***In vitro* fusion between *Saccharomyces cerevisiae* secretory vesicles and cytoplasmic-side-out plasma membrane vesicles**

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The final step in the secretory pathway, which is the fusion event between secretory vesicles and the plasma membrane, was reconstructed using highly purified secretory vesicles and cytoplasmic-side-out plasma membrane vesicles from the yeast *Saccharomyces cerevisiae*. Both organelle preparations were obtained from a *sec 6-4* temperature-sensitive mutant. Fusion was monitored by means of a fluorescence assay based on the dequenching of the lipophilic fluorescent probe octadecylrhodamine B-chloride (R18). The probe was incorporated into the membrane of secretory vesicles, and it diluted in unlabelled cytoplasmic-side-out plasma membrane vesicles as the fusion

process took place. The obtained experimental dequenching curves were found by mathematical analysis to consist of two independent but simultaneous processes. Whereas one of them reflected the fusion process between both vesicle populations as confirmed by its dependence on the assay conditions, the other represented a non-specific transfer of the probe. The fusion process may now be examined in detail using the preparation, validation and analytical methods developed in this study.

Key words: mathematical analysis, membrane fusion, octadecylrhodamine B-chloride (R18), secretion.

INTRODUCTION

Membrane protein transport (and sorting) within and outside of the eukaryotic cell boundary is a complex process based on multiple budding steps of carrier vesicles from one compartment and their specific fusion with the next compartment [1–3]. In the last decade a primary goal for molecular cell biologists has been to elucidate the mechanisms governing these fusion events [4]. But *in vitro* assays that reconstitute membrane trafficking events have provided comparatively little knowledge to a field strongly supported by streams of elegant molecular biology and whole-cell studies [5,6].

An extensive background of knowledge on the secretory pathway has thus emerged from studies using *Saccharomyces cerevisiae*, a well-known lower eukaryote, which offers great versatility for genetic manipulation [7]. In yeast, secretion is a spatially directed event, and the proteins involved in intracellular transport, recognition and fusion events have for the most part been identified, although the specific mechanisms underlying their mode of action remain poorly understood. It is in this context that *in vitro* fusion analysis may provide a complement to our current understanding of this important cellular process.

In the yeast *S. cerevisiae*, cell-free membrane fusion studies between endoplasmic reticulum and Golgi vesicles have been conducted [8,9], as well as homotypic vacuolar fusion [10], but the last step of the secretory pathway remains unexplored, largely due to technical problems.

On one hand, secretion in the yeast *S. cerevisiae* is a rapid and constitutive process, where secretory vesicles (SVs) fuse with the plasma membrane soon after they are formed in the Golgi, without transient accumulation. The use of temperature-sensitive mutants that are blocked at specific points of the secretory pathway [11] permits accumulation of SVs and their subsequent purification for *in vitro* studies [12,13]. In this work, the tem-

perature-sensitive mutant *sec 6-4*, which has an altered component of the exocyst complex, responsible for the targeting of SVs to the appropriate site on the yeast plasma membrane [14], was used to accumulate and purify the SVs.

On the other hand, the study of *in vitro* fusion between SVs and the plasma membrane also requires a highly purified plasma membrane vesicle preparation, displaying the cytoplasmic surface outwards. In this respect, a method for the isolation of inverted and sealed plasma membrane vesicles, i.e. cytoplasmic-side-out plasma membrane vesicles (PMVs) [15], has provided the other necessary element for the study of exocytotic fusion events.

Many fusion studies dealing with biological membranes have used the octadecylrhodamine B-chloride (R18) dequenching assay [16]. This probe can be incorporated into biological membranes at self-quenching concentrations. Upon fusion, the probe undergoes a dilution effect and the dequenching signal can be measured in real time. Although this method has been widely used to report membrane fusion, it has been shown that other forms of probe dilution are also possible. Probe-transfer mechanisms such as aggregation, collision, probe diffusion or the action of lipid-transfer proteins have already been described [16–18]. The fluorescence signal due to these events must therefore be identified and isolated from the true fusion-mediated probe dilution. In 1994 Cobaleda and colleagues [19] applied the SIMFIT computer package to the study of the fusion kinetics between Newcastle disease virus and erythrocyte ghosts. They found that the dequenching curves could be split into two exponential equations that reflected different processes: a real fusion process and a non-specific probe transfer.

