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Endocytosis in *Saccharomyces cerevisiae*: internalization of enveloped viruses into spheroplasts

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When vesicular stomatitis virus was incubated with *Saccharomyces cerevisiae* spheroplasts at 37°C, part of the virus was internalized by the spheroplasts as shown by the following criteria. (i) The spheroplast-associated virus was protected from proteinase K digestion, which releases surface-bound virus by degrading the envelope glycoproteins. (ii) The spheroplast-associated virus was resistant to mild Triton X-100 treatment, which readily solubilizes the virus. The same results were obtained with Semliki Forest virus. Internalization of the two viruses followed linear kinetics up to 90 min at 37°C. Internalization was concentration- and temperature-dependent. At 11°C no uptake could be detected for at least 2 h. Homogenization and organelle fractionation protocols were designed for the *S. cerevisiae* spheroplasts to study the compartments into which the virions were internalized. Three compartments containing both marker viruses could be separated in density gradients. One coincided with vacuole markers, one banded at a slightly higher and one at a similar density to the plasma membrane markers. Thus, *S. cerevisiae* spheroplasts appear to have the capability of endocytosing particulate markers like viruses. The companion paper describes internalization of two soluble macromolecules, α-amylase and fluorescent dextran, into intact cells.

Key words: endocytosis/membrane traffic/*Saccharomyces cerevisiae*/Semliki Forest virus/vesicular stomatitis virus

Introduction

The eucaryotic cell contains membrane-bound compartments, organelles, which have their own stuctural and functional identity. In the animal cell many organelles appear to communicate with each other by vesicular traffic (Palade, 1983). Vesicles pinch off from the membrane of the donor compartment and fuse with that of the acceptor compartment, thus transporting soluble and membraneous material. Exocytosis delivers material from the endoplasmic reticulum *via* the Golgi complex to the exterior of the cell. Endocytosis captures material from the external milieu *via* coated vesicles and endosomes to, for example, lysosomes (Steinman *et al.*, 1983). Procaryotic cells do not have organelles, and thus have no comparable membrane traffic. In the lower eucaryote *Saccharomyces cerevisiae* exocytosis operates according to principles similar to those in the animal cell (Schekman and Novick, 1982). At the present it is not clear whether ingoing membrane traffic occurs in the yeast cell. Internalization of proteins by yeast cells and spheroplasts has been studied previously (Schlenk and Dainko, 1965; Yphantis *et al.*, 1967; Ottolenghi, 1967; Shvila *et al.*, 1969). Though inconclusive, the data suggested that yeast might have endocytotic capacity. *S. cerevisiae* has a prominent cell wall consisting mainly of glucan chains and intercalating mannoproteins (Ballou, 1976; Schekman and Novick, 1982). To overcome the problems caused by the cell wall, notably restriction of diffusion, I decided to start by looking whether spheroplasts would internalize extracellular markers. In the absence of the cell wall, enveloped viruses could be used as internalization markers. They have been successfully used to study endocytosis in animal cells (Helenius *et al.*, 1980; Helenius and Marsh, 1982). In the present paper I show that vesicular stomatitis virus (VSV) and Semliki Forest virus (SFV) were internalized into intracelullar compartments of *S. cerevisiae* spheroplasts. In the companion paper soluble macromolecule markers were used to show that internalization was not restricted to spheroplasts but occurred also in cells with an intact cell wall.

Results

Preparation of spheroplasts

S. cerevisiae was grown to early logarithmic phase and digested with zymolyase to degrade the cell wall for exposure of the underlying plasma membrane. Spheroplasts rather than intact cells were used for virus uptake studies, since the cell wall cannot be penetrated by virions. The yeast cells were labelled with [^{3}H]mannose to optimize the zymolyase digestion conditions (Figure 1). An incubation of 45 min at 37°C or 30°C with 20 μg/ml of the enzyme released the maximum amount of [^{3}H]-mannose label from the cells. The label is incorporated into mannoproteins which reside in the cell wall and are released by zymolyase (Ballou, 1976). About 38% of the label could not be released from the spheroplasts, representing, most likely, the internal pool of the label, plasma membrane glycoproteins and perhaps remnants of the cell wall components. Spheroplasts collected after 45 min of incubation at 37°C with zymolyase were used throughout the present study. At the light microscopic level

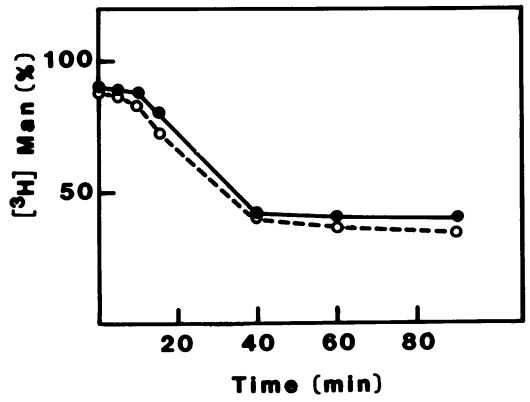

Fig. 1. Optimization of spheroplasting conditions. *S. cerevisiae* cells were labeled with [^{3}H]mannose and subjected to zmyolyase digestion (20 μg/ml) at 37°C (○——○) or at 30°C (●——●). At different time points, released cell wall components were separated by centrifugation for 5 min at 3000 r.p.m. from cells which were counted for radioactivity. The radioactivity lost from the cells was recovered in the supernatant.

