Immunolocalization of Kex2 Protease Identifies a Putative Late Golgi Compartment in the Yeast *Saccharomyces cerevisiae*

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Abstract. The Kex2 protein of.the yeast *Saccharomyces cerevisiae* is a membrane-bound, Ca^{2+}-dependent serine protease that cleaves the precursors of the mating pheromone α-factor and the M_1 killer toxin at pairs of basic residues during their transport through the secretory pathway. To begin to characterize the intracellular locus of Kex2-dependent proteolytic processing, we have examined the subcellular distribution of Kex2 protein in yeast by indirect immunofluorescence. Kex2 protein is located at multiple, discrete sites within wild-type yeast cells (average, 3.0 ± 1.7/mother cell). Qualitatively similar fluorescence patterns are observed at elevated levels of expression, but no signal is found in cells lacking the *KEX2* gene. Structures containing Kex2 protein are not concentrated at a perinuclear location, but are distributed throughout the cytoplasm at all phases of the cell cycle. Kex2-containing structures appear in the bud at an early, premitotic stage. Analysis of conditional secretory (*sec*) mutants demonstrates that Kex2 protein ordinarily progresses from the ER to the Golgi but is not incorporated into secretory vesicles, consistent with the proposed localization of Kex2 protein to the yeast Golgi complex.

In contrast to mammalian cells, neither the structure nor distribution of the Golgi complex has been defined in *Saccharomyces cerevisiae* ("yeast"). In mammalian cells, individual Golgi cisternae serve as sites of progressive modification of the Asn-linked and Ser/Thr-linked oligosaccharides present on proteins arriving from the ER (Dunphy and Rothman, 1985; Kornfeld and Kornfeld, 1985). By analogy, extension of both Asn-linked oligosaccharides and Ser/Thr-linked mannose residues is thought to occur in the Golgi equivalent of the yeast cell (Kukuruzinska et al., 1987), although enzymes responsible for such modifications have not been localized to a specific structure in yeast. Structures resembling the stacked cisternae of the mammalian Golgi complex accumulate in cells containing the conditional lethal secretory mutations *sec7* and *sec14* (Novick et al., 1980). These mutants also accumulate intracellular pools of secretory proteins bearing oligosaccharides with characteristic post-ER modifications (Novick et al., 1981; Esmon et al., 1981; Stevens et al., 1982; Brada and Schekman, 1988). The formation of novel structures in mutant cells under restrictive conditions implies that these structures are aberrant and thus are not representative of the normal yeast Golgi complex. Definitive characterization of the yeast Golgi complex requires the use of specific markers, preferably integral membrane proteins associated with Golgi cisternae.

The yeast Kex2 protease is an integral membrane protein required for processing pro-α-factor, the precursor of the secreted peptide mating pheromone α-factor (Julius et al., 1984*b*; Fuller et al., 1988; 1989*a*). Based on the effects of conditional secretory (*sec*) mutations on proteolytic processing of pro-α-factor, Julius and co-workers suggested that processing occurs either in a "late" (or *trans*) compartment of the yeast Golgi or in secretory vesicles (Julius et al., 1984*a*). At 37°C, the *sec7* mutant, thought to block transport between successive Golgi compartments (Franzusoff and Schekman, 1989), primarily accumulated pro-α-factor. In the *sec1* mutant, in which fusion of secretory vesicles with the plasma membrane is blocked (Novick and Schekman, 1979), processing to mature α-factor was complete. Retention of greater than 97% of Kex2 protease within an intracellular compartment(s), even when the enzyme was overproduced to high levels, supports the view that Kex2 protein is a resident Golgi processing enzyme rather than a component of secretory vesicles (Fuller et al., 1989*b*). In this study, we have used indirect immunofluorescence to identify the subcellular distribution of Kex2 protein and, by implication, the Golgi complex.

Materials and Methods

Materials and Reagents

Leupeptin, pepstatin A, E64, and α-factor were from Peninsula Laboratories (Belmont, CA). Additional protease inhibitors, 4',6-diamidino-2-phenylindole (DAPI)[1], and common chemicals were from Sigma Chemical

1. *Abbreviations used in this paper*: Anti-Kex2 Ab, affinity purified anti-Kex2 antibodies; DAPI, 4',6-diamidino-2-phenylindole; RT, room temperature; TGN, *trans*-Golgi network.

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Table I. Yeast Strains Used

Strain	Genotype	Source
W303-1A	*MAT a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein (Columbia University, New York)
W303-1B	*MAT α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein (Columbia University)
BFY104	W3031A × W303-1B	This study
BFY125	BFY104 *kex2Δ2::TRP1/kex2Δ::HIS3*	This study
CWY2-5A	*MAT a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 sec11-7 kex2Δ2::TRP1*	This study
CWY1-3C	*MAT α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 sec18-1 kex2Δ2::TRP1*	This study
KRY30-2C	*MAT α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 sec1-1 kex2Δ2::HIS3*	This study
KRY31-6C	*MAT α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 sec7-1 kex2Δ2::TRP1*	This study
KRY33-6D	*MAT α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 sec14-3 kex2Δ2::TRP1*	This study
ISY1-26A	*MAT α his4 leu2-3,112 ura3-52 pho80 sec1-1*	R. Schekman (University of California, Berkeley, CA)

Co. (St. Louis, MO). Reacti-Gel HW-65F and BCA protein assay reagents were from Pierce Chemical Co. (Rockford, IL). Goat anti-rabbit IgG, rabbit anti-goat IgG, and goat anti-rabbit IgG-alkaline phosphatase conjugate were from Zymed Laboratories (San Francisco, CA). Other antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Alkaline phosphatase reagents were from Bio-Rad Laboratories (Richmond, CA). Zymolyase 100T and BSA were from ICN Radiochemicals (Irvine, CA). Multi-well immunofluorescence slides were from Polysciences Inc. (Warrington, PA). Rabbit antiserum was raised against a β-galactosidase-(pro-α-factor) fusion protein (as described by Rothblatt and Meyer, 1986). Rabbit antiserum directed against the Kar2 protein was kindly provided by J. Vogel and M. Rose (Princeton University, Princeton, NJ).

Strains, Plasmids, and Culture Conditions

Yeast strains are listed in Table I. Congenic *sec* mutant strains were created by crossing PBY408A (*MATα sec11-7*), RSY11 (*MATα sec18-1*), SF821-8A *MATa sec7-1*), SF292-2A (*MATα sec14-3*), and ISY1-26A (*MATα sec1-1*) (from R. Schekman and co-workers, University of California, Berkeley, CA), with W303-1A, W303-1B, or a derivative and backcrossing segregants two or three additional times. The final cross was to a strain in which the entire *KEX2* structural gene was deleted (*kex2Δ2*) and replaced with the *HIS3* or *TRP1* gene. Plasmid pBM258 (gift of M. Johnston, Washington University, St. Louis, MO) consists of a 685-bp EcoRI–BamHI fragment containing the *GAL10-GAL1* intergenic region (Johnston and Davis, 1984) cloned between the EcoRI and BamHI sites of centromere vector YCp50 (*CEN4 ARS1 URA3*) (Rose et al., 1987). Plasmid pBM-KX22 consists of a 3.3-kb BamHI–BamHI fragment containing the *KEX2* structural gene (Fuller et al., 1989*a*) inserted into pBM258, placing *KEX2* under *GAL1* promoter control. Plasmid pBM743 (gift of M. Johnston) differs only slightly from pBM258 and served as a vector control. Plasmid YCp-KX212 consists of a 4.3-kb BamHI–BamHI fragment from plasmid pAB-KX212 (Fuller et al., 1989*a*), in which the *KEX2* structural gene is under the control of the *TDH3* promoter, cloned into the BamHI site of YCp50. Cells containing YCp-KX212 over express Kex2 protein about 20-fold relative to wild-type (data not shown). In pG5-*MFα1*, the prepro-α-factor structural gene (Kurjan and Heskowitz, 1982) is placed under the control of the *TDH3* promoter on multicopy vector pAB23 (Fuller et al., 1989*a*), resulting in substantial overproduction of pro-α-factor (Bevan, A., and R. S. Fuller, unpublished results).

