

Identification of Coated Vesicles in *Saccharomyces cerevisiae*

SUSETTE C. MUELLER and DANIEL BRANTON

Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138. Dr. Mueller's present address is Department of Cell Biology and Anatomy, Johns Hopkins School of Medicine, Baltimore, Maryland 21205

**ABSTRACT** Clathrin-coated vesicles were found in yeast, *Saccharomyces cerevisiae*, and enriched from spheroplasts by a rapid procedure utilizing gel filtration on Sephacryl S-1000. The coated vesicles (62-nm diam) were visualized by negative stain electron microscopy and clathrin triskelions were observed by rotary shadowing. The contour length of a triskelion leg was 490 nm. Coated vesicle fractions contain a prominent band with molecular weight of ~185,000 when analyzed by SDS PAGE. The presence of coated vesicles in yeast cells suggests that this organism will be useful for studying the function of clathrin-coated vesicles.

The function of clathrin-coated vesicles has been studied most extensively in relation to the selective uptake of proteins (6, 14, 16), but coated vesicles may also be involved in the secretion and intracellular transfer of newly synthesized or recycled proteins and membranes (5, 16, 17). Coated vesicles have been isolated from tissues specialized in secretion (13), and they have also been visualized in secretory cells by electron microscopy (3, 4, 8, 16).

Because baker's yeast, *Saccharomyces cerevisiae*, lends itself to genetic and molecular biology manipulations, it could be an ideal eucaryotic cell in which to investigate the secretory role of coated vesicles. Schekman and collaborators (18) have described the use of yeast mutants to study the path of protein secretion, but initial attempts to identify coated vesicles in *Saccharomyces cerevisiae* have not been successful (R. Schekman, personal communication). Although electron microscopy of intact cells led to the first identification of coated vesicles in mosquito oocytes (15) and several fungal species (1, 7), none of the many electron microscopic studies of yeast have reported seeing coated vesicles in the intact cell. We therefore sought for coated vesicles in yeast homogenates.

Pearse (12) first purified coated vesicles from porcine brain homogenates and named the principal coat polypeptide clathrin. The assembly unit of the coat is a triskelion shaped molecule (21) that comprises three clathrin heavy chains,  $M_r = 180,000$ , and three clathrin light chains,  $M_r \approx 30,000$ – $35,000$  (22). The distinctive morphology of triskelions has been visualized by low angle rotary shadowing (21) and by negative staining (9, 21). Using the approaches developed with brain coated vesicles, we have now identified coated vesicles and triskelions in yeast cells.

## MATERIALS AND METHODS

Zymolyase 60,000 was obtained from Miles Laboratories, Inc. (Elkhart, IN), Sephacryl S-1000 from Pharmacia, Inc. (Piscataway, NJ), yeast extract and

Bacto-peptone from Difco Laboratories, Inc. (Detroit, MI), and RNAase (Sigma II, A, protease-free), morpholine-ethane-sulfonic acid (MES),<sup>1</sup> glucose, sorbitol, Tris, dithiothreitol (DTT), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Materials for SDS PAGE were from Bio-Rad Laboratories (Richmond, CA).

*Saccharomyces cerevisiae* RX-11 (a gift from R. Schmidt, Freie Universität, Berlin) or *S. cerevisiae* BJ926 (a gift from E. Jones, Carnegie-Mellon Institute) were grown to  $\sim 8 \times 10^7$  cells/ml at 30°C with shaking (125 r/min) in 1 liter batches. Cells from 3 liters were sedimented by centrifugation (5 min, 1,000 g) and washed twice with distilled water. The cells were resuspended and incubated in 1 liter of 0.8 M sorbitol, 10 mM Tris pH 7.5, 10 mM CaCl<sub>2</sub>, 2 mM DTT, 0.6 mM PMSF, and 10 µg/ml Zymolyase 60,000 at 30°C for 30–45 min shaking at 80 r/min. Spheroplast formation was monitored using phase contrast microscopy.