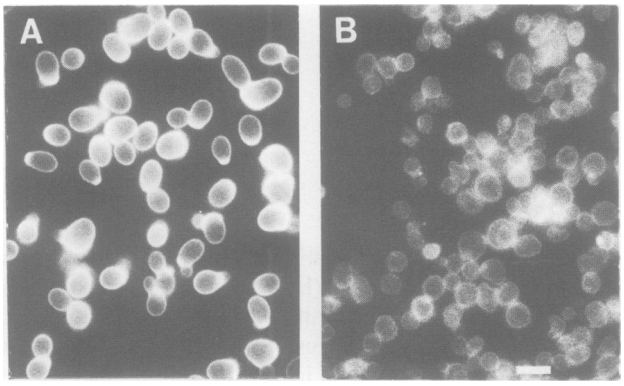

Fig. 2. Morphology of spheroplasts. *S. cerevisiae* cells (**A**) and spheroplasts (**B**) were fixed with formaldehyde and stained with FITC-conjugated concanavalin A. Bar: 5 μm, magnification x870.

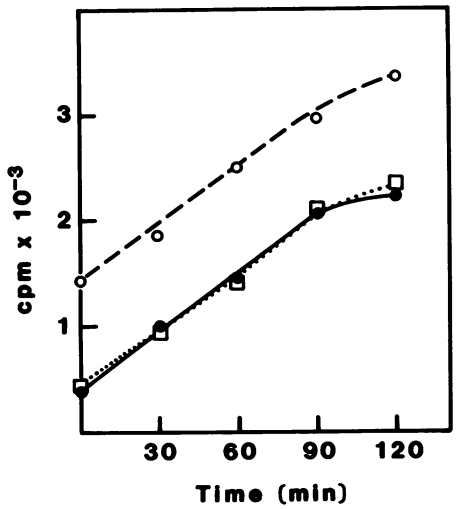

Fig. 3. Association of VSV with spheroplasts. 1.6 x 10^6 c.p.m. of [^{35}S] methionine-labeled VSV was mixed with 48 μg of non-labeled VSV (virus protein) and incubated with 4800 x 10^6 spheroplasts in 3.2 ml YPD-sorbitol at 37°C. At different time points, duplicate samples of 100 μl (150 x 10^6 spheroplasts) were removed and suspended into 1 ml of ice-cold PBS-sorbitol, washed twice with 1 ml of cold PBS-sorbitol and counted for spheroplast-associated viral radioactivity (○— —○) by lysing them in a Triton-containing scintillation cocktail. Parallel duplicate samples were digested after the washes with 0.5 mg/ml of proteinase K for 15 min at 0°C (●——●), or treated with 0.1% Triton X-100 for 5 min at 23°C (□. . . .□) and counted then for radioactivity.

the spheroplasts appeared as a reasonably homogeneous population (Figure 2).

Internalization of VSV by spheroplasts

The enveloped viruses VSV and SFV were chosen to serve as internalization markers, since they can be efficiently destroyed while residing at the exterior of cells. Thus, protection from destruction would suggest internalization to have occurred. Spheroplasts were incubated at 37°C with [^{35}S]methionine-labeled VSV in buffered growth medium containing 1.2 M sorbitol. Samples were taken at different time points, the spheroplasts were washed and counted for viral radioactivity. There was an increase of spheroplast-associated radioactivity with time. The increase was linear up to 90 min whereafter it started to level off (Figure 3). There was a considerable background of viral radioactivity at 0°C which could not be washed off. Proteinase K digestion in the cold is known to degrade the envelope glycoprotein G of VSV, thus releasing bound virions from the

Table I. Association of [^{35}S]methionine-labeled VSV with spheroplasts

Material	Fraction of ^{35}S radioactivity
Medium	90%
1. Wash	5%
2. Wash	<1%
3. Wash	<1%
Spheroplasts after third wash	4%
Medium of protease digest	2%
Wash	0%
Protease-digested washed spheroplasts	2%

500 x10^6 spheroplasts were incubated in 0.5 ml of YPD-sorbitol with 430 x 10^6 c.p.m. of [^{35}S]methionine-labeled VSV (5 μg of viral protein). After 90 min at 37°C, duplicate samples of 150 μl were taken, 1 ml of cold PBS-sorbitol was added followed by centrifugation for 3 min at 3000 r.p.m. The supernatant (Medium) was counted for radioactivity. The spheroplasts were then washed three times with 1 ml of cold PBS-sorbitol and the supernatants (wash 1–3) were counted. Thereafter the spheroplasts were lysed and counted for radioactivity. A parallel set of samples was, after the above manipulations, resuspended into 100 μl of PBS-sorbitol and digested at 0°C with 0.5 mg/ml of proteinase K for 15 min. 1 ml of PBS-sorbitol was added followed by centrifugation. The supernatant (medium of protease digest) was counted for radioactivity. The spheroplasts were washed once with 1 ml of PBS-sorbitol and the supernatant as well as the lysed spheroplasts were counted for radioactivity.