Cultures were grown at 30°C unless otherwise indicated. Synthetic complete medium containing 2% glucose but lacking uracil (−Ura) was used to select plasmid-containing strains (Sherman et al., 1986). Plasmids were introduced into yeast strains as described (Burgers and Percival, 1987).

Affinity Purification of Anti-Kex2 Antibodies

Rabbit antiserum against fusion protein LacZ-KXR (Fuller et al., 1989*b*) was affinity purified to reduce background staining. A "nonspecific" resin was made with a "heterologous" LacZ fusion protein, LacZ-KX4Δ6, consisting of 250 residues of the lumenal domain of Kex2 protein fused to *Escherichia coli* β-galactosidase (Fuller, R. S., unpublished results). A "specific" resin was made using fusion protein LacZ-KXR, consisting of the COOH-terminal 100 residues of Kex2 protein fused to β-galactosidase (Fuller et al., 1989*b*). LacZ fusion proteins (50% pure), prepared as described (Fuller et al., 1989*a*), were coupled to Reacti-Gel HW-65F by the manufacturer's suggested methods. Coupling efficiency was ⩾50%, and resins contained about 5 mg fusion protein/ml. Resins, stored at 4°C in buffer A (50 mM Na·Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM NaN_3, 1 mM benzamidine·HCl, 0.5 mM PMSF), were washed just before use with 15 ml 6 M guanidinium·HCl, 10 ml buffer A, 15 ml buffer B (50 mM Na·Hepes, pH 7.5, 4.5 M $MgCl_2$, 0.5 mM benzamidine·HCl, 0.5 mM PMSF) and 25 ml buffer A.

A dialyzed ammonium sulfate (0.282 g per ml) precipitate of rabbit anti-KXR serum (20 ml) was passed over a 2.5-ml column of the nonspecific (LacZ-KXΔ4) resin, and the flow-through loaded onto a 2.5-ml column of the specific (LacZ-KXR) resin. The specific column was washed with 10 ml buffer A plus 1 mg/ml BSA; 10 ml buffer A plus 1 M guanidine·HCl and 1 mg/ml BSA; and 15 ml buffer A plus 1 mg/ml BSA. Affinity-purified anti-Kex2 antibodies (anti-Kex2 Ab) were eluted with 10 ml buffer B containing 1 mg/ml BSA. 0.5 ml fractions were collected and immediately dialyzed against buffer A at 4°C. Fractions containing anti-KXR reactivity were identified by ELISA (recovery was 60%), made 10% (vol/vol) in glycerol, frozen in liquid N_2 and stored at −80°C. Thawed samples stored at 4°C gave consistent immunofluorescence for ⩾6 mo.

Immunoblotting

Log phase yeast cells (suspended in 50 mM Na·Hepes, pH 7.5, 250 mM NaCl, 10% glycerol, 10 mM EDTA, 0.5 mM PMSF, 1 mM benzamidine·HCl, 100 μM tosyl-phenylalanyl chloromethylketone, 100 μM tosyllysyl chloromethylketone, 5 μM E64) were lysed by vortexing with glass beads. Samples (125 μg protein) were denatured with 1% (wt/vol) SDS and 5% (vol/vol) 2-mercaptoethanol, subjected to electrophoresis on an 8% polyacrylamide-SDS gel, and the gel electroblotted onto Millipore HA membrane using the Transblot (Bio-Rad Laboratories). The membrane was probed with a 1:200 dilution of anti-Kex2 Ab, and developed using goat anti-rabbit alkaline-phosphatase conjugate according to the TransBlot Instructions.

Indirect Immunofluorescence and Sandwich Amplification

Our methods were modifications of published methods (for review see Pringle et al., 1989). Yeast cells were fixed by adding, to cultures, potassium phosphate (pH 6.5) to 0.1 M and formaldehyde to 4% (vol/vol). After 30 min with agitation, cells were pelleted (100 *g*, 10 min), resuspended in secondary fixation buffer (0.1 M potassium phosphate, pH 6.5, 4% formaldehyde) and gently shaken at room temperature (RT) for 1 h. Cells to be permeabilized with SDS were incubated for 12 h in secondary fixation buffer.

Fixed cells were washed and resuspended in SHA buffer (1.0 M sorbitol, 0.1 M Na·Hepes, pH 7.5, 5 mM NaN_3) and stored at 4°C for up to 2 wk. To remove the cell wall, fixed cells were incubated in SHA buffer containing 2-mercaptoethanol (0.2%) and Zymolyase 100T (15 μg/ml) for 30 min at 30°C with occasional inversion. Fixed spheroplasts were harvested (3000 *g*, 5 min) and permeabilized by resuspension in SHA buffer containing 0.1% (vol/vol) Triton X-100 for 5 min at RT. Alternatively, fixed spheroplasts were resuspended in SHA buffer containing 0.5% SDS and incubated at RT for 5 min (Rothman et al., 1990). After washing twice with SHA buffer, spheroplasts were stored at 4°C for no longer than 24 h.

To reduce background staining, antibodies were preadsorbed against fixed, permeabilized spheroplasts. Antibodies were diluted into WT buffer (50 mM Na·Hepes, pH 7.5, 150 mM NaCl, 0.5 mg/ml BSA, 1% nonfat dry milk, 0.1% Tween 20, 5 mM NaN_3) containing $2–4 \times 10^8$ spheroplasts/ml, rotated for 1 h at RT, and cells removed by centrifugation. Anti-Kex2 Ab was preadsorbed against *kex2Δ2* spheroplasts. Anti-(pro-α-factor) antiserum was preadsorbed against *MATa* spheroplasts. Preadsorption of the anti-Kar2 antiserum was not necessary.

Fixed, permeabilized spheroplasts (∼10^6 per well) were adsorbed to polylysine-coated multiwell slides for 15 min, then washed (WT buffer). Wells were incubated (60–90 min) with 15 μl primary antibody diluted into WT buffer (1:50–1:100 for anti-Kex2 Ab, 1:50 for anti-[pro-α-factor] antiserum, and 1:3,000 for anti-Kar2 antiserum) and then washed five times (WT buffer). Sandwich amplification of the signal in cells expressing Kex2 protein at the wild-type level was achieved by serial incubation with 0.5 μg/ml goat anti–rabbit IgG, 1 μg/ml rabbit anti–goat IgG, 2 μg/ml goat anti–rabbit IgG, and 4 μg/ml rabbit anti–goat $F(ab')_2$ conjugated to either Texas red or fluorescein, with 5 washes (WT buffer) between incubations. For cells expressing the *KEX2* gene from plasmid pBM-KX22 or YCp-KX212, only one additional incubation was performed, using 1 μg/ml goat anti–rabbit $F(ab')_2$-Texas red. For DAPI staining, 1 μg/ml DAPI was included in the third of the last set of washes and was allowed to incubate at RT for 15 min.

After the final washes, coverslips were applied with mounting solution and sealed. Fields of cells were observed using either a Zeiss Universal fluorescence microscope or a Zeiss Axiophot Photo-Microscope microscope (Zeiss, Oberkochen, Germany) and photographed with Tmax 400 film (Eastman Kodak Co., Rochester, NY) or Ektachrome P800/1600 slide film, developed at ASA 1,600. For quantitative analysis of the distribution of fluorescence, 35-mm slides taken at three planes of focus for each field were projected, diameters of the mother and bud measured, nuclear morphology noted, and the distinct spots of Kex2 immunofluorescence quantified.

Fractionation of Yeast Membranes

Yeast membranes from strain ISY1-26A[pJ2B] were fractionated and assayed as described (Holcomb et al., 1987). Assays for the Kex2 protease were performed as described (Fuller et al., 1989*b*).