In the present paper, the lipophilic fluorescent dye R18 is used to study the fusion process between SVs and PMVs. The experimentally obtained dequenching signal was analysed using the SIMFIT computer package. The response of the fusion process to variations in temperature, target-membrane con-

Abbreviations used: DTE, dithioerythritol; %Fd, percentage of fluorescence dequenching; PMV, cytoplasmic-side-out plasma membrane vesicle; R18, octadecylrhodamine B-chloride; SNARE, soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor; SV, secretory vesicle.

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centration, cation composition of the medium and proteolytic attack has been examined. In addition, the fusion reactions have been observed using electron-microscopic techniques, to complement and validate the results obtained by spectrofluorimetry.

MATERIALS AND METHODS

Materials

Reagents for media were purchased from Pronadisa, Madrid, Spain. Lyticase, trypsin, ATP, creatine phosphate and creatine phosphokinase were from Sigma (St. Louis, MO, U.S.A.). Percoll and Density Marker beads were from Pharmacia Biotech Sverige (Uppsala, Sweden). R18 was from Molecular Probes (Eugene, OR, U.S.A.). The anti-R18 antibody was from Euromedex (Souffelweyersheim, France) and the goat anti-rabbit Protein A-gold was from British Biocell International (Cardiff, Wales, U.K.). HMSF136 (see below) was received from the Yeast Genetic Stock Center (Department of Molecular and Cell Biology, Berkeley, CA, U.S.A.).

Yeast strain and medium

The *S. cerevisiae* strain used in this study was HMSF136 (*MAT a*, *sec 6-4*, *pet* or *rho*⁻), a late secretory mutant. It was grown in YPD [2% (w/v) glucose, 2% (w/v) Bacto-peptone and 1% (w/v) Bacto-yeast extract].

Purification of PMVs

Highly purified PMVs were prepared as described by Menéndez et al. [15]. Briefly, HMSF136 was grown at 25 °C to late exponential phase and harvested by centrifugation. PMVs were obtained by a combination of osmotic lysis, differential centrifugation and separation in an aqueous dextran/polyethylene glycol two-phase system.

Purification of SVs

SVs were purified using a combination of two different methods [12,13]. HMSF136 strain was grown at 30 °C to early exponential phase. Cells were pelleted, resuspended in fresh low-glucose medium [0.2% (w/v) glucose] and equilibrated at the restrictive temperature, 37 °C. Incubation was continued for 2.5 h to allow accumulation of post-Golgi vesicles. The biomass was harvested by centrifugation and diluted in protoplast production medium (PPM; 5 mM Tris/HCl, pH 7.5/0.7 M sorbitol) until the suspension reached an attenuation of 0.6 units at 800 nm when diluted 10-fold. The volume of the suspension was then measured and mixed with one quarter of the volume of a 300 units · ml⁻¹ lyticase solution [20]. Finally, 6.5 mM dithioerythritol (DTE) was added and the suspension incubated at 37 °C for 1 h with occasional shaking. From this point, all steps were carried out at 4 °C. The protoplast suspension was harvested by centrifugation at 3000 g for 5 min, washed with PPM and finally resuspended in 25 mM Mes/Tris, pH 6.5, 0.7 M sorbitol, 5 mM EDTA, 1 mM PMSF and 1 mM DTE. Glass beads (0.425–0.6 mm diameter) were added and the protoplasts were lysed in a mixer (Braun Multimix MX32) at the lowest speed, by five bursts of 20 s each with 10 s breaks. The crude homogenate was clarified by centrifugation at 3000 g for 10 min. The supernatant was centrifuged at 100 000 g for 1 h to obtain a microsomal fraction. This microsomal fraction was resuspended in resuspension medium (RM; 25 mM Mes/Tris, pH 6.5, 0.7 M sorbitol and 1 mM DTE). The microsomal fraction (700 µl) was layered on top of a 50% (w/v) Percoll gradient (7 ml). The gradient was formed *in situ* by centrifugation at 28 000 g for 15 min. The gradient was fraction-

ated from the top and the density of each fraction was determined by using Density Marker beads. The fractions containing SVs were pooled and centrifuged at 85 000 g for 2 h with the aim of concentrating and removing the Percoll. The membrane band placed on top of a Percoll pellet was collected and then diluted once with RM. The SV fraction was stored under liquid nitrogen.