cell surface (Matlin *et al.*, 1983; Pesonen and Simons, 1983). When spheroplasts were digested with proteinase K in the cold after incubation with [^{35}S]methionine-labeled VSV, 71% of the background was removed (Figure 3, Table I). Protection of the viral radioactivity from proteinase K digestion suggests that the virus was internalized. The radioactivity which could be released by proteinase K represented virions bound to the spheroplast surface. The amount of [^{35}S]methionine-labeled VSV internalized in 1 h at 37°C was ~1.2% of the added virus per 10^8 spheroplasts (range 0.9–1.6%, n = 8, conditions as in Figure 3). Similar results were obtained for [^{35}S]methionine-labeled SFV.

Protection against Triton X-100 treatment was used as another criterion for internalization. Mild Triton X-100 treatment solubilizes the VSV membrane with the G and M proteins, releasing the nucleocapsid containing the N, NS and L proteins together with the RNA genome (Carroll and Wagner, 1979). When VSV was fused with the spheroplast plasma membrane, and the spheroplasts were thereafter treated with Triton X-100, G protein was extracted almost quantitatively, leaving the other viral components associated with spheroplasts (M.Makarow, in preparation). Thus, if the association between VSV and spheroplasts were to be binding, Triton X-100 would release all viral components and solubilize the virus. If the association were to be fusion of virus with spheroplasts plasma membrane, only G protein would be removed. In the case of internalization all virus components should be protected against the detergent treatment. Similar treatment of mammalian cells with Triton X-100 results in removal of lipids, but retention of most of the plasma membrane proteins with the cytoskeletal framework and the morphology of the cells (Ben-Ze'ev *et al.*, 1979). Spheroplasts were incubated with [^{35}S]methionine labeled VSV at 37°C as before, treated with Triton X-100 and counted for radioactivity. The amounts of viral radioactivity associated with the spheroplast were similar to those after proteinase K digestion (Figure 3). Triton X-100-treated spheroplasts retained their morphology and could be gently pelleted, presumably due to remnants of cell wall components. When the Triton X-100-treated spheroplasts were lyz-

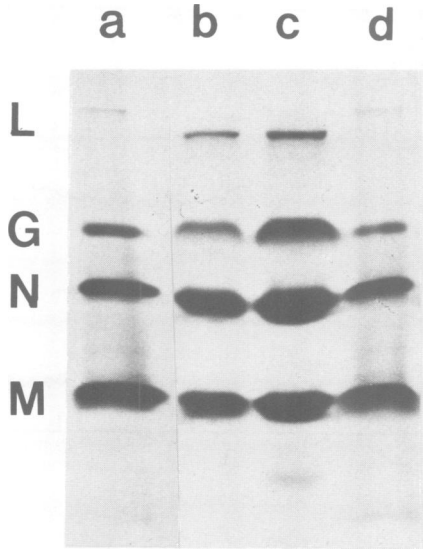

Fig. 4. SDS-PAGE of spheroplast lysates after internalization of [^{35}S]methionine-labeled VSV. 150 x 10^6 spheroplasts were incubated with 200 000 c.p.m. of [^{35}S]methionine-labeled VSV (1 μg of virus protein) in 100 μl of YPD-medium for 1 h (**lane b**) or for 2 h (**lane c**) at 37°C, washed and treated with Triton X-100 to remove external virus (see text), lysed and subjected to SDS-PAGE. **Lanes a** and **d**: marker [^{35}S]methionine-labeled VSV. About 5000 c.p.m. (**lanes a, b** and **d**) and 10 000 c.p.m. (**lane c**) were applied.