Results

Characterization of Affinity-purified Anti-Kex2 Antibody

Consistent with previous results (Fuller et al., 1989*b*), affinity-purified antibody (hereafter, "anti-Kex2 Ab") recognized a 135-kD band in an immunoblot of total protein from *KEX2* wild-type diploid strain BFY104 (Fig. 1, lanes *2* and *4*). This band was absent in strain BFY125 (Fig. 1, lane *1*), which is homozygous for a deletion of the entire *KEX2* structural gene, but otherwise isogenic to BFY104. As expected, greater amounts of the 135-kD band were found in strains overexpressing the *KEX2* gene (Fig. 1, lanes *3* and *5*). These results demonstrate that, to the limits of detection, Kex2 protein is the sole protein recognized in yeast cells by the affinity-purified antibody.

Localization of Kex2 Protein by Indirect Immunofluorescence

Kex2 protease is inabundant, comprising <1 part in 10^5 of total protein, equivalent to ∼10^2–10^3 molecules per cell

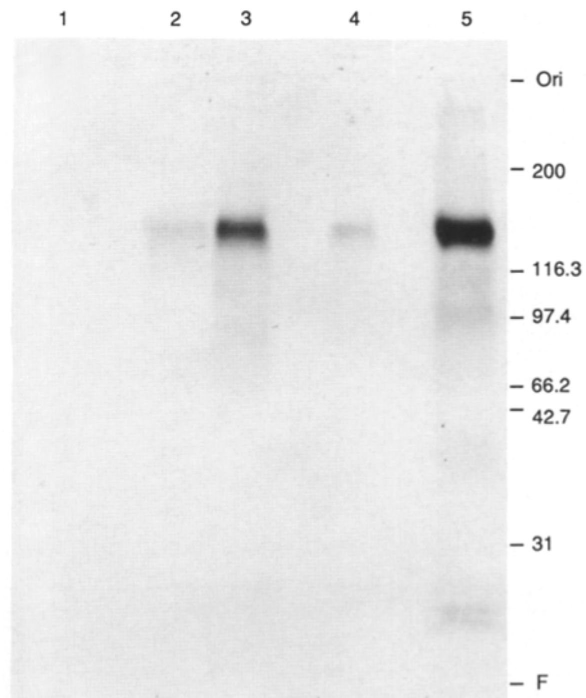

Figure 1. Kex2 protein is the sole protein recognized by affinity-purified anti-Kex2 Ab. Shown is an immunoblot of total protein (125 μg) from strains BFY125[pBM743] (lane *1*), BFY104[pBM 743] (lane *2*), BFY104[pBM-KX22] (lane *3*), BFY104[pAB23] (lane *4*), BFY104[pAB-KX22] (lane *5*), developed using affinity-purified anti-Kex2 Ab. Plasmid pAB-KX22 causes greater than 100-fold overproduction of Kex2 protease (Fuller et al., 1989*a*). Faster migrating bands, which do not appear in the extract from the *kex2Δ2/kex2Δ2* strain, probably represent proteolytic degradation fragments (Fuller et al., 1989*a*). Strains were grown in medium containing 2% glucose (lanes *1*, *4*, and *5*) or 2% galactose/0.1% glucose (lanes *2* and *3*).

(Fuller et al., 1989*a*). When standard yeast immunofluorescence methods proved ineffectual, we modified the "sandwich method" (Schulze and Kirschner, 1987) to permit detection of Kex2 protein at the wild-type level. Diploid cells lacking Kex2 protein because of a homozygous deletion of the structural gene (Fig. 2 *A*) and diploid cells homozygous for the wild-type *KEX2* gene (Fig. 2, *B* and *E*) were both stained by this "sandwich-amplification" method. Comparison of the two populations of stained cells revealed a punctate pattern of Kex2 protein-specific immunofluorescence that suggested localization of the protease to multiple bodies distributed throughout the yeast cell. By shifting the plane of focus through individual cells, what appeared to be a single spot of fluorescence in one focal plane was occasionally the cross section of a larger structure. More generally, the spots were discrete. The apparent sizes of the stained organelles varied from <0.1 to ∼0.2 times the diameter of the maternal yeast cells. Although loci of staining were sometimes near the periphery of the cell, staining of the plasma membrane was not observed. Kex2-specific fluorescence was not associated consistently with either the major vacuolar structures identified by differential interference contrast microscopy (Fig. 2 *D*) or the nucleus (or mitochondria) as identified by DAPI staining (Fig. 2 *F*). Kex2-specific fluorescence was frequently found within buds of premitotic cells, clearly

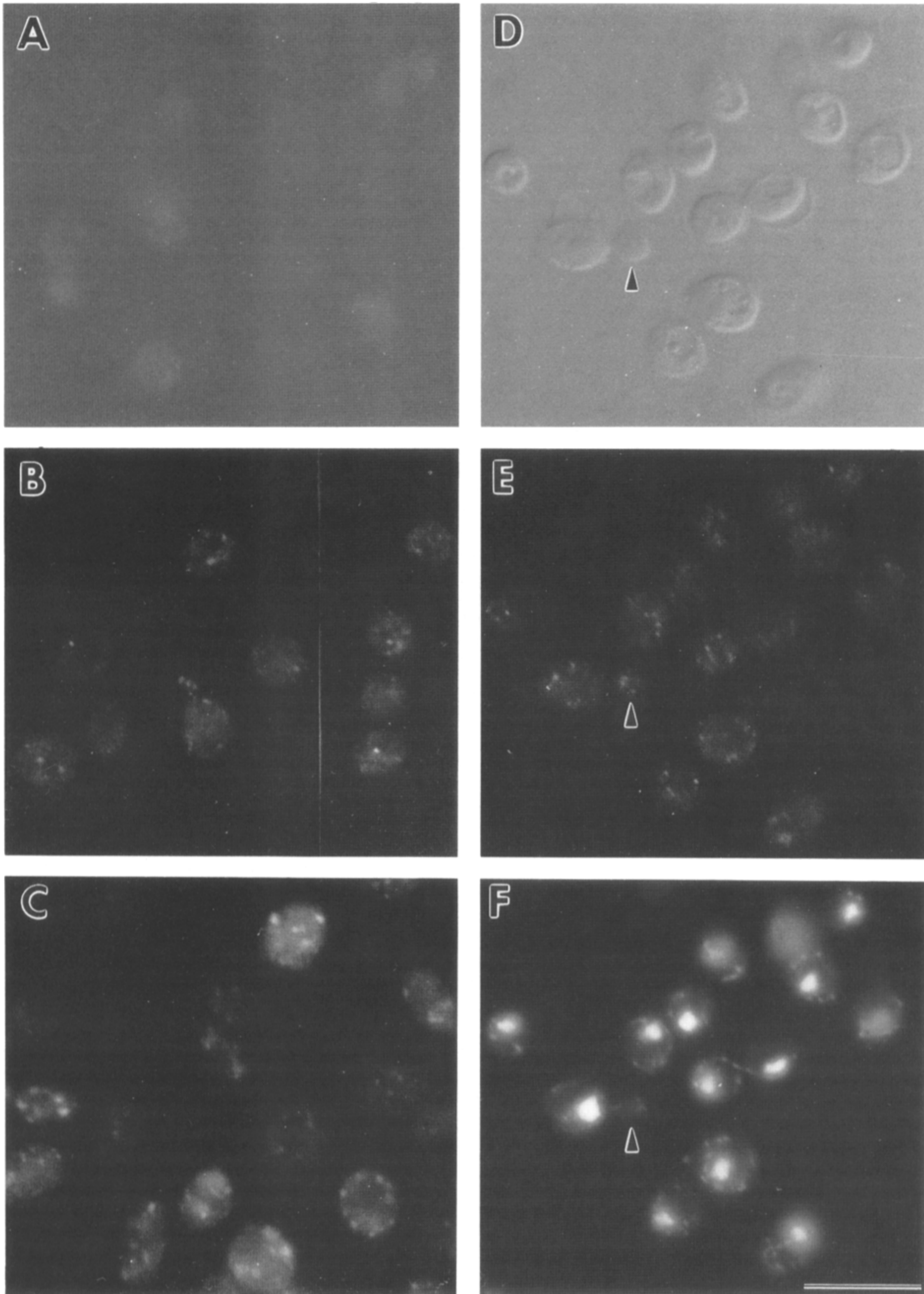

Figure 2. Localization of Kex2 protein in wild-type cells by amplified indirect immunofluorescence. In *A*, *B*, and *D–F*, the signal was amplified as described in Materials and Methods. (*A*) The diploid *kex2* null strain BFY125; (*B*, *D–F*) the diploid *KEX2* wild-type strain BFY104[pBM743] (*KEX2/KEX2*); (*C*) the diploid *KEX2* wild-type strain carrying a galactose-inducible *KEX2* gene on a centromere plasmid, BFY104[pBM-KX22]. In *C*, cells were grown in medium containing 2% galactose/0.1% glucose, resulting in a 15-fold elevated level

