Stoichiometry and kinetics of transport vesicle fusion with Golgi membranes

Joachim Ostermann^{*,‡}

European Molecular Biology Laboratory, Cell Biology Program, Meyerhofstrasse 1, 69117 Heidelberg, Germany

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The *in vitro* complementation assay established by Rothman and co-workers continues to be an important tool to study intra-Golgi transport. In this study, kinetic modeling is used to identify four main parameters that, together, explain the basic features of an assay that is a modification of the original assay. First, the assay signal depends on the ratio of Golgi membranes to transport intermediates in the assay. Secondly, an inactivation rate describes how the activity of transport intermediates decreases over time. Thirdly, the rate at which transport intermediates irreversibly bind to Golgi membranes is measured independently of membrane fusion, thus allowing a quantitative distinction between these two steps. Fourthly, a single rate constant describes the remaining reactions, which result in membrane fusion. This approach of kinetic modeling of experiments is generally applicable to other *in vitro* assays of cell biological phenomena, permitting quantitative interpretations and an increased resolution of the experiments.

INTRODUCTION

In vitro reconstitution of transport-coupled membrane fusion was first accomplished by Rothman and co-workers with membranes of the Golgi apparatus (Fries and Rothman, 1980). A complementation assay was designed by incubating secretory cargo-containing Golgi membranes from a glycosylation-deficient cell line with Golgi membranes from wild-type cells. Transport between these membranes would result in glycosylation. Recently we showed that one of the reactions reconstituted in this assay is the retrograde transport of resident enzymes from wild-type membranes to the glycosylation-deficient membranes (Love *et al.*, 1998; Lin *et al.*, 1999). Functional transport vesicles in this pathway can be generated *in vitro* (Lanoix *et al.*, 1999). Their formation requires coat proteins (COP1), which are also required for a full round of intra-Golgi transport (Ostermann *et al.*, 1993).

By substituting transport vesicles for wild-type Golgi membranes, the assay is greatly simplified. Only one reaction generates an assay signal, which is the fusion of Golgi enzyme-containing transport vesicles with vesicular stomatitis virus glycoprotein (VSV-G)-containing Golgi membranes. Other transport reactions or direct fusion of Golgi membranes are impossible as no wild-type Golgi membranes other than transport vesicles are present. Isolated COP1-derived transport vesicles should constitute an ideal basis for examining the transport events that take place after their formation, namely docking and fusion. This would then produce a detailed functional examination of the many cytosolic and membrane components that have been implicated in Golgi trafficking. However, both docking and fusion of vesicles are measured through the subsequent complementation of the glycosylation deficiency of the membrane with which the vesicles fuse. At first glance, preceding steps may be difficult to separate from the final glycosylation step. However, a higher resolution view of these events can be obtained once a mathematical model of the process is developed. Fitting the model to experimental data allows calibration of the parameters that determine how fast and strong an assay signal is generated. These are the rates at which vesicles dock and fuse under the conditions of the specific *in vitro* situation. The reasoning leading to this model is presented to illustrate how mathematical models can be used to examine other cellular events, be it *in vitro* or *in vivo*.

RESULTS

Fusion of a single vesicle with lec1 membranes completes glycosylation in the fused compartment

The *in vitro* complementation assay used in this study detects fusion between two membrane-bound compartments: Golgi membranes from glycosylation-deficient CHO cells and COP1-derived vesicles

*Corresponding author. Tel: +49 6221 387 289; Fax: +49 6221 387 512; E-mail: Joachim.Ostermann@embl-heidelberg.de

‡Present address: Department of Cell Biology and Anatomy, McGill University, Montreal, Quebec, Canada H3A 2B2

Fig. 1. Endo H resistance of VSV-G after incubation with wild-type Golgi-derived vesicles. **(A)** ^{35}S -labeled VSV-G-containing lec1 Golgi were incubated with the indicated amounts of vesicles. At the end of the incubation, membranes were pelleted and digested with Endo H. Proteins were separated by gel electrophoresis, and VSV-G was visualized by autoradiography. **(B)** Vesicles and lec1 Golgi were incubated at 37°C for the indicated times. **(C)** At the indicated time, one aliquot was put on ice. Another aliquot received 5 mM BAPTA and was further incubated at 37°C for a total of 1 h.

from rat liver Golgi. The glycosylation-deficient membranes were from $\beta 1,2$ -N-acetylglucosaminyltransferase 1 (GlcNAc-T1)-deficient lec1 cells, which were infected with VSV to express the viral glycoprotein (VSV-G). Isolated membranes were then incubated with isolated COPI-derived vesicles generated from rat liver Golgi (Lanoix *et al.*, 1999) shown to contain high amounts of Golgi-derived glycosylation enzymes including GlcNAc-T1. Upon docking and fusion, the vesicles transfer glycosylation enzymes to the Golgi membranes. The resulting modifications of the two N-linked oligosaccharides attached to VSV-G are then detected by determining incorporation of radiolabeled GlcNAc (Balch *et al.*, 1984), or through the increased resistance to deglycosylation by endoglycosidase H (Endo H) as a result of a conversion from high mannose to complex N-linked oligosaccharides (Fries and Rothman, 1980).