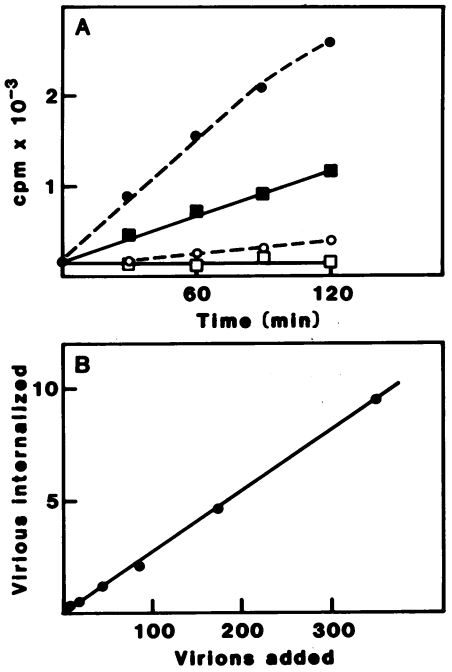

Fig. 5. Characteristics of virus internalization. (**A**) Temperature-dependency. 1800 x 10^6 spheroplasts in 1.2 ml of YPD-sorbitol were incubated with 680 000 c.p.m. of [^{35}S]methionine-labeled VSV (12 μg of protein) at 37°C (●), 30°C (■), 23°C (○) or 11°C (□). Duplicate samples of 100 μl were removed at different time points and counted for spheroplast-associated radioactivity after external proteinase K digestion. (**B**) Concentration-dependency. 0 – 18 μg of unlabeled VSV (protein) was mixed with a constant amount (120 000 c.p.m.) of [^{35}S]methionine-labeled VSV and incubated with 200 x 10^6 spheroplasts in 100 μl of YPD-sorbitol for 90 min at 37°C. 1 μg of VSV corresponds to 4000 x 10^6 particles (Cartwright *et al.*, 1972). The spheroplasts were then treated with proteinase K to remove external virus and counted for radioactivity. From the spheroplast-associated viral radioactivity, the number of internalized virions per spheroplast were calculated and plotted against the number of virus particles added per spheroplast.

ed and subjected to SDS-PAGE, the major viral polypeptides M, N and G were found to be present in the lysate in ratios similar to those in the virus (Figure 4). These results strongly suggest that VSV was internalized by the spheroplasts.

Some characteristics of the uptake of VSV into spheroplasts are shown in Figure 5. Internalization was clearly temperature-dependent. At 30°C the uptake rate was ~35% of that at 37°C. At 23°C internalization started after a lag of 30 min and proceeded slowly thereafter. At 11°C no internalization could be detected for at least 2 h (Figure 5A). Internalization was also dependent on the concentration of VSV in the incubation medium. When spheroplasts were incubated with 10 – 350 virus particles per spheroplast, a constant percentage (2.3 – 2.9) of the added virus was internalized (Figure 5B).

Homogenization of spheroplasts

Triethanolamine and EDTA-containing buffers have been successfully used to lyse animal cells under isotonic conditions (Stahn *et al.*, 1970; Harms *et al.*, 1980). This method was adapted for *S. cerevisiae* spheroplasts, which require 1.0 – 1.2 M sorbitol to be preserved against hypotonic lysis.

Spheroplasts were suspended in TEA-buffer (10 mM triethanolamine and 1 mM EDTA in 10 mM acetic acid, pH 7.2) containing different concentrations of sorbitol, passed 10 times through a small Eppendorf-tip and assayed for lysis. Figure 6A shows that, in the presence of triethanolamine, spheroplasts could be lysed in a sorbitol concentration as high as 0.8 M. Efficient hypotonic lysis occurred only in 0.4 M sorbitol. Lysis by triethanolamine in 0.8 M sorbitol was rapid, being maximal within 15 min at 23°C (Figure 6B). Lysis of *S. cerevisiae* was dependent on prior removal of the cell wall. Intact cells were resistant to lysis. About 50% of the cell wall had to be removed before efficient homogenization was possible (Figure 6C). The yield of lysed spheroplasts varied between 57 and 80% in different experiments. However, when the homogenates containing some unbroken spheroplasts were subjected to centrifugation in the presence of triethanolamine, the rest of the spheroplasts were lysed during the run, resulting in a virtually quantitative homogenization (see below).

Intracellular localization of virus by organelle fractionation

The plasma membrane. A fractionation protocol was designed to separate the vacuole and the plasma membrane compartment to study the subcellular distribution of internalized virus. [^{35}S]-Methionine-labeled VSV was fused with the spheroplast plasma membrane to serve as a marker for this compartment. A similar protocol has been used to mark the plasma membrane of MDCK cells for subcellular fractionation (Pesonen *et al.*, 1984a, 1984b). After fusion the spheroplasts were washed to remove unfused virus, homogenized and subjected to fractionation in self-forming Percoll density gradients. The viral radioactivity in the fractions revealed a major peak containing 90% of the label banding at the density of 1.065 g/cm^3 (Figure 7A). Two minor peaks of 5% each banded at densities of 1.075 and 1.19 g/cm^3. Though the G protein represents only some 15% of the ^{35}S radioactivity of VSV it is very likely that the peak banding at the density of 1.065 g/cm^3 in Figure 7A represents the plasma membrane compartment. The nucleocapsid released by Triton X-100 treatment from VSV migrated in the six top-most fractions when mixed with spheroplast lysates prior to density fractionation (not shown). Moreover, the plasma membrane could also be labeled with

