplasmic matrices from mechanical shock associated with centrifugal washes. It was found that cytoplasmic matrices rapidly disintegrate if cell walls are removed completely. It is conceivable, therefore, that some proteins retained during our extraction procedure are wall-associated rather than matrix associated.

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References

1. Adams, A. E. M., and J. R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant Saccharomyces cerevisiae. J. Cell Biol. 98:934–945.

2. Bravo, R., J. V. Small, S. J. Fey, P. M. Larsen, and J. E. Celis. 1982. Architecture and polypeptide composition of HeLa cytoskeletons: modification of cytoarchitectural polypeptides during mitosis. J. Mol. Biol. 154:121–143.

3. Breuer, H.-J., J. Ferguson, T. A. Peterson, and S. I. Reed. 1983. The isolation and transcriptional characterization of three genes which function at start, the controlling event of the S. cerevisiae cell division cycle: CDC36, CDC37 and CDC39. Mol. Cell. Biol. 3:881–891.

4. Brown, S., W. Levinson, and J. A. Spudich. 1976. Cytoskeletal elements of duck embryo fibroblasts revealed by detergent extraction. J. Supramol. Struct. 5:119–130.

5. Byers, B., and L. Goetsch. 1974. Duplication of spindle plaques and integration of the yeast cell cycle. Cold Spring Harbor Symp. Quant. Biol. 39:123–131.

6. Bücker-Throm, E., W. Dunte, L. H. Hartwell, and T. R. Manney. 1973. Reversible arrest of haploid yeast cells at the initiation of DNA synthesis by a diffusible sex factor. Exp. Cell Res. 76:99–110.

7. Capco, D. G., K. M. Wan, and S. Pennman. 1982. The nuclear matrix: three-dimensional architecture and protein composition. Cell. 29:847–858.

8. Denis, C. L., J. Ferguson, and E. T. Young. 1983. mRNA levels for the fermentative alcohol dehydrogenase decrease upon growth on a nonfermentable carbon source. J. Biol. Chem. 258:1165–1171.

9. Dutcher, S. K., and L. H. Hartwell. 1982. The role of S. cerevisiae cell division cycle genes in mammalian foci. Genetics. 100:175–184.

10. Greer, C., and R. Scheckman. 1982. Actin from Saccharomyces cerevisiae. Mol. Cell. Biol. 2:1270–1278.

11. Hartwell, L. H. 1967. Macromolecule synthesis in temperature-sensitive mutants of yeast. J. Bacteriol. 93:1663–1670.

12. Hartwell, L. H., J. C. Culotti, J. R. Pringle, and B. J. Reid. 1974. Genetic control of the cell division cycle in yeast. (Science (Wash. D.C.).) 183:46–51.

13. Heuser, J. E., and M. W. Kirschaer. 1980. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. J. Cell Biol. 86:212–234.

14. Hitchcock, S. E., L. Carlsson, and U. Lindberg. 1976. Depolymerization of F-actin by deoxynucleotide 1. Cell. 531–542.

15. Jensen, R., G. F. Sprague, and I. Herskowitz. 1983. Regulation of yeast mating type inter-conversion: feedback control of HO gene expression by the mating type locus. Proc. Natl. Acad. Sci. USA. 80:3035–3039.

16. Johnston, G. C., J. R. Pringle, and L. H. Hartwell. 1977. Coordination of growth with cell division in the yeast Saccharomyces cerevisiae. Exp. Cell Res. 105:79–88.

17. Johnstone, M., and R. W. Davis. 1984. Sequences that regulate the divergent GAL-GAL10 promoter in Saccharomyces cerevisiae. Mol. Cell. Biol. 4:1440–1448.

18. Katsumoto, T., and T. Inoue. 1985. Simultaneous stereoscopic observation of the cytoskeleton and the membrane organization of saponin-extracted and critical-point-dried culture cells. J. Electron Microsc. Tech. 2:517–518.

19. Kilbourn, J. J., H. A. Strack, J. Gladfelter, J. Pringle, and J. L. Walker. 1983. A simplified method for measuring cytoarchitecture of S. cerevisiae. J. Cell Biol. 98:934–945.

20. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.

21. Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. Nature (Lond.). 285:249–255.

22. Less, R. L., R. T. Ransom, R. Kaufman, and S. Pennman. 1977. A cytoskeletal structure with associated ribonucleoproteins obtained from HeLa cells. Cell. 10:67–78.

23. Lorincz, A. T., and S. I Reed. 1984. Primary structure homology between the product of the yeast cell division control gene CDC28 and vertebrate oncogenes. Nature (Lond.). 307:183–185.

24. March, D. C., J. P. Park, and P. Cuatrecasas. 1974. A simplified method for cytochrome b6f expression in yeast. J. Cell Biol. 64:149–152.

25. Nasmyth, K. A., and S. I. Reed. 1980. The isolation of genes by complementation in yeast: the molecular cloning of a cell cycle gene. Proc. Natl. Acad. Sci. USA. 77:2119–2123.

26. Novick, P., and D. Botstein. 1985. Phenotypic analysis of temperature-sensitive yeast actin mutants. Cell. 40:405–416.

27. Peterson, T. A., J. Yochem, B. Byers, M. F. Nunn, P. H. Duesberg, R. F. Doolittle, and S. I. Reed. 1984. Relationship between the yeast cell cycle genes CDC4 and CDC36 and the ets sequence of oncogenic virus E26. Nature (Lond.). 309:556–558.

28. Ritter, L., and F. Soloman. 1986. Components of microtubular structures in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. 83:2468–2472.

29. Pringle, J. R. 1975. Methods for avoiding proteolytic artefacts in studies of enzymes and other proteins from yeast. Methods Cell Biol. 12:149–185.

30. Reed, S. I. 1980. The selection of S. cerevisiae mutants defective in the start event of cell division. Genetics. 93:561–577.

31. Reed, S. I. 1982. Preparation of product-specific antisera by gene fusion: antibodies specific for the product of the yeast cell cycle gene CDC28. Gene. 20:253–263.

32. Reed, S. I., M. A. de Barros Lopes, J. Ferguson, J. A. Hadwiger, J. Y. Ho, R. Horwitz, C. A. Jones, A. T. Lorincz, M. D. Mendenhall, T. A. Peterson, S. L. Richardson, and C. Wittenberg. 1985. Genetic and molecular analysis of division control in yeast. Cold Spring Harbor Symp. Quant. Biol. 50:627–634.

33. Reed, S. I., J. Ferguson, and J. C. Gruppo. 1982. A preliminary characterization of the transcriptional and translational products of the yeast cell division cycle gene CDC28. Mol. Cell. Biol. 2:412–425.

34. Pringle, J. R. 1975. Methods for avoiding proteolytic artefacts in studies of enzymes and other proteins from yeast. Methods Cell Biol. 12:149–185.

35. Reed, S. I. 1980. The selection of S. cerevisiae mutants defective in the start event of cell division. Genetics. 93:561–577.
ments with altered sequences constructed in vitro. Proc. Natl. Acad. Sci. USA. 76:4951-4955.
38. Schliwa, M. 1982. Action of cytochalasin D on cytoskeletal networks. J. Cell Biol. 92:79-91.
39. Schliwa, M., and J. van Blerkom. 1981. Structural interaction of cytoskeletal components. J. Cell Biol. 90:222-235.
40. St. John, T. P., and R. W. Davis. 1981. The organization and transcription of the galactose gene cluster of Saccharomyces. J. Mol. Biol. 131:41-53.
41. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.
42. Vale, R. D., B. J. Schnapp, T. S. Reese, and M. P. Sheetz. 1985. Movement of organelles along filaments dissociated from the axoplasm of the squid giant axon. Cell. 40:449-454.
43. Webster, R. E., E. Henderson, M. Osborn, and K. Weber. 1978. Three-dimensional electron microscopical visualization of the cytoskeleton of animal cells: immunoferritin identification of actin- and tubulin-containing structures. Proc. Natl. Acad. Sci. USA. 75:5511-5515.
44. Zoller, M. J., and M. Smith. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned in M13 vectors. Methods Enzymol. 100:468-500.