Fluorescence dequenching assay for membrane fusion

Lipid mixing between SVs and PMVs was followed by the increase in fluorescence resulting from dilution of the self-quenched probe R18 [16]. SVs were labelled with the fluorescent lipophilic probe R18 by injection during vortexing into an aliquot containing SVs to 3 mol% of R18 with respect to total phospholipid. The suspension was left at 37 °C for 20 min and the unbound probe was removed by centrifugation at 85 000 g at 4 °C for 30 min. Labelled SVs and unlabelled PMVs were mixed at a 1.4:8 protein ratio, which corresponded to a 1:4 phospholipid ratio. Lipid mixing was monitored at 30 °C in a final volume of 50 µl. R18 fluorescence was measured continuously at 560 nm excitation and 590 nm emission wavelengths, using a Shimadzu RF-540 spectrofluorimeter.

Unless otherwise indicated, 1.4 µg of protein of labelled SVs were typically added to fusion buffer [25 mM Mes-Tris, pH 6.5, 0.7 M sorbitol, 150 mM potassium acetate, 0.25 mM CaCl₂, 5 mM magnesium acetate, 0.1% (w/v) BSA, 1 mM DTE, 1 mM ATP, 40 mM creatine phosphate and 192 units · ml⁻¹ creatine phosphokinase]. After a setting period of 11 min, unlabelled PMVs were added in a small volume (5–10 µl), mixed with the labelled suspension and fluorescence monitored for further 60 min. The fusion reaction was stopped by addition of Triton X-100, to reach a final concentration of 0.5% (v/v), which results in maximal probe dilution. The fluorescence increase was measured as the difference with respect to the background fluorescence of labelled SVs.

To normalize the data, fusion was measured as the percentage of fluorescence dequenching (% Fd). At any point in time, it was calculated according to the following relationship:

$$\% \text{Fd} = [(F - F_0)/(F_\infty - F_0)] \times 100$$

where F_0 and F are the fluorescence intensities at the time when the target membranes are added after the equilibration period and at a given time point, respectively. F_∞ is the fluorescence after disruption of the membranes by detergent [21]. The data are given as means $\pm$ S.E.M.

Data analysis

Previously obtained % Fd data were fitted to different models by non-linear regression with the SIMFIT computer package, version 3.1 (for simulation, curve-fitting and statistical analysis; developed by W.G. Bardsley, University of Manchester, Manchester, U.K.; www.simfit.man.ac.uk). The best fit was chosen according to the statistical data provided by the program, among which the regression coefficient value was included.

Electron microscopy

SV and PMV fractions were obtained as described above and used for fusion assays under the same conditions as for the spectrofluorimetric assays. Fusion was stopped by adding 1% (w/v) glutaraldehyde prepared in cacodylate buffer (50 mM Caco, pH 7.2, 1.25 mM CaCl₂, 1.25 mM MgCl₂, 70 mM KCl and 0.7 M sorbitol) at room temperature. For standard transmission electron-microscopic observation, the samples were then centrifuged at 80 000 g at 4 °C for 30 min. The pellets were

washed with the same buffer and post-fixed in 1% (w/v) osmic acid supplemented with 1.5% (w/v) potassium ferrocyanate in the same buffer without sorbitol on ice for 1 h. The pellets were then washed with distilled water and stained with 0.5% (w/v) uranyl acetate at room temperature for 30 min. Samples were dehydrated in a series of ethanol and embedded in Epon. Ultrathin sections were obtained from a Reichert Ultracut S (Leica) and contrasted with ethanolic uranyl acetate and Reynold's lead citrate. Immunogold labelling of invertase was carried out according to the method of Kleijmeer et al. [22] by fixing the vesicle preparations with 4% (w/v) paraformaldehyde in 100 mM phosphate buffer, followed by centrifugation as above and suspension in 2% paraformaldehyde at room temperature for several days. The pellets were quenched with PBS/glycine and embedded in 7.5% gelatine in 100 mM phosphate buffer before being infiltrated with 2.1 M sucrose in PBS and mounted on specimen holders before freezing in liquid nitrogen. Ultrathin cryosections were made and labelled with polyclonal anti-invertase antibodies supplied kindly by Dr Eitan Bibi (Weizmann Institute, Rehovot, Israel) followed by Protein A-gold 10 nm (purchased from Dr J. W. Slot, Utrecht University, Utrecht, The Netherlands). Finally, the cryosections were embedded in a film of methylcellulose/uranyl acetate solution.