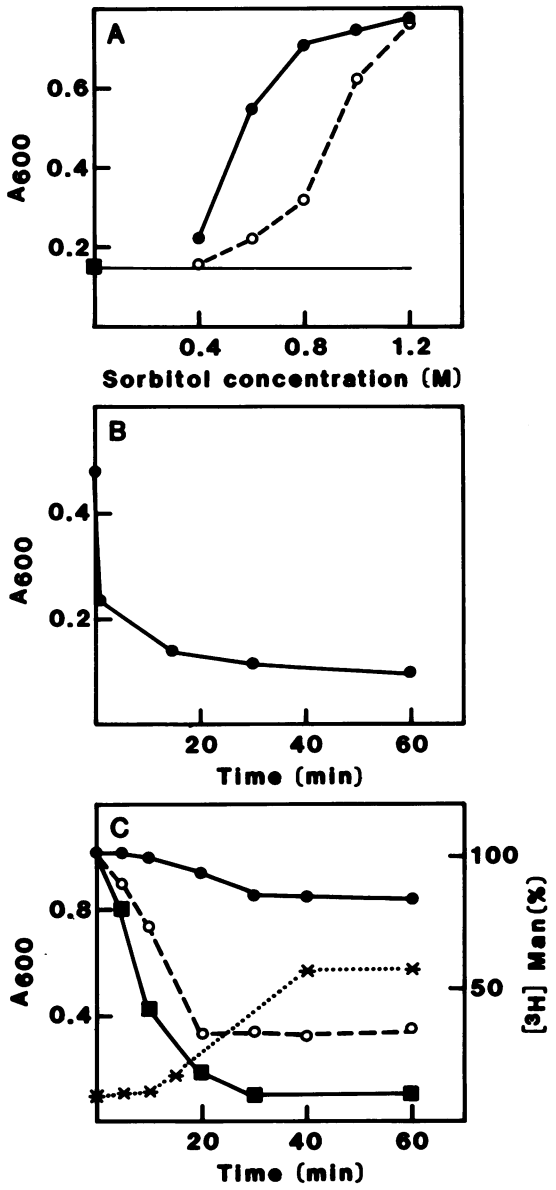

Fig. 6. Homogenization of spheroplasts with triethanolamine.
(A) Dependency on sorbitol concentration. 20 x 10^6 spheroplasts were resuspended into 1.0 ml of TEA-buffer containing different concentrations of sorbitol (○- - -○). Parallel control samples were resuspended in sorbitol lacking triethanolamine (●——●). The preparations were passed 10 times through a small Eppendorf pipette tip, incubated for 15 min at 23°C and assayed for absorbance at 600 nm. One control sample was lysed with 0.2% SDS in water (■). (B) Time-dependency. Spheroplasts were resuspended into TEA-buffer containing 0.8 M sorbitol, passed through the Eppendorf pipette tip as before, incubated for various periods of time at 23°C and assayed for absorbance at 600 nm. (C) Dependency on removal of the cell wall. Cells were labeled with [^{3}H]mannose and subjected thereafter to zymolyase digestion as described in Materials and methods. At different time points, parallel samples were removed and resuspended into 1.2 M sorbitol (●——●), into TEA-buffer containing 0.8 M sorbital (○ – – ○) or into 0.2% SDS (■—■), passed through an Eppendorf pipette tip as before, incubated for 5 min at 23°C and assayed for absorbance at 600 nm. Parallel samples were taken for centrifugation to determine the percentage of ^{3}H-label released by zymolyase into the supernatant (*....*).