Spheroplasts were sedimented by centrifugation (15 min, 200 g) and washed twice with 0.8 M sorbitol, 100 mM MES pH 6.5, 0.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.2 mM DTT, 0.6 mM PMSF at 4°C. The spheroplasts were then hypotonically lysed in 500 ml of 100 mM MES pH 6.5, 0.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.02% NaN<sub>3</sub>, 0.2 mM DTT, 0.6 mM PMSF, 0.1 mg/ml RNAase for 30 min at 30°C on a rotary shaker.

The yeast lysate was centrifuged for 30 min, 4°C, at 21,000 g. The pellet was discarded and the supernatant centrifuged for 60 min at 100,000 g, 4°C. The resulting high speed membrane pellet was resuspended in an isolation buffer containing 100 mM MES pH 6.5, 0.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.02% NaN<sub>3</sub>, 0.2 mM DTT (Buffer A in reference 12) with 0.6 mM PMSF and homogenized in a glass Dounce homogenizer.

The resuspended high speed pellet was loaded onto a 1.5 × 10<sup>7</sup> cm Sephacryl S-1000 column pre-equilibrated in isolation buffer. Fractions were eluted at 16 ml/h, 5.5 ml each, and monitored at OD<sub>280</sub>.

Negative staining was performed by floating a carbon film (3–4 nm thick) onto a solution containing freshly diluted (in isolation buffer) membrane samples by the technique of Valentine et al. (23). The carbon film was transferred to a drop of 2% uranyl acetate, picked up on a 400-mesh copper grid, and blotted from the edge with filter paper.

For rotary shadowing, samples from the S-1000 column fractions that contained coated vesicles were diluted 1:11 in 70% glycerol, 155 mM ammonium acetate, pH 6.5 resulting in a final protein concentration of ~20–30 µg/ml. Samples in glycerol were sprayed onto freshly cleaved mica and rotary shadowed at an angle of 5.5° with platinum-carbon (19).

<sup>1</sup> Abbreviations used in this paper: DTT, dithiothreitol; MES, morpholino-ethane-sulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

Negatively stained and rotary shadowed samples were photographed using a Philips 301 microscope operating at 60 or 80 kV.

Polypeptides were analyzed on SDS polyacrylamide gradient gels (6–15%) using the discontinuous buffer system of Laemmli (10). Gel samples were solubilized at 37°C for 20 min. Gels were stained using Coomassie Brilliant Blue R250. To estimate what fraction of the total protein the putative clathrin constituted, polypeptide bands were excised from the Coomassie-Blue stained gels and the stain was eluted from the gel pieces with 25% (vol:vol) pyridine in H<sub>2</sub>O as described by Fenner et al. (2). The absorbance of the eluted stain was measured at 605 nm.

## RESULTS

When the high speed pellet from a yeast homogenate was examined after negative staining, structures appearing to be coated vesicles were sometimes observed (Fig. 1*a*) but they were frequently aggregated with other particles. This was true whether we examined the pellet from strain RX-11 or strain BJ926. Preliminary attempts to purify these coated vesicles by sedimentation in sucrose gradients were unsuccessful, probably because of the extensive aggregation that occurs at pH 6.5, the pH required to stabilize coated vesicles.

Better separation of coated vesicles was obtained when the high speed pellet was fractionated on a Sephacryl S-1000 column (Fig. 2, which shows the elution profile typical of strain BJ926). The elution profile from strain RX-11 was similar except that the second peak, fractions 34–38, was absent.

Material eluting in the void volume of the S-1000 column contained large, smooth vesicles (average size 190 nm). Fractions collected just prior to the coated vesicle fractions contained smooth vesicles with average size 90 nm ± 31 nm (SD). An example from strain RX-11 is shown in Fig. 1*b*. The fractions observed to contain coated vesicles (fractions 28–36) were pooled and concentrated by centrifugation (strain RX-11, Fig. 1*c*). The average size of the coated vesicles obtained was 62 ± 8 nm (SD) in both strains. Smooth vesicles contaminating these fractions were, on average, 81 nm ± 19 nm (SD). Fractions eluting just after the coated vesicles contained smooth vesicles with average diameter 37 nm ± 0.3 nm (SD) and various smaller particles (strain BJ926, Fig. 1*d*). The peak of 37-nm vesicles (second peak, fractions 34–38 in Fig. 2) was evident in strain BJ926 but not in RX-11 cells. Thus, the coated vesicle fractions from the wild type RX-11 strain shown in Fig. 1, *a–c* are less contaminated with smooth vesicles (Fig. 1*d*) seen in the corresponding fractions from strain BJ926.