Table II. Quantitation of Sites of Kex2 Immunofluorescence in Wild-type Cells

Ratio of Bud/Mother Volume		Percent of cells having buds containing:		Number of Kex2⁺ spots in:			
				Mother		Bud	
		Nucleus	≥1 spot	Mean	SD	Mean	SD
%*	*n*						
0	310	0	0	2.8	1.8	0	0
0.1–2.5	14	0	7	3.1	2.1	0.07	0.27
2.6–13	18	0	72	3.7	1.9	0.83	0.62
14–30	27	13	100	3.3	1.7	1.9	1.2
31–50	30	38	93	4.0	2.2	2.3	1.5
>50	14	71	71	2.8	2.0	1.8	1.5

* In both Tables II and III, the long and short diameters of the mother cells and buds were measured from projections of differential interference contrast images and the volumes were calculated assuming an ellipsoid shape.

demonstrating the lack of association of Kex2-containing structures with the nucleus. Qualitatively indistinguishable results were obtained when Kex2 protein was overproduced 15-fold (Fig. 2 *C*), although overproduction eliminated the need for sandwich amplification. Thus, sandwich amplification enhanced the signal in wild-type cells about 10-fold.

Punctate fluorescence was never observed in cells lacking Kex2 protein. A low level of diffuse fluorescence observed in *kex2Δ* cells presumably corresponded to the background level of staining (Note that Fig. 2 *A* is overexposed several-fold relative to the other panels). Additional controls confirmed the specificity of staining. Use of preimmune serum in place of anti-Kex2 antibody or omission of anti-Kex2 antibody also resulted in only background staining. Preincubation of anti-Kex2 Ab with a 10-fold molar excess of fusion protein LacZ-KXR, but not with an unrelated fusion protein, completely eliminated punctate staining (data not shown).

Distribution of Kex2-containing Structures through the Cell Division Cycle

The mammalian Golgi complex is a dynamic organelle that fragments during mitosis, presumably to aid its segregation to daughter cells. During interphase, the organelle coalesces at one or a few perinuclear regions (Lucocq et al., 1989; Kreis, 1990). Because of the presumed Golgi localization of Kex2 protease, we were surprised at the highly dispersed distribution of Kex2-specific immunofluorescence and its apparent lack of association with the nucleus. Results of a statistical analysis of the distribution of sites of Kex2 staining throughout the cell cycle of wild-type cells is presented in Table II and Fig. 3 *A*. Nuclear morphology and the ratio of bud and mother cell volumes are indicative of cell cycle position. As indicated by the large SDs, there was substantial variation in the number of Kex2-containing bodies from cell to cell (range: 0–10 in mother cells; 0–6 in buds). However, the average number of Kex2-specific bodies per mother cell (3.0 ± 1.7) remained essentially constant during the cell cycle. There was, therefore, no evidence for the coalescence of Kex2-containing organelles at any stage of the cell cycle during logarithmic growth.

Similar results were obtained with cells overproducing Kex2 protein 15-fold (Table III, Fig. 3 *B*), although the average number of Kex2-staining spots was higher (6.3 ± 2.8). The difference between the average number of observed sites of Kex2-specific staining in wild-type and overproducing cells is probably exaggerated because of the somewhat greater difficulty in distinguishing weakly staining spots in wild-type cells from background. Therefore our determination of the average number of structures in wild-type cells is a minimum estimate.

In both wild-type and overproducing cells, Kex2-specific fluorescence did not appear within buds until a threshold size was reached. Fewer than 8% (5% in the overproducer) of buds with a volume <2.5% that of the mother cell contained Kex2-specific staining, whereas ≥90% (95% in the overproducer) of buds with volumes >3% that of the mother cell did contain Kex2 immunofluorescence. Above this threshold size, the average number of Kex2-containing structures in the emerging bud increased roughly in proportion to bud volume, and in the largest buds reached a value ~60% that of the mother cell. Nevertheless, appearance of Kex2-containing organelles in the bud clearly preceded nuclear division, underscoring the independence of these organelles from association with the nucleus.

Because failure of the Kex2-containing organelle to coalesce during logarithmic growth might be a consequence of the rapid rate of division, cells arrested in the G_1 phase of the cell cycle were examined. Three methods were used to arrest cells in G_1: (*a*) nitrogen starvation; (*b*) growth to stationary phase; and (*c*) treatment of haploid *MATa* cells with α-factor. The first two methods yield cells arrested at the same early point in G_1, analogous to G_0 in mammalian cells (Iida and Yahara, 1984). After starvation for nitrogen, cells still contained multiple Kex2-containing bodies (Fig. 4 *B*). Frequently, these structures were close to the cell periphery, probably because of the presence of large vacuoles typical of starved cells. Localization of Kex2-specific staining in stationary phase cells was qualitatively similar (data not shown).

The mating pheromone α-factor causes haploid *MATa* cells to arrest at a later stage in G_1. Pheromone-treated

of Kex2 protein as measured by enzymatic assay (Fuller et al., 1989*a*). *D* and *F* are the same field of cells as *E* visualized by differential interference contrast microscopy and DAPI staining, respectively. Arrowheads in *D*, *E*, and *F* indicate a premitotic bud which has several spots of Kex2-specific fluorescence. Bar, 10 μm.

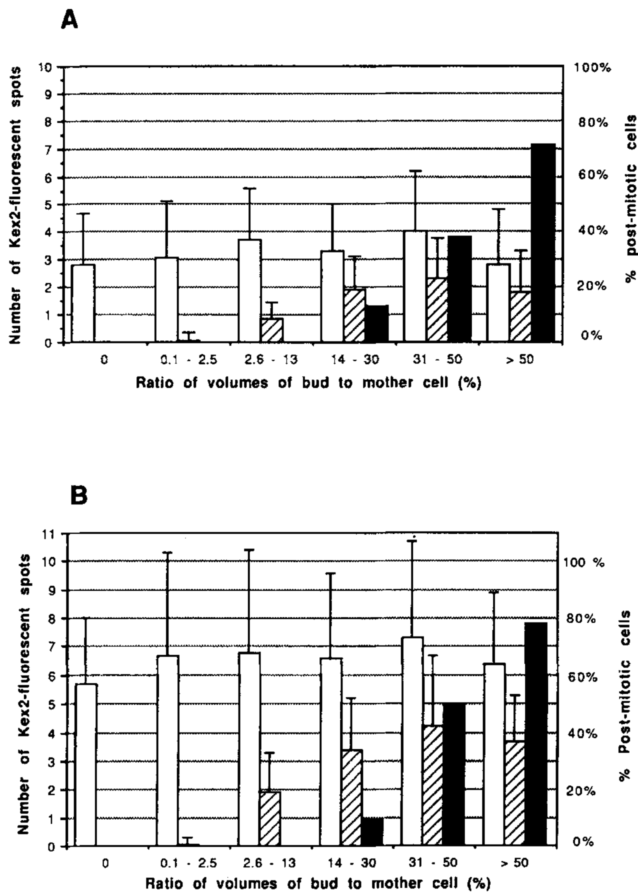

Figure 3. Distribution of Kex2-containing organelles through the cell cycle in wild-type and Kex2-overproducing cells. For each interval of bud to mother cell volume ratios, the average numbers of Kex2-specific fluorescent spots in the mother cell (□) and the bud (▨), and the percentage of cells which are postmitotic (■) are plotted. (*A*) Wild-type cells (BFY104) in which the signal was amplified by the sandwich method. (*B*) Cells overproducing Kex2 protein 15-fold BFY104[pBM-KX22]. Error bars indicate one SD of the mean.