Immunolabelling of the fluorescent probe R18 was carried out using the whole-mount technique as follows: fusion reaction products were fixed with 2% (w/v) paraformaldehyde and aliquots of this mixture were directly placed on Formvar carbon-coated grids for binding. The grids were washed with PBS/glycine and then incubated for 10 min on 10% (w/v) fetal calf serum. The grids were then incubated for 30 min with the anti-R18 antibody in PBS supplemented with 5% (w/v) fetal calf serum. After rinsing with PBS plus 0.2% (w/v) fetal calf serum, the grids were incubated for 20 min with goat anti-rabbit Protein A-gold coupled to 10 nm gold. Then, the grids were rinsed and fixed in 1% (w/v) glutaraldehyde in PBS for 5 min. After rinsing with distilled water the grids were embedded in a thin film of methylcellulose/uranyl acetate, pH 4, as described in [23]. All the grids were observed using a Philips CM120 transmission electron microscope at 80 kV.

Protein and enzyme assays

Protein concentration was determined by the method of Bradford [24] with BSA as standard. In the case of SVs, Percoll was removed from the fractions prior to protein detection [25]. Proton pumping was measured as described by Menéndez et al. [15]. Reduction in Acridine Orange absorption was monitored at 495 nm in reaction medium (10 mM Mes/Tris, pH 6.0, 140 mM KCl, 0.1 mM EDTA, 20 μ M Acridine Orange, 1 mM DTE, 0.7 M sorbitol, 1 mM sodium azide, 0.1 mM ammonium molybdate, 50 mM KNO₃ and 4 mM MgCl₂), in the presence of 10–25 μ g of membrane protein. The mixture was preincubated for 5 min at room temperature and the reaction was started by addition of 2 mM NaATP. Invertase activity was used as a marker to determine the purity of SVs. The enzyme activity was assayed at 37 °C for 10 min as described by Goldstein and Lampen [26]. To preserve the integrity of the SVs, 0.7 M sorbitol was included in the reaction medium. Each reaction tube contained between 1 and 2 μ g of protein. The percentage of intact sealed vesicles is directly related to the percentage of latent activity of the enzyme. For this purpose, the enzyme activity was assayed in the presence and absence of 0.002% (w/v) Triton X-100 and the percentage of latent activity calculated as in [27]: latent activity (%) = [(activity in the presence of detergent –

activity in the absence of detergent)/activity in the presence of detergent] $\times$ 100. The data are given as means $\pm$ S.E.M.

RESULTS

Functional characterization of the subcellular components participating in the fusion event

Characterization of the functional state of the isolated subcellular components was conducted to ascertain that they were functionally competent to undertake *in vitro* fusion. Isolated SVs showed a high degree of purity and integrity, as confirmed through the specific invertase activity and the latency tests on the luminal marker invertase, as well as electron-microscopic observation (see below). As displayed in Table 1, purified SVs showed a 50-fold enrichment of the enzymic marker activity with respect to the crude homogenate. The high latency values displayed by this fraction confirmed that SVs were sealed. The preservation of functional properties by highly purified SVs was also tested by their ability to accumulate protons through H⁺-ATPase proton pumping, an activity that is specifically inhibited by vanadate [28]. When the membrane bilayer is intact, this activity results in vesicle lumen acidification, which can be monitored with Acridine Orange [29], as shown in Figure 1. Previously published similar functional assays have already demonstrated that highly purified

Table 1 Invertase activity as a marker of purified SVs

Invertase-specific activity is expressed as μ mol of glucose/mg of protein per min. Fold purification is calculated with respect to the crude homogenate (CH). Latency is considered as a marker of vesicle integrity and is calculated as the percentage of the total enzyme activity that is contained in the lumen of the SVs, as described in the Materials and methods section. MF, microsomal fraction. $n = 4$.

Fraction	Invertase activity (μ mol of glucose/mg of protein per min)	Latency	Fold purification
CH	0.186 $\pm$ 0.028	8	1
MF	1.940 $\pm$ 0.950	43	10
SVs	9.222 $\pm$ 3.375	81	50

Figure 1 Membrane sealing

Proton pumping displayed by the SVs in the absence (●) or presence (○) of 0.1 mM Na₃VO₄. Protein amount, 25 μ g.