It is striking that a large number of the coated vesicles seem to have lost their coats during the negative staining procedure (Fig. 1*c*). Some cages seem to have disassembled, forming a ring of coat material on the carbon film around the vesicle. (Calf brain coated vesicles do not lose their coats during negative staining even when prepared at dilute concentrations.) The naked vesicles (strain RX-11, Fig. 3*a*) (40 nm ± 6 nm) are similar in appearance to brain coated vesicles that have been purposely stripped of their clathrin coats (20).

We reasoned that yeast coated vesicles may fall apart because of proteolysis. In calf brain coated vesicles, both the clathrin light chains and a 100,000-dalton membrane protein were very susceptible to proteolytic digestion. When the 100,000-dalton protein of intact calf brain coated vesicles was selectively cleaved with elastase, the clathrin heavy chains remained intact but the clathrin-coats tended to fall off the vesicle during negative staining (our unpublished observation). To guard against the possibility of proteolysis in yeast preparations, the BJ926 mutant cell line was used. These cells have only 5–10% of wild type protease activity (E. Jones, personal communication). PMSF was included routinely in all of the buffers, including the spheroplast wash buffer, since the wall digesting enzyme preparation contains protease contaminations. No proteolysis was detected by SDS PAGE in brain coated vesicles when they were incubated with the crude yeast fractions for at least 2 h at 37°C. But even with the use of the BJ926 cell line and PMSF, many coated vesicles still fell apart on the carbon film (strain BJ926, Fig. 3*b*).