cells continue to grow without initiating bud formation, resulting in cells of drastically altered morphology having one or more elongated projections that are the presumed sites of cell wall and membrane growth (Field and Schekman, 1980). Like log phase and G_0-arrested cells, *MATa* cells treated with α-factor for 3 h (Fig. 4 *C*) exhibit punctate Kex2 fluorescence. A dramatically altered pattern was observed 6 h after treatment with pheromone, when cells had one or two highly elongated projections (Fig. 4, *D–F*). Kex2 fluorescence was often concentrated at the very tips of the projections, frequently without any apparent staining in the body of the cell. Even in these cells, however, the region of concentrated staining at the projection tip comprised multiple, distinct Kex2-containing structures.

Effects of sec Mutations Imply Golgi-Localization of Kex2 Protein

To establish the identity of the compartment containing Kex2 protein, localization of the protein was assessed by immunofluorescence in *sec* mutant strains (Schekman, 1985). If Kex2 protein ordinarily resides in the Golgi complex, then the *sec11* mutation, which blocks signal peptide cleavage and results in retention of secretory proteins in the ER (Böhni et al., 1988), and the *sec18* mutation, which interferes with transport between the ER and Golgi complex (Kaiser and Schekman, 1990), should dramatically alter localization of newly synthesized Kex2 protein. Mutations such as *sec7* or *sec14*, which interfere with transport between Golgi compartments or with formation of secretory vesicles from the Golgi complex (Novick et al., 1981; Franzusoff and Schekman, 1989), might have no effect or only a subtle effect on the distribution of the protein.

Expression of the *KEX2* gene on plasmid pBM-KX22 in congenic *sec* mutant strains deleted of the chromosomal *KEX2* gene was induced by galactose after shifting cultures to the restrictive temperature (37°C). When expression of Kex2 protein was induced in a *sec11-ts* mutant under restrictive conditions (Fig. 5, *A* and *B*), Kex2-specific staining was observed in a circumnuclear, peripheral, and filamentous pattern characteristic of yeast ER (Rose et al., 1989), rather than in the typical punctate pattern. At the restrictive temperature in a *sec18-ts* mutant, Kex2 protein was distributed in a pattern similar to that found in the *sec11-ts* strain, except that the peripheral and filamentous staining was more prominent than circumnuclear staining (Fig. 5, *C* and *D*). As a control, the *sec18-ts* cells were also stained with antibody against the Kar2 protein, a resident of the ER lumen (Rose et al., 1989). Although principally circumnuclear in wild-type cells (Rose et al., 1989) and in the *sec18-ts* cells grown at 25°C (data not shown), a peripheral and filamentous pat-

Table III. Quantitation of Sites of Kex2 Immunofluorescence in Cells Overexpressing Kex2 Protein 15-fold

Ratio of Bud/Mother Volume		Percent of cells having buds containing:		Number of Kex2⁺ spots in:			
				Mother		Bud	
		Nucleus	≥1 spot	Mean	SD	Mean	SD
%*	*n*						
0	103	0	0	5.7	2.3	0	0
0.1–2.5	18	0	5.6	6.7	3.6	0.06	0.24
2.6–13	25	0	92	6.8	3.6	1.9	1.4
14–30	21	9.5	95	6.6	3.0	3.4	1.8
31–50	20	50	95	7.3	3.4	4.2	2.5
>50	24	78	100	6.4	2.5	3.7	1.6

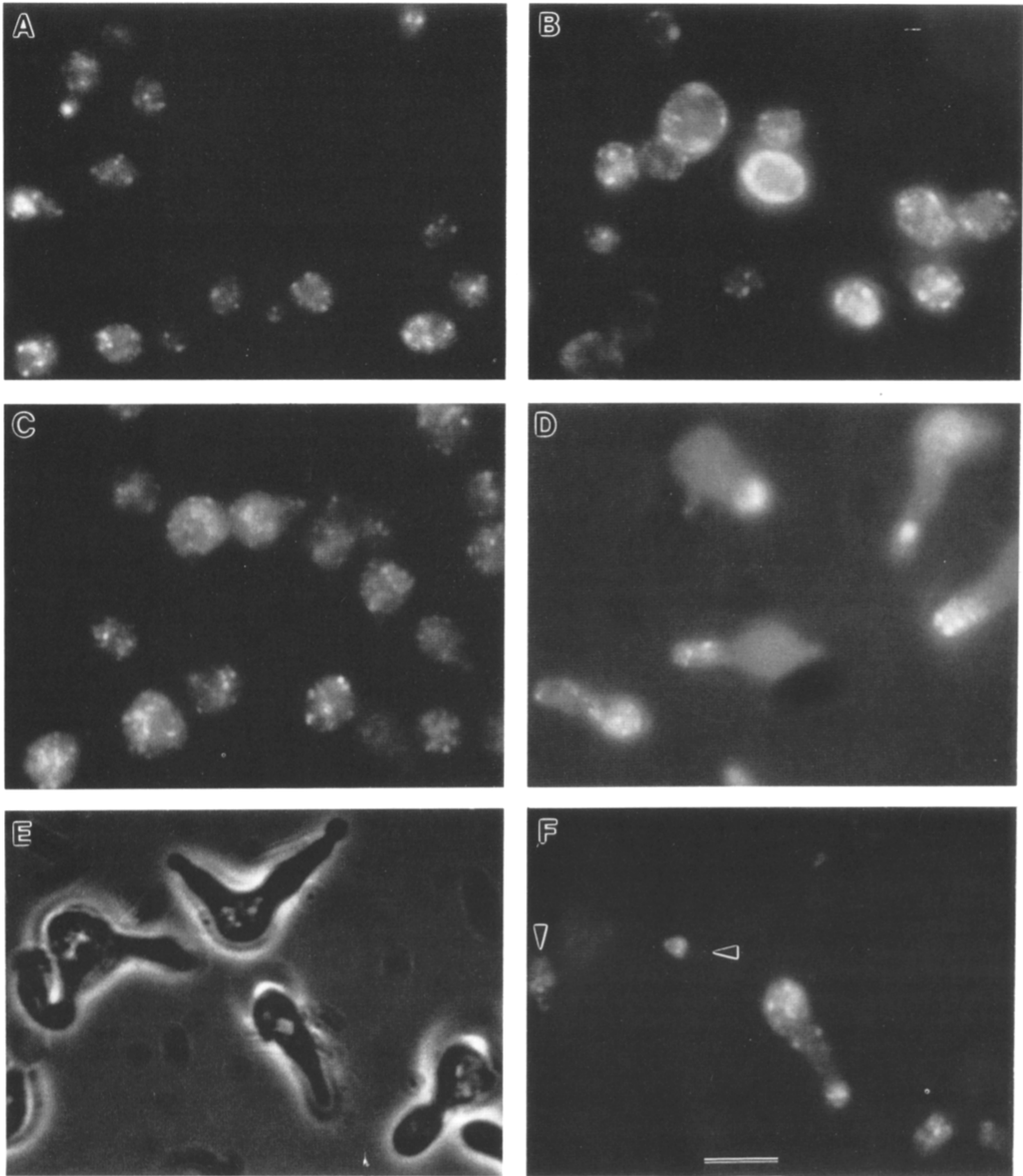

Figure 4. Kex2 immunofluorescence in early and late G_1-arrested cells. Early G_1 arrest (G_0) by nitrogen starvation. (*A* and *B*) Strain W303-1A containing plasmid YCp-KX212 was grown in YNB-Ura medium (Johnston et al., 1977) and a sample of the log phase culture was prepared for immunofluorescence (*A*). The remainder of the culture was washed and resuspended in YNB-Ura-N medium (lacking ammonium sulfate), grown for 24 h, and then fixed and prepared for immunofluorescence (*B*). Late G_1 arrest by α-factor (*C–F*); synthetic α-factor was added to a final concentration of 3.75 μg/ml (2.2 μM) to a log phase culture of strain W303-1A containing plasmid YCp-KX212, and samples were fixed and prepared for immunofluorescence after 3 h (*C*) and 6 h (*D–F*). (*A–D*, and *F*) Kex2-immunofluorescence; (*E*) phase contrast view of field in *F*. Arrowheads in *F* indicate fluorescence at the tips of two projections that arose from one cell. Bar, 10 μm.