Figure 2 Comparative invertase secretion studies between protoplasts and intact cells

Percentage of secreted invertase in protoplasts (●) and intact cells (○). 100% secreted invertase corresponds to 0.005 and 0.0012 units for 10^9 initial protoplast and intact cells, respectively ($n = 3$).

PMVs, obtained by two-phase partitioning, are also functionally competent. In addition, they are oriented with the cytoplasmic surface facing outward, with a frequency of higher than 85% [15].

Since protoplast production by enzymic digestion results in cellular modifications, including cell polarity loss [30], preliminary studies were aimed at confirming that the membrane preparations obtained from protoplasts were functionally competent for fusion despite the loss of polarity. For this purpose, invertase secretion in protoplasts and intact cells was compared [31]. These experiments showed that after a short lag time, protoplasts secreted invertase at a rate comparable with that observed in intact cells (Figure 2). This indicated that the secretory pathway was fully functional, despite the loss of polarity. Consequently, it could be assumed that PMVs and SVs obtained from protoplasts could be considered competent for intracellular fusion studies.

Validation of the fusion assay

Fusion between the two vesicle populations was monitored using the self-quenching lipophilic fluorescent dye R18, as an indicator of lipid mixing [16]. The probe was typically incorporated into SV membranes at 3 mol% of R18 with respect to total phospholipid. Under these conditions, which were adopted as the experimental baseline, the fluorescence intensity of the probe was quenched to 70–80% of the maximum obtainable on addition of detergent (see the Materials and methods section). Exposure of labelled SVs to unlabelled PMVs resulted in a concomitant increase in fluorescence intensity, which was monitored in real time. The kinetics of probe dilution observed at 30 °C are shown in Figure 3. As the fusion reaction progressed, the increasing fluorescence gradually stabilized, approaching a saturation level of 20–30% after 5 h with respect to the maximum obtainable with detergent addition.

When a lipid analogue is used to measure a fusion process some control experiments are needed to distinguish between a real fusion process and a non-specific probe transfer caused by probe diffusion, collisions, aggregation processes [16,17] or the action of lipid-transfer proteins [18]. In 1994 Cobaleda and co-workers analysed the fusion kinetics between Newcastle disease virus and erythrocyte ghosts using the SIMFIT computer package [19]. Besides fitting the experimental data to different non-linear equations, the program also determines the quality of the

Figure 3 Kinetics of R18-dequenching curve

R18-labelled SVs (1.4 μ g of protein) were mixed with an excess of unlabelled PMVs at 30 °C and the fluorescence increase was monitored for 300 min. (A) Experimental data (○) were transformed to %Fd versus time, and the transformed data fitted with the SIMFIT computer package to different exponential models: a simple exponential equation (eqn 2, see text; dashed line) or the sum of two exponential terms (eqn 3, see text; solid line). (B) Representation of each exponential equation: ●, fast process; ○, slow process; $n = 3$.

fitting. Applying the same analysis to our system, i.e. the fusion between SVs and PMVs, the experimental values of % Fd could be fitted to the following exponential equation:

$$\% \text{ Fd} = A[1 - \exp(-kt)]$$

where A is the maximum value (the asymptotic value) of % Fd, k is the constant that determines the slope of the ascending part of the curve and t is time in min.

When experimental data were fitted to one exponential equation the obtained regression coefficient was 0.984. But when the same data were fitted to the sum of two exponential equations the value for this coefficient was 1. The differences between both fittings are plotted in Figure 3(A). Thus, this result allows for the splitting of the % Fd data into two different but simultaneous processes, expressed as:

$$\% \text{ Fd} = A_1[1 - \exp(-k_1t)] + A_2[1 - \exp(-k_2t)]$$

The first exponential describes a rapid process where k_1 is $0.105 \pm 0.008 \text{ min}^{-1}$ and the value for A_1 is $10.04 \pm 0.646 \%$. The second exponential expresses a slower process where the value for k_2 is $0.013 \pm 0.001 \text{ min}^{-1}$ and A_2 is $14.56 \pm 0.546 \%$. Therefore, two independent processes can be distinguished: a fast process with a high value for k_1 reaching the asymptotic part of the curve in a very short time and a slow process with a minor slope but a higher value for A_2 . Both exponential curves are plotted separately in Figure 3(B), showing that, in brief time periods, the fast process predominates over the slow one.