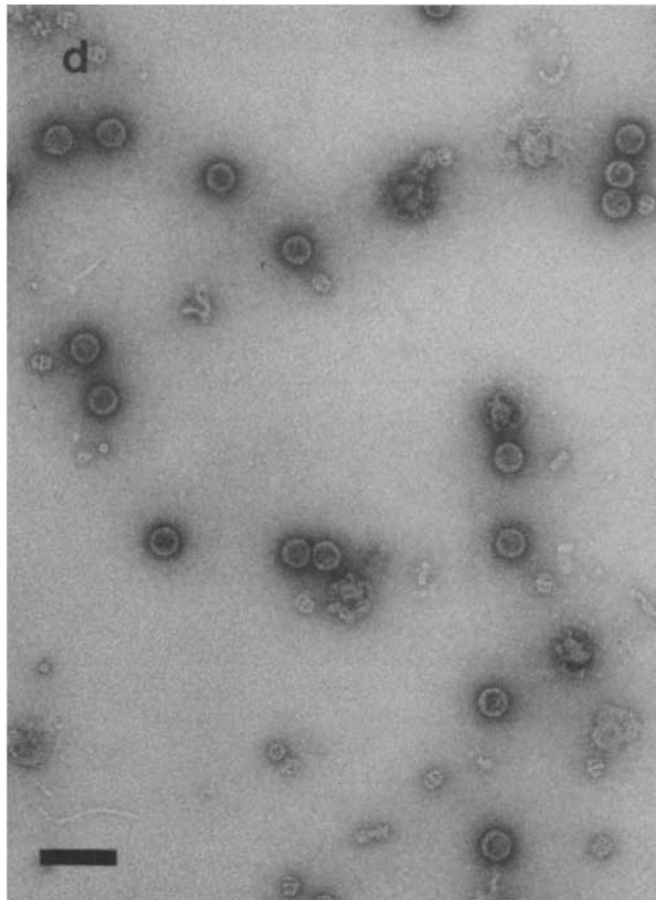
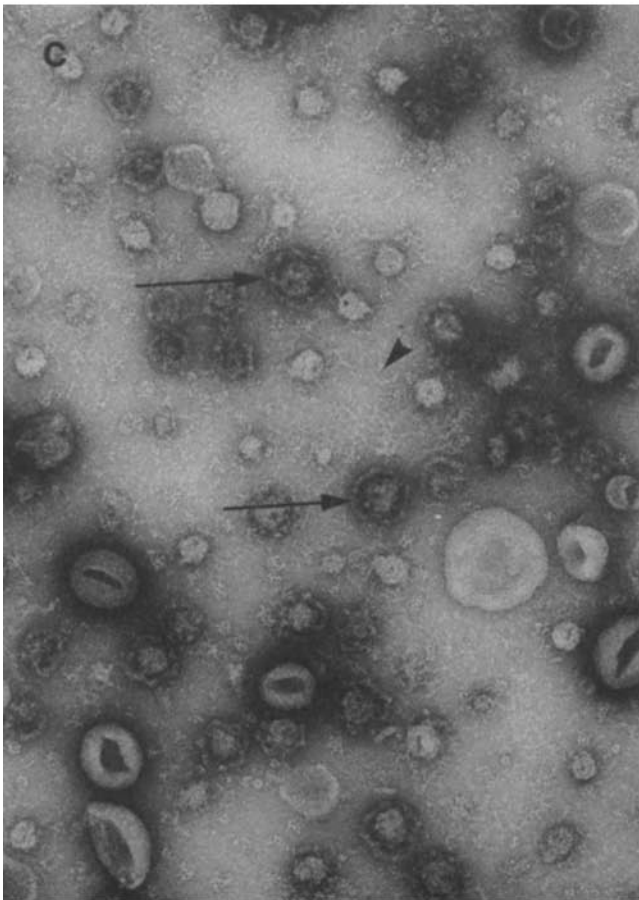
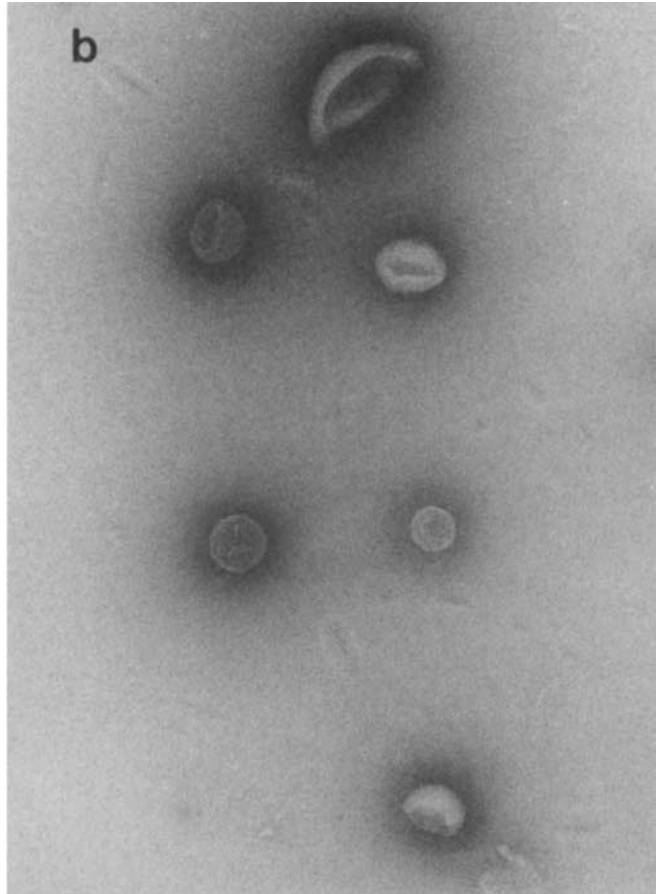
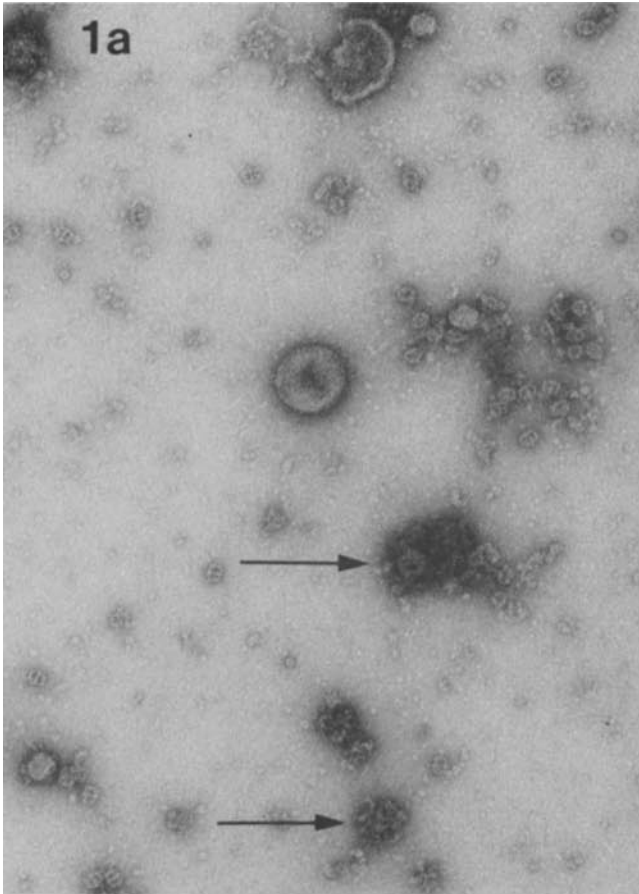
As with brain coated vesicles, yeast coated vesicles disassembled into triskelions and naked vesicles when they were mixed with glycerol, sprayed onto mica, and rotary shadowed (strain BJ926, Fig. 3*c*). The contour length of the triskelion leg of yeast was 490 Å ± 35 Å (for example, S-1000 fraction 30, strain BJ926, Fig. 4), whereas that reported for brain triskelions was 445 Å ± 23 Å (21).