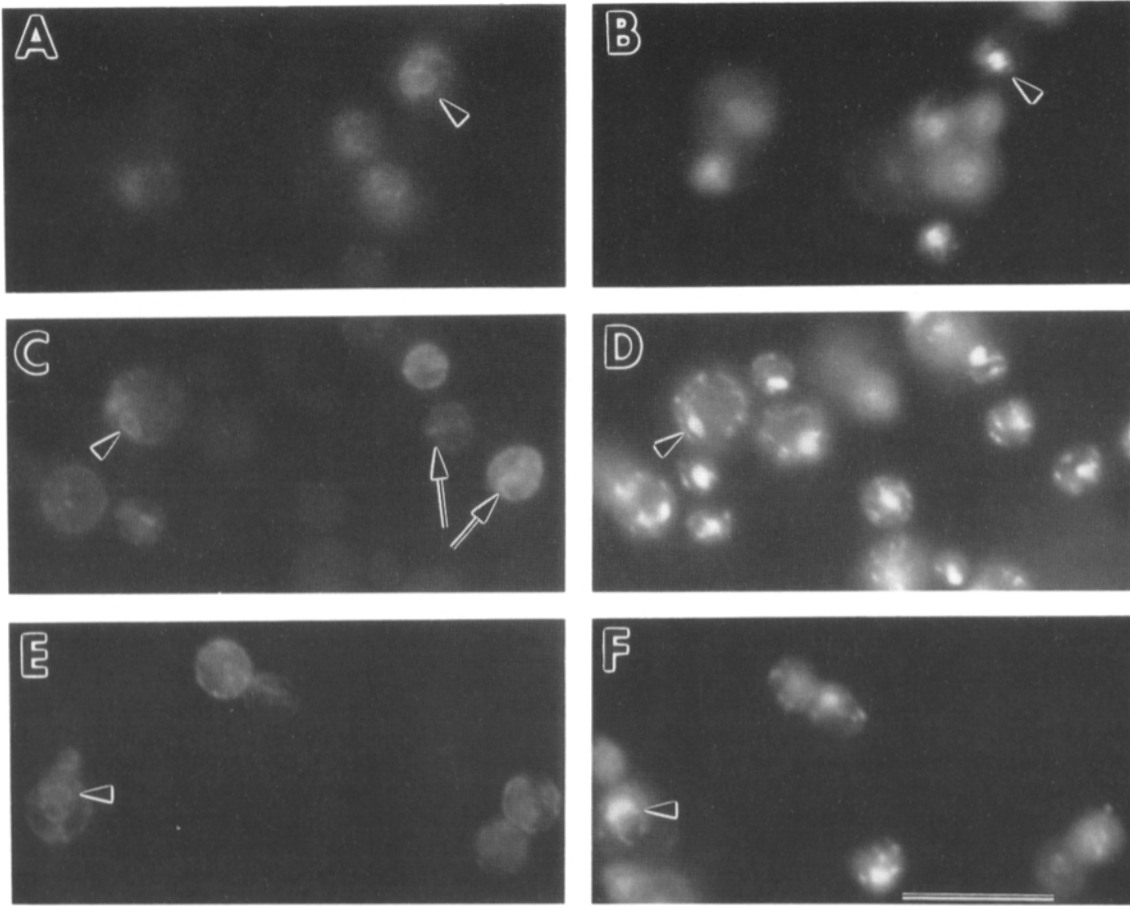

Figure 5. The *sec11* and *sec18* mutations prevent normal localization of Kex 2 protein. Log phase cultures of *sec* mutant strains containing plasmid pBM-KX22, were grown at 25°C in SC-Ura medium containing 2% raffinose and lacking glucose, and then shifted to 37°C to impose the secretory block. After 30 min, galactose was added to a final concentration of 3% to induce the *GAL1* promoter. 3 h after the shift to 37°C, cells were fixed and prepared for immunofluorescence. (*A* and *B*) CWY2-5A[pBM-KX22] (*sec11*); (*C–F*) CWY1-3C[pBM-KX22] (*sec18*). *A* and *C* show anti-Kex2 immunofluorescence, while *E* shows anti-Kar2 immunofluorescence. *B*, *D*, and *F* are DAPI micrographs that correspond to *A*, *C*, and *E*, respectively. Arrowheads indicate nuclear envelope fluorescence in *A*, *C*, and *E*, and nuclear DNA fluorescence in *B*, *D*, and *F*. The arrows in *C* indicate filamentous fluorescence. Bar, 10 μm.

tern of Kar2-specific staining predominated in the *sec18-ts* strain at the restrictive temperature (Fig. 5, *E* and *F*), as had been found for Kex2 protein (Fig. 5, *C* and *D*).

Induction of Kex2 protein synthesis in *sec7-ts* and *sec14-ts* mutants at the restrictive temperature (Fig. 6) resulted in punctate fluorescence which differed only subtly from that observed in congenic wild-type cells at 37°C (data not shown). The average number of Kex2-staining spots per cell in the *sec7-ts* strain at 37°C was similar to that found in wild-type cells, but some spots appeared to be unusually large. In *sec14-ts* cells at the restrictive temperature, the average number of Kex2-staining bodies appeared to be greater than that found in the congenic wild-type control at 37°C. These effects of the *sec7* and *sec14* mutations on localization of Kex2 protein are consistent with steady state retention of the protein within a compartment of the yeast Golgi.

Kex2 Protein Does Not Enter Secretory Vesicles

Intracellular retention of Kex2 protein requires the COOH-terminal cytosolic tail of the protease and a functional clathrin heavy chain gene (Fuller et al., 1989*b*; Payne and Schekman, 1989). Net intracellular retention of Kex2 protein might result from transport to the cell surface via secretory vesicles followed by rapid, clathrin-mediated endocytotic recycling to a Golgi compartment. Temperature-sensitive mutations in the *sec1* gene cause accumulation of 80–100-nm secretory vesicles (Novick and Schekman, 1979; Novick et al., 1981). Within 15 min after shifting to the restrictive temperature (37°C), secretory vesicles accumulate in a polarized fashion within or near the bud of *sec1* mutant cells (Novick, P., personal communication). Although the *sec1* mutation does not interfere with processing of pro-α-factor, it does prevent release of the mature pheromone, indicating that α-factor is secreted via *SEC1*-dependent vesicles (Julius et al., 1984*a*). We therefore examined the effect of a temperature-sensitive allele of the *SEC1* gene both on the localization of Kex2 protein and, as a control, on its substrate, pro-α-factor. If transport of Kex2 protein to the cell surface were an obligatory step in its intracellular transport, then the *sec1* mutant block should result in redistribution of Kex2 protein to the bud.