Figure 4 Influence of the target membrane concentration on both processes

All assays were carried out in the presence of 1.4 µg of labelled SV protein and the indicated amount of PMV protein, at 30 °C for 60 min. Shown are the (A) fast process and (B) slow process of each experimental dequenching curve; $\triangle$, 4 µg; $\blacksquare$, 5.3 µg; $\circ$, 6 µg; $\bullet$, 8 µg; $\square$, 16 µg; $n = 4$.

Figure 5 Temperature-dependence of each process

Labelled SVs (1.4 µg of protein) were mixed with unlabelled PMVs (8 µg of protein) at different temperatures. The obtained dequenching curves of R18 were split into a slow and a fast component. Resulting %Fd values for the fast ($\bullet$) and slow ($\circ$) process at the end of the assay are shown ($n = 4$).

To ascertain the biological meaning of each process we performed a series of assays known to specifically influence fusion events between biological membranes.

In the first instance, the fusion pattern observed with increasing amounts of target membrane was studied and, consequently, the optimal PMV concentration for the fusion assays determined. For this purpose, labelled SVs were incubated with increasing amounts of PMVs and the data of each experimental curve were analysed as described above. The kinetic curves are shown in Figure 4. In the first exponential term, i.e. the fast process, the asymptotic value of the curve increases with increasing amounts of target membrane, reaching a maximum value at 8 µg of

Figure 6 Influence of salts on the fusion process

Labelled SVs (1.4 µg of protein) were mixed with unlabelled PMVs (8 µg of protein) and the assay was run at 30 °C for 60 min. The dequenching curves of R18 obtained without (dashed lines) or with (solid lines) salts (150 mM potassium acetate, 5 mM magnesium acetate and 0.25 mM CaCl₂) were split in slow ($\circ$) and fast ($\bullet$) components ($n = 4$).

Figure 7 Effect of trypsinization of SVs and PMVs on the fast and slow processes

R18-labelled SVs (1.4 µg of protein) and PMVs (8 µg of protein) were separately preincubated at 30 °C for 10 min with 0.014 and 0.08 µg · µl⁻¹ trypsin, respectively (dashed lines; $n = 4$). Assays were run for 60 min and the obtained experimental data were split into fast ($\bullet$) and slow ($\circ$) processes using the SIMFIT computer package. The same mixture without trypsin was run at 30 °C for 60 min and used as a control (solid lines; $n = 4$).

protein (Figure 4A). This saturation pattern entails that this fast process is kinetically limited.

In contrast with the results obtained in the fast process, the second exponential component (or slow process) increases almost linearly with increasing amounts of target protein and no saturation was observed at or beyond 8 µg of target membrane (Figure 4B). Thus it could be reflecting a non-specific process more attributable to a probe-transfer mechanism between both populations.

In the second instance, it is known that membrane-fusion events between biological membranes are temperature-dependent [19,32,33]. The influence of a temperature change in both processes was studied following their kinetics in a range between 4 and 30 °C (Figure 5). In these experiments, the slow process increased linearly with increasing temperature, while the fast process increased exponentially between 20 and 30 °C, with a more moderate variation from 5 to 20 °C. Again, it is the fast process that is more affected by the change in temperature.

Thirdly, in other systems, the requirement for moderate ionic strength has been reported to be needed for fusion to occur [33,34]. When the assay was carried out in a salt-free medium the fast process was inhibited by 80%, while the slow process followed a pattern similar to control assays, even with slight

Figure 8 Morphological characterization of fusion between SVs and PMVs

The fractions were mixed in salt-free medium or fusion buffer and incubated for 20 min at 30 °C. The samples were centrifuged and the pellets were processed for electron microscopy. **(A)** SVs and **(B)** PMVs are shown. **(C)** In the absence of salts no fusion is observed, **(D)** whereas in fusion buffer some SVs appear to be docked to a PMV. **(E)** Immunolocalization of invertase, as described in the Materials and methods section, reveals that, in the absence of salts the marker is not transferred to PMVs. **(F)** In fusion buffer, however, docking leads to fusion as evidenced by immunolabel transfer to the PMV lumen. In addition, the fluorescence probe R18 was immunolocalized by whole-mount immunogold in fusion and non-fusion conditions. **(G)** R18 is only confined to clusters of SVs (white arrowhead) under non-fusogenic conditions with no labelling of PMVs (black arrowheads) but, under fusogenic conditions, **(H)** the probe spreads over the surface of PMVs. Scale bars, 100 nm.

increases (Figure 6). Thus, the fast process is highly dependent on the cationic composition of the medium, while the slow process is even slightly enhanced by the reduced cationic composition of the medium.