When examined by SDS PAGE, the Sephacryl column fractions containing the maximum concentration of electron microscopically visible coated vesicles coincided, in both strains RX-11 and BJ926, with the maximum density of a Coomassie Blue-stained band that always ran slightly behind the corresponding clathrin band from brain coated vesicles (strain BJ926, Fig. 5). Polypeptides with approximate apparent molecular weights of 110,000, 90,000, 60,000–50,000, 37,000, and 34,000 also co-purified with the coated vesicles. Other polypeptides with approximate apparent molecular weights of ~220,000 and ~80,000 did not co-purify with the peak of the coated vesicle fraction but were present, presumably as contaminants.

Spectrophotometry of pyridine extracts of the Coomassie-stained bands showed that the ~185,000-mol wt polypeptide comprises <0.3% of the protein contained in the crude supernatant of the low speed spin (Fig. 5, lane *c*), ~3.0% in the high speed pellet (lane *e*), and 12.0% in the pooled coated vesicle fractions (29–34, lanes *g–e*) in strain BJ926. Estimates based on these percentages and the measured protein concentrations applied to the gels indicate that ~1 mg of the 185,000-dalton clathrin polypeptide was obtained from 2 × 10<sup>11</sup> cells of either strain (4 liters of mid log-phase culture) after processing through the S-1000 column).

Coated vesicles comprised ~80% of the vesicles visible in the pooled, negatively stained coated vesicle fractions from strain RX-11 and a considerably lesser percentage of the vesicles in the pooled fractions from strain BJ926. If we

FIGURE 1 Negatively stained yeast membrane fractions. (a) The high speed membrane pellet from strain RX-11. Possible coated vesicle profiles (arrows) are obscured by contaminating particles. (b) Material from RX-11 that eluted at the equivalent of fraction 22 of the elution profile shown in Fig. 2. The average size of the smooth vesicles was 90 nm. (c) Pooled coated vesicle fractions from RX-11, the equivalent of fractions 28–36 of Fig. 2. Note that in addition to many coated vesicles (solid arrows) and some larger smooth vesicles (81 nm ± 19 nm) there are many vesicles that we presume have lost their coats (40 nm ± 6 nm). Coat material is observed in the background adjacent to these stripped vesicles (arrowhead). (d) Fraction 34 from yeast strain BJ926. This fraction from BJ926 contains many smooth vesicles (37 nm ± 0.3 nm) not seen in strain RX-11 preparations as well as other smaller particles common to both preparations. Bar, 100 nm. × 100,000.



assume that clathrin in yeast constitutes 65% of the coated vesicle protein, as it does in calf brain coated vesicles, then our preparations with 12% of the protein in clathrin (such as obtained from strain BJ926, Fig. 5) must contain 80% contaminating protein.