In the *sec1-ts* strain at 25°C, punctate and diffuse anti-(pro-α-factor) immunostaining was distributed at a similar density in both mother cells and buds (Fig. 7 *B*). This pattern presumably represents pro-α-factor in transit through multiple secretory compartments (ER, Golgi, and secretory vesi-

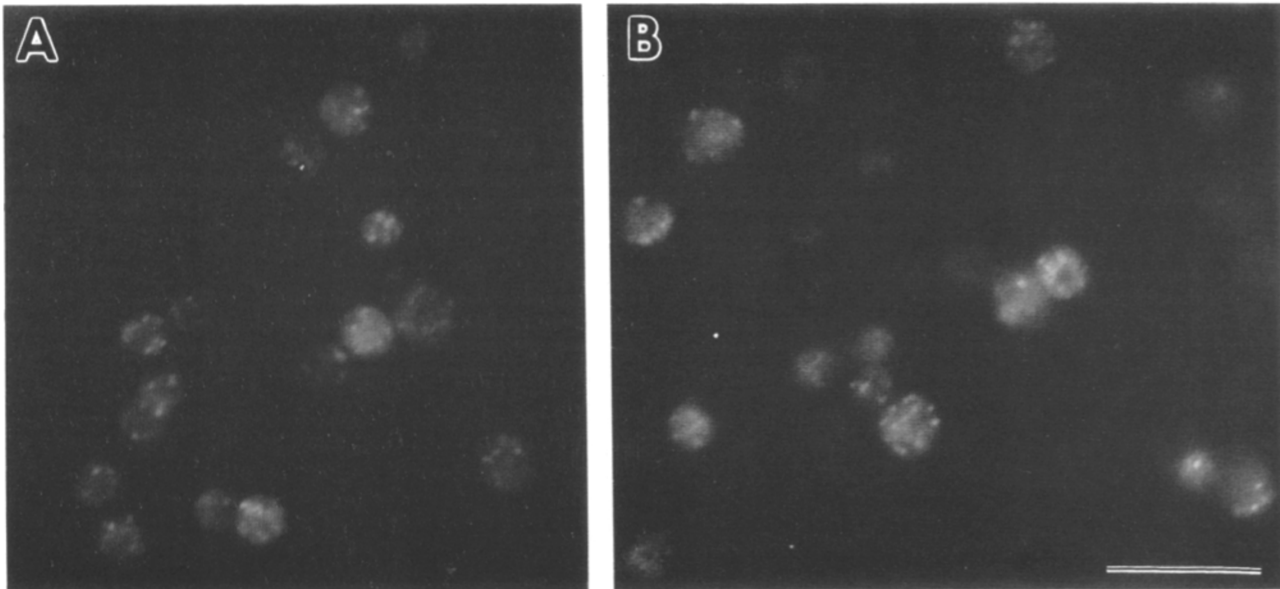

Figure 6. Accumulation of Kex2 protein at the *sec7* and *sec14* arrest points. The cells were treated as in Fig. 5. (*A*) KRY31-6C[pBM-KX22] (*sec7*); (*B*) KRY33-6D[pBM-KX22] (*sec14*). Bar, 10 μm.

cles). Shifting to 37°C for 20 min resulted in a dramatic accumulation of pro-α-factor in the bud, as expected for a secretory vesicle marker (Fig. 7 *D*). In contrast, punctate fluorescence was observed for Kex2 protein at both temperatures, without a shift to the bud at 37°C (Fig. 7, *A* and *C*). Thus, unlike α-factor, Kex2 protein does not appear to travel to the cell surface in *SEC1*-dependent secretory vesicles.

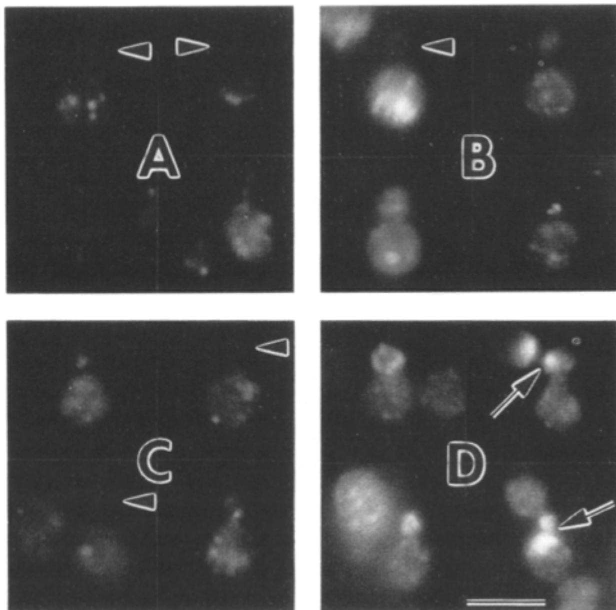

Figure 7. Unlike pro-α-factor, Kex2 protein does not accumulate within secretory vesicles in a *sec1* mutant. KRY30-2C (*MATα sec1-ts kex2Δ*) cells, harboring either pBM-KX22 (*A* and *C*, Kex2p overproducer) or pG5-MFα1; (*B* and *D*, pro-α-factor overproducer) were grown at 25°C in galactose medium. Aliquots were fixed before (*A* and *B*) and after (*C* and *D*) a 20 min shift to 37°C, and were processed for anti-Kex2 (*A* and *C*) or anti-(pro-α-factor) (*B* and *D*) immunofluorescence. Representative micrographs of fluorescent cells in which the mother and bud were in the same focal plane are presented. Arrowheads indicate buds invisible because of low fluorescence intensity relative to mother cells. In *D*, arrows indicate concentrations within buds of (pro-α-factor)-specific immunofluorescence. Bar, 5 μm.

We used a second, independent approach to determine whether Kex2 protease might accumulate in secretory vesicles at later times. Organelles from *sec1* spheroplasts shifted to 37°C for 90 min were fractionated by Percoll gradient centrifugation (Holcomb et al., 1987). At the permissive temperature, the majority of Kex2 activity appeared in a peak near the top of the gradient, with a shoulder of activity extending to higher densities (Fig. 8 *A*). The low-density peak represents the bulk of intracellular membranes including ER, marked by NADPH cytochrome C reductase, and a small amount of acid phosphatase activity, presumably present in ER and Golgi membranes (Holcomb et al., 1987). At the restrictive temperature, a large peak of acid phosphatase activity appeared at a position of higher density near the bottom of the gradient, indicating sedimentation of the enzyme in accumulated secretory vesicles. However, no shift of Kex2 proteolytic activity (or of NADPH cytochrome C reductase) into this peak was observed. A decrease of total Kex2 activity was seen at 37°C, and is consistent with our observation of both a decrease in the rate of synthesis of Kex2 protein and an increase in its rate of degradation upon shifting cells from 30 to 37°C (data not shown). In agreement with the conclusion that Kex2 protein and acid phosphatase reside in distinct compartments at 37°C, the two activities also behaved differently in earlier steps in the fractionation. Although nearly all of the Kex2 activity in an initial cleared lysate ("low speed supernatant" in Holcomb et al., 1987) was membrane associated (>70% sedimented at 100,000 *g*), only 27% of the Kex2 activity pelleted at 12,000 *g*. In contrast, 76% of the acid phosphatase activity in the low speed supernatant sedimented at 12,000 *g*.