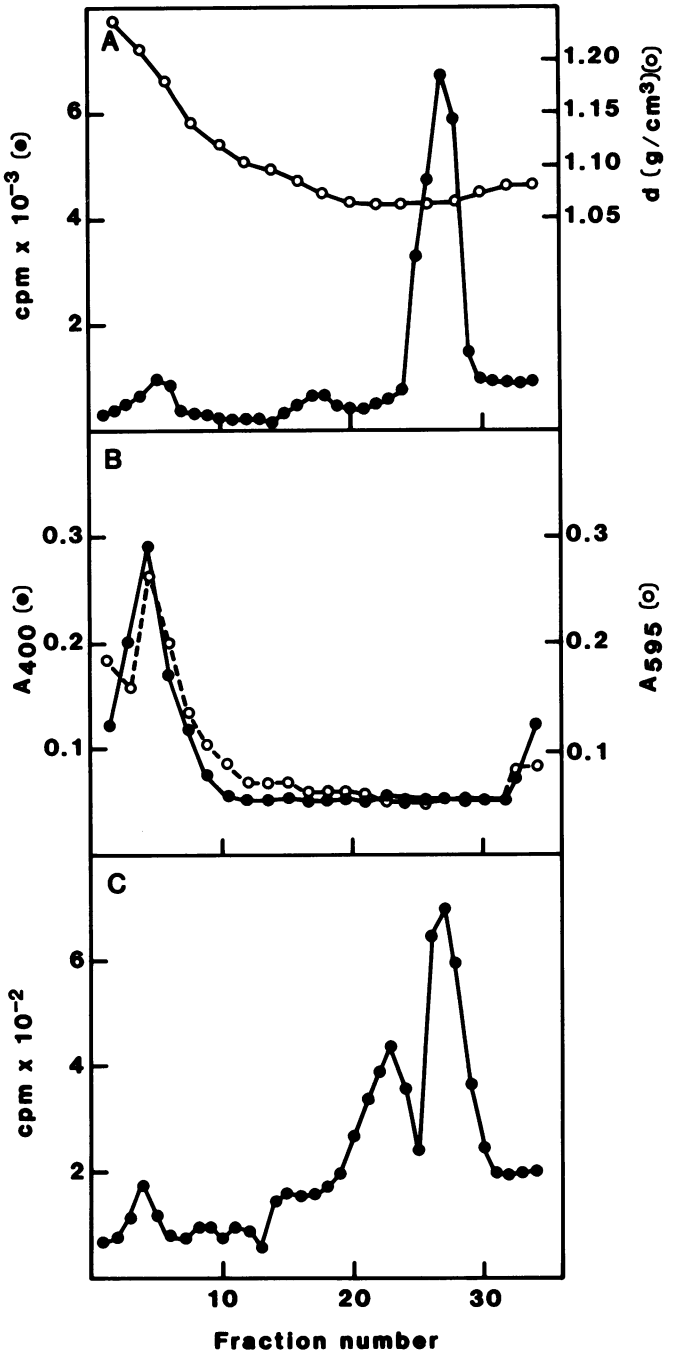

Fig. 7. Subcellular fractionation of spheroplasts with internalized virus.
(A) Plasma membrane. [^{35}S]Methionine-labeled VSV was fused with the spheroplast plasma membrane (200 x 10^6 spheroplasts). Non-fused virions were removed and the spheroplasts were homogenized for fractionation in 20% Percoll density gradients. From the 360 μl fractions, 250 μl were used for counting of viral radioactivity (●——●) (recovery 70–95%) and 100 μl to determine the density (○——○) by weighing. (B) Vacuole. Non-specific protease (○— —○) (recovery 83–91%) and α-mannosidase (●——●) (recovery 77–100%) activities were asayed from the total fractions of 540 μl. The profiles of two parallel gradients (each 400 x 10^6 spheroplasts) are drawn in the same panel (C) Subcellular compartments with internalized [^{35}S]methionine-labeled VSV. 1 x 10^6 c.p.m. of [^{35}S]methionine-labeled VSV (2 μg of protein) was incubated with 200 x 10^6 spheroplasts for 90 min in 100 μl of growth medium, extracellular virus was removed and the spheroplasts were lysed for fractionation. The 360 μl fractions were counted for viral radioactivity (recovery 70–90%).

[^{3}H]concanavalin A. The lectin was bound to the spheroplast surface (Figure 2) in the cold prior to homogenization, followed by density gradient fractionation. The label coincided with the peak of viral radioactivity at the density of 1.065 g/cm^3 shown in Figure 7A (data not shown). [^{3}H]Mannose-labeled cell wall components, which were released from cells by zymolyase digestion, floated on top of the gradient (not shown).

When intact spheroplasts were subjected to centrifugation in

the density gradients, about half of them were lyzed during the run, due to triethanolamine in the gradients. The surviving spheroplasts were sedimented to the bottom (not shown). Since there was very little viral radioactivity in the bottom fractions of gradients containing the lysates of VSV-fused spheroplasts (Figure 7A), those spheroplasts which survived the homogenization procedure (20–43%, see above) appeared to have lysed during the run. Thus, the material in the gradients represented virtually the whole population of spheroplasts subjected to homogenization, without any selection.

The vacuole. To find the position of the vacuolar compartment in the gradients of spheroplast lysates, two markers were assayed, non-specific protease activity and α-mannosidase activity. Both activities have been reported to be perhaps exclusively located in the vacuole (Wiemken *et al.*, 1979). About 90% of the protease activity was in a peak at the density of 1.205 g/cm^3 (Figure 7B). Five to fifteen percent of the activity floated in the top fractions. Most of the α-mannosidase activity coincided with the protease activity and only 3–11% was found in the top fractions. The activities in the top fractions probably represented enzymes escaped from broken vacuoles. Since the proteases are soluble molecules, the vacuole was apparently well preserved during the separation procedure. Internalization of virus into the spheroplasts (see below) did not alter the migration of the plasma membrane or vacuole markers in the gradients.

Subcellular distribution of internalized virus. [^{35}S]Methionine-labeled VSV was incubated with spheroplasts for 90 min at 37°C as before, the spheroplasts were digested with proteinase K in the cold to remove extracellular virus, homogenized and subjected to fractionation in Percoll density gradients. Three main peaks were revealed by viral radioactivity. The first peak (I; fr.4) containing 4–18% of the radioactivity banded at a density of 1.21 g/cm^3 (Figure 7C). Peak I coincided with the vacuole markers. About 25–35% of the radioactivity banded as peak II (fr. 23) corresponding to the density of 1.065 g/cm^3. The major peak (III) (fr. 27) contained 60–70% of the radioactivity and banded very close to the plasma membrane markers. The R_f values (peak fraction/total number of fractions) of peak II varied in six experiments between 0.603 and 0.686 with an average of 0.64. The R_f values of peak III were 0.711–0.800 with an average of 0.74. Peak III appeared not to coincide exactly with the plasma membrane markers. The average R_f value for the plasma membrane was 0.76 (0.730–0.803). Essentially the same results were obtained using [^{35}S]methionine-labeled SFV.