## DISCUSSION

Our results show that coated vesicles can be enriched from a wild type and a protease deficient yeast mutant by a rapid procedure utilizing gel filtration. In one step, gel filtration of the crude membrane extract rapidly removed many contaminating particles and membranes. This technique has the added advantage of avoiding osmotic stress on the vesicles that is introduced by sucrose density gradient separations. And, at the pH required to stabilize coated vesicles (pH 6.5), yeast membranes aggregate extensively, particularly in the presence of sucrose.

Column fractions containing coated vesicles were highly enriched with a polypeptide that we assume is yeast clathrin but that always migrated somewhat more slowly than brain clathrin, with an approximate apparent molecular weight of

185,000. Clathrin from higher plants has also been observed to have a molecular weight greater than that of mammalian clathrin (11). The somewhat greater apparent molecular

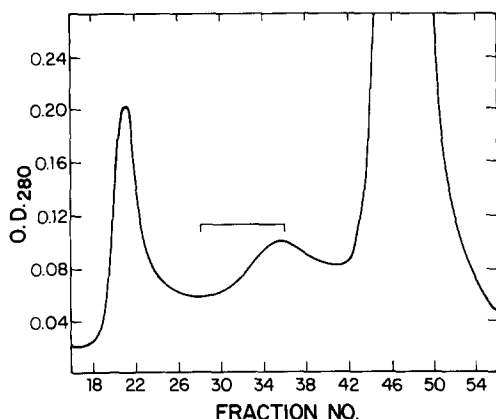


FIGURE 2 Sephacryl S-1000 elution profile of the yeast membrane fractions. The resuspended high speed membrane pellet from *S. cerevisiae* BJ926 was loaded onto an S-1000 column. The column was eluted with Buffer A (12) and 5.5-ml fractions were collected. Coated vesicles were detected by negative staining in fractions 28–36. The elution profile using RX-11 was similar except that the second peak (fractions 32–38) was much less pronounced.