Discussion

We have identified the intracellular secretory compartment that contains the yeast Kex2 protease by indirect immu-

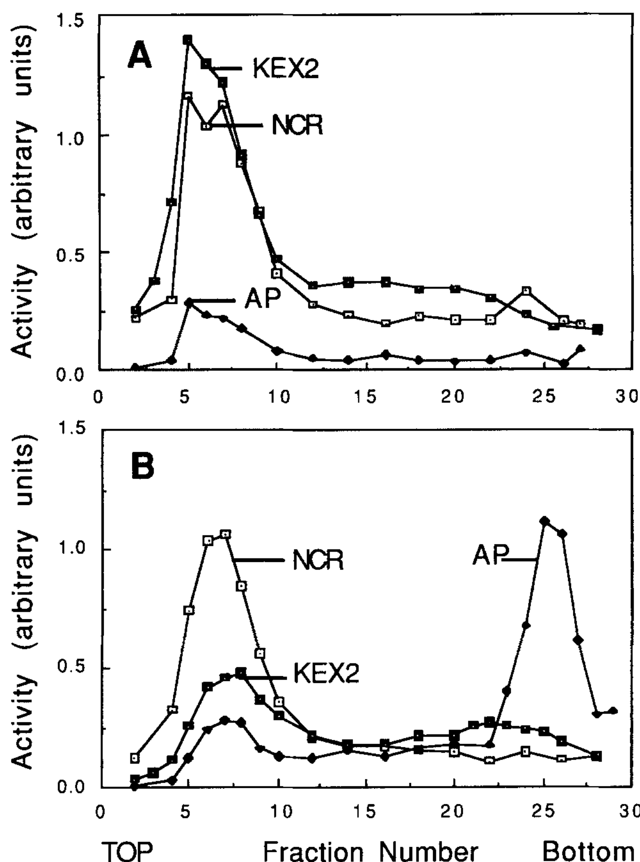

Figure 8. Separation of membranes containing Kex2 proteolytic activity from secretory vesicles by Percoll gradient fractionation. A "high-speed" pellet fraction (12,000 *g* for 60 min), containing secretory vesicles was prepared from yeast strain ISY1-26A[pJ2B] at 24 (*A*) or 37°C (*B*) as described (Holcomb et al., 1987). Multicopy plasmid pJ2B results in about 10-fold overproduction of Kex2 protease (Julius et al., 1984*b*; Fuller et al., 1989*a*). The high-speed pellet was fractionated on a 62-ml Percoll gradient (initial concentration, 22%, wt/vol) in a rotor (model Ti45; Beckman Instruments, Inc., Palo Alto, CA) at 29,000 rpm for 30 min as described (Holcomb et al., 1987) and 31 fractions (2 ml) were collected. 50 μl samples were assayed for acid phosphatase activity as described (Holcomb et al., 1987) and activities are reported as A_{400} (♦). 20 μl samples were assayed for NADPH cytochrome C reductase activity and activities are reported as $A_{550} \times 2.5$ (□). 10 μl samples were assayed for Kex2 activity as described (Fuller et al., 1989*a*) and the total Kex2 activity per fraction $\times 10^{-3}$ is reported (■).

nofluorescence. By a number of criteria, previous studies have suggested that Kex2 protease occupies a late compartment of the Golgi complex. Thus, we were surprised by the distribution of the enzyme into multiple, discrete organelles that lack the perinuclear association of mammalian Golgi complexes. Analysis of these structures during the cell cycle indicated no phase in which perinuclear clustering occurred. However, studies of localization of Kex2 protease in both wild-type and several *sec* mutant strains demonstrated that the enzyme was not present in the ER, secretory vesicles, the vacuole, or the plasma membrane. Taken together, our findings strongly suggest that the Kex2-containing structure is indeed an element of the yeast Golgi apparatus. The dispersed nature of the Kex2-containing compartments is consistent with the failure, in EM studies of yeast cells, to detect stacked cisternae, or any other vesicular complex, at a consistently perinuclear location (Novick et al., 1981; Svoboda and Necas, 1987; Redding, K., and R. S. Fuller, unpublished observations).

The effects of the *sec11* and *sec18* mutations demonstrate that Kex2 protein normally progresses from the ER to the Golgi complex. Indeed, the *sec18* mutation prevents processing of pro-α-factor (Julius et al., 1984), and both the *sec11* and the *sec18* mutations block post-ER processing of glycosyl chains present on Kex2 protein (Wilcox, C. A., and R. S. Fuller, manuscript submitted for publication). Conversely, the absence of Kex2 protease from secretory vesicles is apparent from the failure of the *sec1* mutation to perturb the normal distribution of Kex2 protein. These results also argue against obligatory transport of Kex2 protease to and recycling from the plasma membrane. Degradation of Kex2 protein in secretory vesicles en route to the plasma membrane can also be ruled out. The lifetime of the protease is long compared to the rate of secretion of mature α-factor, and degradation of Kex2 protein both at 30 and at 37°C requires vacuolar proteases and occurs in the vacuole (Wilcox, C. A., K. Redding, and R. S. Fuller, manuscript in preparation).

The punctate distribution of Kex2 protein accumulated at the *sec7* and *sec14* mutant blocks is consistent with localization of the enzyme to an element of the yeast Golgi apparatus. Indeed, we have found that 80% of the sites of Kex2-specific immunofluorescence coincide precisely with sites of localization of Sec7 protein (Franzusoff et al., 1991). Punctate fluorescence has also been observed for Ypt1 protein, whose homologue is thought to be localized to the cytosolic face of the Golgi structure in mammalian cells (Segev et al., 1988). The more subtle effects of the *sec7* and *sec14* mutations on the Kex2-staining pattern may reflect both alterations in Golgi structure and the accumulation of Kex2 protein in earlier Golgi cisternae. Indeed, both the *sec7* and *sec14* mutations prevent maturation of the Ser/Thr-linked oligosaccharides of Kex2 protein, implying that the protein normally enters later Golgi compartment(s) in which these modifications occur (Wilcox, C. A., and R. S. Fuller, manuscript submitted for publication).

By analogy to mammalian cells, the compartment in which Kex2 protein resides might be the yeast equivalent of the *trans*-Golgi network (TGN). Efficient intracellular retention of Kex2 protease requires both its COOH-terminal cytosolic tail and a functional gene encoding clathrin heavy chain, suggesting interaction of Kex2 protein with coated pits (Fuller et al., 1989*b*; Payne and Schekman, 1989). In mammalian cells, clathrin is associated with coated pits and vesicles not only at the plasma membrane, but also at the TGN (Brodsky, 1988). In neuroendocrine cells, processing reactions analogous to those catalyzed by Kex2 protease proceed in nascent secretory granules that form at the TGN. The nascent granules contain a partial clathrin coat that is absent from mature granules (Tooze and Tooze, 1986; Orci et al., 1987), suggesting the possibility of clathrin-dependent recycling of some granule components to the TGN. Thus, given the absence of Kex2 protease from secretory vesicles, the most likely role of clathrin in intracellular retention of the enzyme might be either in static retention of Kex2 protein in the TGN, or in recycling of the enzyme back to the TGN from closely associated nascent secretory vesicles.

The perinuclear localization of the mammalian Golgi ap-

paratus is probably due to association with microtubules, because disruption of the microtubule network results in dispersal of Golgi structures throughout the cytoplasm (reviewed in Kreis, 1990). Thus, although the significance of the constitutively dispersed nature of the yeast Golgi apparatus is unclear, it might be explained by a lack of stable association of the organelle with microtubules. Certain other aspects of Golgi function must be highly conserved, given that two mammalian factors have been identified as functional homologues of yeast *SEC* gene products (Wilson et al., 1989; Clary et al., 1990).

Like the Kex2-containing compartment, mitochondria (Stevens, 1981), vacuoles (Weisman et al., 1987), and ER (Preuss, D., J. Mulholland, and D. Botstein, personal communication) appear within newly formed buds soon after bud emergence. Appearance of the Kex2-containing compartment in small buds may be due to segregation of existing organelles or to *de novo* assembly of structures. The clustering of Kex2-specific fluorescence in the tips of pheromone-induced projections may provide a system for analyzing this process. In such cells, Kex2-fluorescence was frequently absent from the cell body, strongly suggesting transport of existing Golgi compartments from the cell body to the projection (examples in Fig. 4, *D* and *F*). Clustering of the Kex2-compartment at the projection tip may be required for segregation of the organelle to the new daughter cell that forms at the tip upon recovery from pheromone-induced G_1 arrest. Indeed, clustering of Kex2-specific fluorescence frequently coincided with the apparent migration of both nuclei and mitochondria into projections (data not shown).

The sandwich-amplification procedure used here to detect Kex2 protein in wild-type cells may be generally useful for visualizing inabundant antigens in yeast. This procedure has been used successfully to visualize both the *VPS1* (Rothman et al., 1990) and *SIR2* gene products (Pillus, L., and J. Rine, personal communication).

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