Finally, the participation of proteins in both processes was tested by pretreating SVs and PMVs separately with trypsin. Trypsinization resulted in 80% inhibition of the fast process, while the slow process did not show significant variations

(Figure 7). These results imply that only the fast process and not the slow one is mediated by proteins.

Electron microscopy

Electron microscopy observations of the fusion event were undertaken to confirm the evidence obtained by fluorimetric measurements. Both vesicle populations, SVs and PMVs, were examined separately, showing a high degree of purification and homogeneous vesicle size (Figures 8A and 8B). While SVs had a diameter of 90 nm, PMVs presented the previously described diameter of 300 nm [15].

When both vesicle populations were placed together under non-fusogenic conditions, in a medium lacking salts (RM; Figure 8C), no contact between them was observed. Under fusogenic conditions, i.e. in fusion buffer, SVs appeared docked to PMVs, with an evident distortion of membrane shape. Single SV–PMV dockings were the most common situation, but multiple SV dockings (two or three) to one PMV were also encountered regularly (Figure 8D). In addition, increased PMV diameter also reflects the advent of multiple fusions taking place, as SV membrane material is incorporated into the original PMV after fusion (Figure 8D; diameter, 340 nm). This feature is consistent of all samples subjected to fusogenic conditions, as can be seen in Figures 8(D) and 8(F), compared with Figure 8(B).

The transfer of vesicle cargo from SVs to PMVs without leakage of vesicle contents was monitored to further confirm that true fusion events were taking place. Figures 8(E) and 8(F) show immunogold labelling of invertase under non-fusogenic and fusogenic conditions respectively (as above). The label is exclusively found in SVs under non-fusogenic conditions, while transfer of the invertase cargo to PMVs is visible under fusogenic conditions, with associated attachment of SVs in many instances (Figure 8F).

An additional demonstration that both membrane populations merged as a result of true fusion events was obtained by following the dilution of the hydrophobic membrane marker R18 by immunogold electron microscopy. Labelled SVs were mixed with unlabelled PMVs. In salt-free medium dye transfer was not observed between labelled and unlabelled vesicles. In this case gold particles were only present in SV clusters, with no labelling of PMVs (Figure 8E). In contrast, Figure 8(F) reveals that the fluorescent dye originally inserted into SVs has spread throughout the membrane of the newly formed fusion product, demonstrating that fusion between labelled SVs and unlabelled PMVs had already occurred.

DISCUSSION

The establishment of an *in vitro* assay procedure with a minimum of interference from unwanted side reactions involved the use of two highly purified populations of membrane vesicles: PMVs and SVs. The former not only exhibited high homogeneity in the orientation of the target membrane surface, but also preserved functional properties, as shown by ATP-dependent proton pumping and a high degree of sealing [15]. SVs were previously accumulated intracellularly in the temperature-sensitive mutant *sec 6-4* [11], and then purified with a high degree of integrity, as shown by the invertase latency tests. They also displayed the capacity to pump protons into the lumen through the H⁺-ATPase, since the proton pump is activated *en route* from the *trans*-Golgi network to the plasma membrane [35]. Proton pumping is not only a good indicator of protein functionality, but also of the preservation of membrane sealing in these subcellular fractions [29]. The high degree of purity of both

membrane preparations was further confirmed by electron microscopy, which is the definitive test for the quality of subcellular membrane preparations [36].

During this study, fusion was measured as an increase in fluorescence caused by the dilution of the probe R18 [16]. This assay has the advantage of being practical, requires low sample quantities, and permits monitoring in real time. Nevertheless, the risk of non-fusion-related probe transfer is a potential drawback of this technique [37,38]. One strategy used by several authors consists of using mathematical models to single out the fusion processes from interfering non-specific probe transfers [19,32,39,40]. The majority of these reports describe at least two simultaneous processes, one of which is attributed to real fusion, while the other one is considered to be non-specific probe transfer. Both processes closely fit exponential model equations [19,32].

In this report, the mathematical analysis has also shown that two processes are occurring simultaneously. However, they follow distinctly different kinetic patterns. The differences reported in this study are qualitative, and more profound than those previously reported, as will be discussed below. The difference may reside in that all previous such analyses reported on virus-mediated membrane fusion, as opposed to intracellular membrane fusion, as in this case.