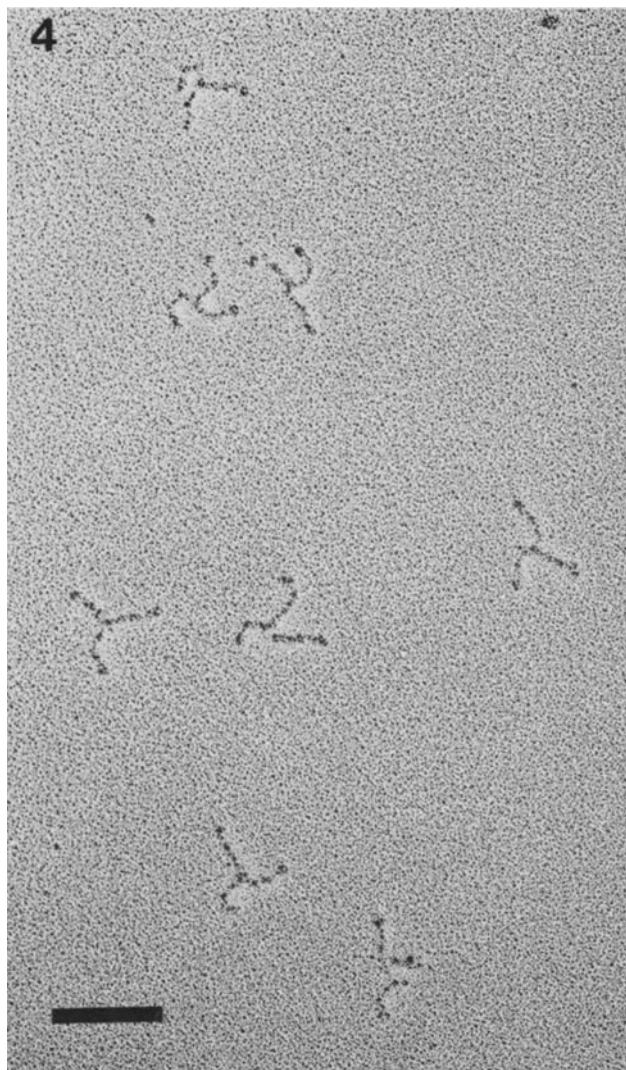


FIGURE 4 Low-angle rotary shadowed yeast triskelions obtained by spraying fraction 30 have leg contour length of  $492 \pm 35 \text{ \AA}$ . Bar, 100 nm.  $\times 150,000$ .

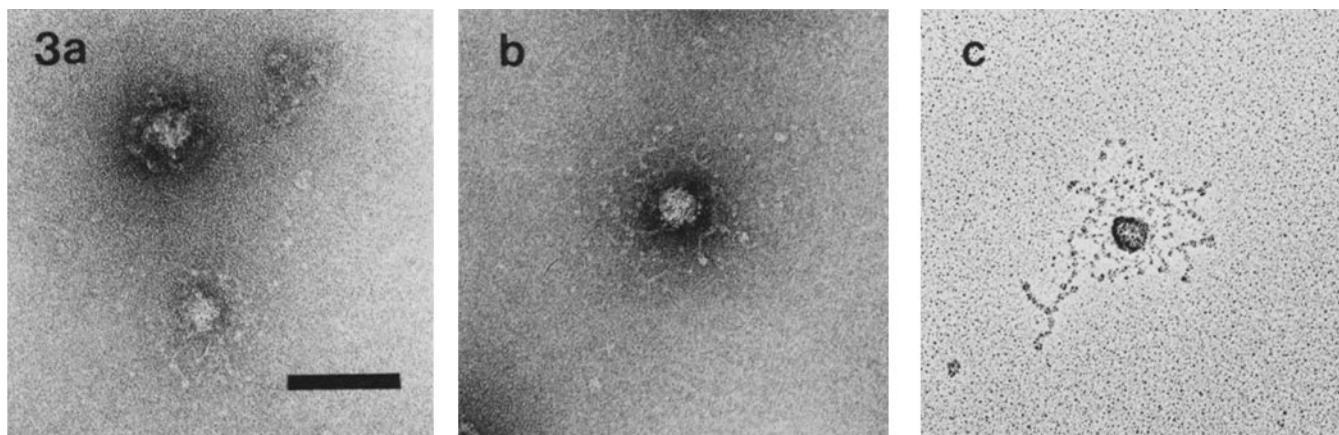


FIGURE 3 Yeast coated vesicles tend to lose their coats during preparation for electron microscopy. (a and b) Negatively stained coated vesicle preparations show vesicles with dissociating coats surrounding them from RX-11 (a) and BJ926 (b). (c) Coated vesicles that are not chemically cross-linked will lose their coats when they are sprayed in glycerol onto mica, dried, and rotary shadowed (BJ926). The disassembled coat material consists of triskelions. Bar, 130 nm.  $\times 150,000$ .