Discussion

Enveloped viruses were used as markers for endocytosis in *S. cerevisiae*, since they have been successfully used to study endocytosis in animal cells (Helenius and Marsh, 1982). The use of viruses as internalization markers required, however, the removal of the cell wall of *S. cerevisiae* to expose the underlying plasma membrane. When incubated with spheroplasts at 37°C, VSV and SFV became protected against degradation with proteinase K and solubilization with Triton X-100. Thus, the viruses resided in locations to which neither the enzyme nor the detergent molecules had access, suggesting that they were internalized by the spheroplasts. The characteristics of the uptake meet those described for endocytosis in the animal cell. Uptake was concentration- and temperature-dependent. The threshold temperature for internalization was 11°C; at 10°C endocytosis ceases in many animal cells (Marsh and Helenius, 1980; Pesonen *et al.*, 1984a; Dunn *et al.*, 1980).

In endocytosis, internalization of markers occurs into intracellular organelles. To study the intracellular distribution of internalized virus, homogenization and subcellular fractionation procedures were adapted for *S. cerevisiae* spheroplasts. Virtually complete lysis was obtained in the presence of triethanolamine and EDTA in high sorbitol concentrations, which preserved at least the vacuole from breaking. In the presence of triethanolamine under isotonic conditions, the animal cell lysosomes remain well protected against lysis (Stahn *et al.*, 1970; Harms *et al.*, 1980). The yeast cell vacuole accounts for many of the functions of the lysosome (Wiemken *et al.*, 1979). The fractionation protocol allowed separation of the vacuole, the plasma membrane and the cell wall remnants. After internalization the virus was found in three compartments. One coincided with the vacuole markers, protease and α-mannosidase, activities. One banded close to the plasma membrane markers. [^{35}S]Methionine-labeled VSV fused into the yeast plasma membrane and [^{3}H]concanavalin A bound to the spheroplast surface prior to lysis served as markers for the plasma membrane. One compartment did not coincide with any of the markers but banded at a higher density than the plasma membrane. About 4–18% of the virus migrated with the vacuole markers after an internalization period of 90 min at 37°C. Either or both of the other two compartments (peaks II and III in Figure 7C) could represent intermediary organelles, operating between the plasma membrane and the vacuole. We are at present studying, with biochemical and electron microscopic techniques, whether internalized VSV fuses with the membrane of this organelles. If that is the case, it would suggest a low pH milieu, which is an important characteristic of the animal cell endosome (Tycko and Maxfield, 1982; Galloway *et al.*, 1983). The fusogenic activity of viral envelope glycoproteins does operate in the yeast system, since VSV can be fused with the *S. cerevisiae* plasma membrane at low pH (M.Makarow, in preparation).

In the animal cell VSV and SFV are adsorbed to the cell surface and concentrated in coated pits. Coated vesicles, containing one or a few viruses, pinch off from the plasma membrane into the cytoplasm. Thereafter the viruses are found in smooth-surface endosomes (Helenius *et al.*, 1980; Marsh and Helenius, 1980). In the low pH milieu the fusogenic activity of the viral envelope proteins is activated and the virion fuses with the membrane of the endosome (White *et al.*, 1980, 1983; Marsh *et al.*, 1983). In this way the nucleocapsid carrying the viral genes is introduced into the cytoplasm to start infection. The envelope proteins implanted into the endosomal membrane end up in the lysosome where they are degraded after fusion of the endosomal membrane with the lysosome membrane (Marsh and Helenius, 1980). From the present data it appears that even *S. cerevisiae* spheroplasts have the capability of internalizing enveloped viruses into intracellular organelles. From the present data it cannot be judged whether the viruses were internalized by fluid phase uptake or adsorptive endocytosis (see accompanying paper). The accompanying paper shows that the capability of endocytosis was not limited to spheroplasts, but occurred also in cells having an intact cell wall. It also appears that endocytosis in spheroplasts was the same phenomenon as endocytosis in cells. Using temperature-sensitive transport-deficient mutant cells, intracellular accumulation of markers was impaired at the restrictive temperature both in cells and in spheroplasts (accompanying paper).

Materials and methods

Growth of yeast cells and preparation of spheroplasts

S. cerevisiae (derivative of S288C) was grown in YPD-medium (growth medium) containing 1% yeast extract (Oxoid, UK), 2% bactopeptone (Difco, USA) and 2% glucose (BHD, UK), at 30°C in a shaker to early logarithmic phase (40 – 150 x 10^6 cells/ml). The cells were collected by centrifugation and washed successively at 23°C with distilled water, 1.2 M sorbitol and 0.1 M Tris in 1.2 M sorbitol with 0.01 M $CaCl_2$, pH 7.56. The cells were suspended into the latter buffer (1000 x 10^6 cells/ml) and zymolyase 60000 (Seikaguku, Japan) was added to a final concentration of 20 μg/ml. After incubation at 37°C the spheroplasts were washed twice with 1.2 M sorbitol and resuspended into the sorbitol (1000 x 10^6 spheroplasts/ml). The spheroplasts require 1.2 M sorbitol in order to be preserved from osmotic lysis. Thus, all media used in conjunction with spheroplasts contained 1.2 M sorbitol unless otherwise stated. [^{3}H]Mannose-labeled cells were prepared by adding 2-[^{3}H]mannose (Amersham International, UK, 11 Ci/mmol) to early logarithmic phase cultures to a concentration of 100 μCi/1000 x 10^6 cells. After an incubation of 2 – 3 h at 30°C, the cells were washed three times with distilled water.