One of the exponential equations describes a fast process that is saturated after only 60 min. In contrast, the second equation reflects a slow process that is only half-way to saturation in the same period of time. In this interval of 60 min, a first-order equation could faithfully describe the slow process. These clearly distinct kinetic traits are accompanied by marked differences in the response to changes in temperature, the concentration of target membrane (PMVs), salt presence or absence, and proteolytic attack, which helped to determine the true biological significance of these two components.

The fast process possesses all the features, and so it can be assigned to a biological fusion phenomenon. The kinetic equation defining the fast process between SVs and PMVs closely resembles that described for intra-Golgi vesicle fusion, $G_1 = G[1 - \exp(-mt)]$ (where G_1 is the concentration that have bound vesicles after time t , G is the concentration of Golgi membranes available for binding vesicles at time 0 and m is the ratio bound functional vesicles/Golgi membranes at time t ; see the supplementary data section of [41]). This kinetic coincidence may reflect the fact that both events probably share mechanistic features, such as the action of soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) complexes.

The involvement of SNARE complexes in intracellular fusion has been well documented [1,2,42], and it is known that membrane fusion is preceded by docking and formation of a *trans*-SNARE complex [10,43]. Thus intracellular fusion events depend, among other things, on the number of free SNAREs available on both vesicle and target membranes. When all free SNARE proteins have been already engaged in SNARE complexes, a saturation of the fusion process should be observed.

The inhibition of the fast process caused by proteolytic digestion indicates that proteins are involved in this event. Moreover, the saturation pattern showed by the fast process with increasing amounts of target membrane could be reflecting the attainment of maximum SNARE complexes possible in the population. When 8 µg of PMVs or less were used in the assay, the limiting factor was the amount of these membranes. On the other hand, when PMVs concentration was increased up to 16 µg or beyond, the observed asymptotic value did not exceed the value obtained with 8 µg of protein. This result implies that the limiting factor in this case was the number of binding sites

present on the SVs. This saturation also shows that no fusion takes place between PMVs or between them and the fusion product of the last round of SV-PMV fusion.

The presence of cations in the medium was also shown to be a determining factor in the fast process. In this system the elimination of cations from the fusion buffer results in the effective blocking of the fast process. Specific effects were observed, moreover, since both monovalent cations (K^+) and divalent cations (Mg^{2+}) cannot be replaced with others, such as Na^+ or Ca^{2+} . Other studies have further demonstrated that cation involvement is not related to the chemical charge of the medium [31]. In this regard, our results are in line with those reported for vacuolar fusion studies by Conradt and collaborators [34].

The evidence discussed above, as well as the pattern of response to a range of temperatures, is consistent with previous reports describing biological fusion processes. In contrast, the slow process showed a pattern of response, which could be ascribed to a physico-chemical event. The temperature response curve of this component shows a steady increase with temperature over and beyond the range of temperatures which are characteristic for biological fusion processes. The slow process could be describing a non-fusion-related probe transfer between both vesicle populations. This non-specific transfer could combine one or several processes already proposed in the literature: spontaneous diffusion through the assay medium between aggregated membranes, the action of lipid transfer proteins or collisions [16,17]. The probe R18 with an aliphatic chain of 17 carbons has a monomer-transfer kinetic of days [16,17]. This fact rules out any measurable spontaneous diffusion of the probe through the assay medium bulk during the assay period, but may still account for a transfer process between membranes which are in close proximity, such as in the case of aggregations and/or collisions. This possibility is supported by the observation that an increase in the amount of target protein present in the assay, which facilitates the fortuitous physical contact or proximity, gives rise to an increase of the slow process throughout the range of concentrations tested in the assay. On the other hand, a drop in temperature, which causes a steady reduction in the probe diffusion coefficient, may partly explain the gradual reduction in probe transfer observed experimentally.

The confirmation that a fusion process between SVs and PMVs is taking place was obtained by electron microscopy observations. Our observations have recorded three of the steps which have been recognized to take place: docking, whereby membrane continuity is disturbed with the presence of highly convex contact zones, the transfer of luminal content without leakage and, finally, fusion, where a single membrane is formed with the mixture of membrane contents [44].

The compounded evidence presented in this paper supports the view that the heterotypic fusion process taking place between SVs and plasma membrane vesicles may be further explored *in vitro* with the assay presented above, and thus supply a valuable complement to molecular and cellular studies.

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