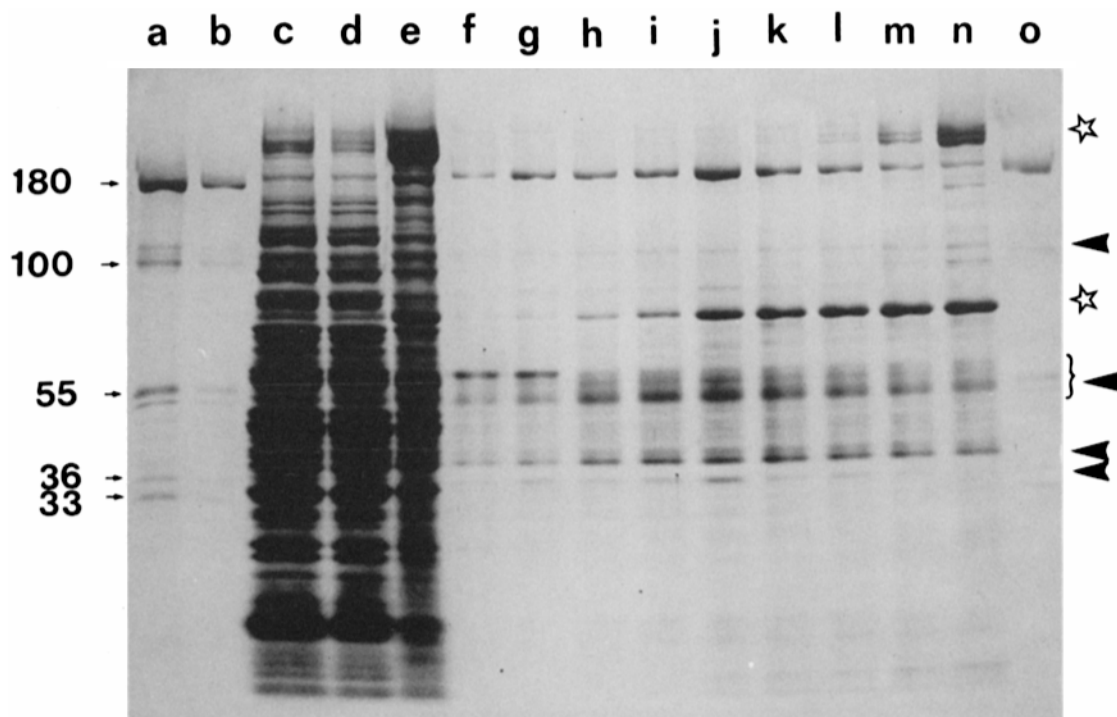


FIGURE 5 SDS PAGE of the crude strain BJ926 yeast fractions and column eluted material. The apparent molecular weights of brain coated vesicle polypeptides (lanes a, b, and o) are compared with the yeast low speed supernatant (lane c), the high speed supernatant (lane d), the high speed membrane pellet (lane e), and column fractions 28–36 (lanes f–n, respectively). The fraction containing the maximum numbers of coated vesicles by electron microscopy coincides with the peak of the 185,000-mol-wt polypeptide in lane j. Polypeptides with approximate molecular weights of 110,000, 90,000, 60,000–50,000, 37,000, and 34,000 (arrowheads) in lanes f–n also co-purified with the coated vesicles, whereas polypeptides with approximate molecular weights of ~200,000 and 80,000 (stars) did not. Values at left indicate the molecular weight  $\times 10^3$ .

weight of yeast clathrin is consistent with the observation that the contour length of a yeast triskelion leg was longer than that of a calf brain triskelion leg, but differences in the mass per unit length of yeast and calf triskelions cannot be ruled out.

Attempts to compare yeast and brain clathrin by two dimensional peptide mapping did not indicate extensive overlap of peptides. A rabbit anti-calf brain triskelion antibody prepared in our laboratory did not bind to either triskelion heavy chains or light chains that had been transferred to nitrocellulose after electrophoresis of SDS polyacrylamide gels.

The diameter of the yeast coated vesicles (62 nm) corresponds to the smallest brain coated vesicles isolated (13). They are the approximate size of Golgi-associated coated vesicles but smaller than coated vesicles usually derived by endocytosis (5). Small coated vesicles associated with Golgi elements have previously been observed by thin section electron microscopy in other fungal cells (1, 7). Thin section studies of yeast cells have not been successful (R. Schekman, personal communication), so that the possible intracellular source of the coated vesicles isolated from yeast is uncertain.

Several proteins appeared to co-purify with coated vesicles from yeast, in addition to the 185,000-dalton polypeptide. More extensive purification of the coated vesicles will help to determine whether these polypeptides are components of triskelions, the vesicle surface, or the vesicle contents. Our simple procedure using an S-1000 column yielded an enriched preparation of yeast coated vesicles. A more complete purification of these coated vesicles should facilitate the study of

these structures in relation to the synthesis and transport of secretory molecules in yeast.

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