Virus preparations

Unlabeled and [^{35}S]methionine-labeled VSV (Indiana serotype) as well as unlabeled SFV and [^{35}S]methionine-labeled SFV were grown in BHK-21 cells and purified as described in Matlin *et al.* (1983), Kääriäinen and Söderlund (1971) and Oker-Blom *et al.* (1983), respectively.

Microscopy of cells and spheroplasts

Fixation of cells and spheroplasts was carried out for 30 min at 23°C in 3% formaldehyde prepared in phosphate-buffered saline (PBS), pH 7.2. In the case of spheroplasts, the fixative contained 1.2 M sorbitol. The preparations were then washed three times with PBS and incubated for 30 min at 23°C in this buffer containing 0.8 mg/ml of fluorescein isothiocyanate-conjugated succinylated concanavalin A (Sigma, USA). After three washes with PBS-BSA the preparations were resuspended in mounting medium (25 mM sodium barbitol, pH 8.6, containing 107 mM NaCl and 50% glycerol). Preparations were applied on microscopic slides pre-treated with 0.1 mg/ml of polylysine and viewed through a Polyvar (Reichert-Jung, Austria) photomicroscope equipped with a 100x oil immersion objective and filters for fluorescein isothiocyanate fluorescence and Nomarski optics.

Gel electrophoresis

Spheroplasts were lysed in 2% SDS, boiled for 5 min, sheared by forcing 10 times through a narrow needle and subjected to gel electrophoresis in 7.5 – 10% gradient polyacrylamide gels in the presence of SDS (SDS-PAGE) according to Laemmli (1970) overnight at 5 mA. The gels were prepared for autoradiography (Bonner and Laskey, 1974) and exposed to X-O-Mat film (Kodak, USA) at −70°C.

Density gradient centrifugation

Spheroplasts were resuspended into 400 μl of TEA-buffer (10 mM triethanolamine and 1 mM EDTA in 10 mM acetic acid buffer, pH 7.2), containing 0.8 M sorbitol, passed 10 times through a small Eppendorf pipette tip and applied on Percoll (Pharmacia, USA) density gradients. The gradients consisted of a 2.0 ml cushion of 20% Percoll and 60% sucrose in TEA-buffer, and 10.0 ml of 20% Percoll in TEA-buffer containing 1.0 M sorbitol. The gradients were run for 1.25 h at 4°C at 15 000 r.p.m. in a JA-17 rotor in a Beckman J2-21M centrifuge. Fractions of 365 or 540 μl were collected starting from the bottom of the gradients.

Marker assays

Plasma membrane. Trace amounts of [^{35}S]methionine-labeled VSV were fused with the spheroplast plasma membrane in a 5-min incubation at pH 4.0 at 37°C. The details of this procedure are described in M.Makarow (in preparation). 260 000 c.p.m. of N-[acetylated-^{3}H]concanavalin A (33 Ci/mmol, Amersham International, UK) was incubated with 200 x 10^6 spheroplasts for 30 min at 0°C in 100 μl of PBS-sorbitol. 30% of the label was bound to the spheroplast surface. The free label was washed off with cold PBS-sorbitol and the spheroplasts were homogenized for fractionation. Prior to fusion with viruses or incubation with the lectin the spheroplasts were treated for 15 min at 0°C with 0.5 mg/ml of proteinase K. This was because the spheroplasts after virus uptake were always digested with proteinase K in the cold to remove surface-bound virions prior to homogenization. Thus, the plasma membranes of all preparations were comparable.

Vacuole. Non-specific protease activity was assayed by adding to the gradient fractions (0.54 ml) 1.46 ml of 50 mM Tris, pH 7.5, 40 μl of 10% Triton X-100 and Hide Powder Azure (Calbiochem, USA). Incubations were for 16 – 19 h at 37°C in a shaker. The samples were filtrated and the absorbance of the filtrate was read at 595 nm (Rinderknecht *et al.*, 1968). The α-mannosidase (EC 3.2.1.24) activity was assayed by adding to the fractions 10 μl of Triton X-100 and *p*-nitrophenyl-α-D-mannopyranoside (Sigma, USA) to a final concentration of 0.5 mg/ml. Incubations were for 2 – 5 h at 37°C. The reaction was stopped by adding 1.0 ml of 0.2 M Na_2CO_3. The samples were centrifuged at 5000 r.p.m. for 5 min and the absorbance of the supernatant was read at 400 nm (Wiemken *et al.*, 1979). Taking a constant amount of spheroplasts (100 x 10^6 per sample) it was found that the protease assay was linear with time for at least 22 h and the α-mannosidase assay for at least 6 h. Using 25 to 400 x 10^6 spheroplasts, for both assays a linear correlation between signal and amount of spheroplasts was obtained.

Other methods and materials

Scintillation counting was carried out in xylene-Nonidet P-40-Permablend cocktail in an LKB-Wallac 1211 Rackbeta counter. Hepes (N-2′-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) was from Sigma, USA, fungal proteinase K from Merck, FRG and [^{35}S]methionine (1460 Ci/mmol) from Amersham International, UK.

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