The Endo H resistance assay allows us to determine which fraction of VSV-G is Endo H resistant, and therefore, which fraction of Golgi membranes have fused with one or more vesicles. When wild-type Golgi-derived vesicles were incubated with VSV-G-containing lec1 Golgi, up to 70% of VSV-G was converted to an Endo H-resistant form (Figure 1A). Most VSV-G-containing membranes therefore fused with the added vesicles. When the number of vesicles is limiting, most Golgi membranes will fuse with either one vesicle or not at all. However, at the end of the incubation, VSV-G is either fully resistant or fully sensitive on both glycans. This suggests that a single vesicle

suffices for complete conversion of all VSV-G molecules present in the cisterna with which the vesicle has fused.

Figure 1B shows the time course of glycosylation. Only after short incubations is VSV-G partially resistant to Endo H. Even under these conditions, some VSV-G had been processed on both glycans in the Endo H-resistant fraction. The rapid formation of fully Endo H-resistant VSV-G suggests that glycosylation occurs within minutes after fusion. The influence of glycosylation on the overall kinetics can also be estimated by blocking fusion with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (BAPTA) (Porat and Elazar, 2000). If fusion is fast and glycosylation slow, then BAPTA should inhibit the reaction only when it is present from the beginning. BAPTA remained inhibitory for at least 15 min into the incubation (Figure 1C), showing that glycosylation is relatively rapid.

Quantitation of transport using $[^3\text{H}]\text{GlcNAc}$ incorporation

In vitro complementation can be reliably quantified by measuring $[^3\text{H}]\text{GlcNAc}$ incorporation into VSV-G. To avoid diluting the specific activity of the added UDP- $[^3\text{H}]\text{GlcNAc}$, endogenous UDP-GlcNAc is removed from cytosol by gel filtration. However, fast uptake of UDP-GlcNAc by Golgi membranes and subsequent glycosylation require micromolar concentrations of UDP-GlcNAc (Hiebsch and Wattenberg, 1992). Therefore, cytosol was prepared with some remaining endogenous UDP-GlcNAc. This resulted in a reduction of the specific activity of UDP- $[^3\text{H}]\text{GlcNAc}$ and hence a lower signal. To compensate, VSV-G was accumulated in the early part of the Golgi by incubating infected cells at 15°C (Saraste and Kuismanen, 1984) before membrane isolation. This yielded a higher signal, presumably since GlcNAc-T1-containing transport intermediates fused more efficiently to early Golgi cisternae (intermediate compartment/*cis* Golgi network) than to later cisternae (Lin *et al.*, 1999).

For each batch of cytosol used, the concentration of UDP-GlcNAc was measured by adding increasing amounts of UDP-GlcNAc to the assay (Figure 2). The reduction of the assay signal resulting from the dilution of the radiolabeled sugar can be expressed as

$$\text{cpm} = \text{cpm}_0 \cdot c_{\text{endogenous}} / (c_{\text{endogenous}} + c_{\text{added}})$$

Using this equation, the endogenous UDP-GlcNAc concentration was determined to be $3.6 \mu\text{M}$. This amount is enough to saturate sugar uptake when using this *in vitro* assay, thereby ensuring glycosylation does not become rate limiting.

The vesicle-to-Golgi ratio can be calculated from the dose–response curve

In order to correctly express and evaluate the mathematical parameters underlying vesicle docking and fusion, it is necessary first to determine how many functional vesicles exist in a given preparation. A kinetic model can be deployed (see supplementary data) to predict the relationship between the assay signal and the added volume of vesicles:

$$G_{\infty}^+ = G \cdot [1 - \exp(-V \cdot c_v^{app})].$$

G_{∞}^+ is the proportion of Golgi membranes (G) that at the end of the incubation contain glycosylated proteins, V is the added

Fig. 2. UDP-GlcNAc concentration in the assay. The indicated concentrations of UDP-GlcNAc were added to transport assays that were otherwise identical and contained 0.3 μM UDP-[^3H]GlcNAc each. The black circles are individual data points from an experiment. The curve is the calculated inhibition. In the experiment shown, $c_{\text{endogenous}}$ is 3.6 μM .

Fig. 4. Determination of the apparent vesicle-to-Golgi ratio after 2-fold dilution. Squares represent incubations that were 2-fold diluted compared with the controls (circles). A dilution was performed by adding a reaction mixture containing all components, except membranes. In the experiment shown, the apparent vesicle concentration is 2.8 μl^{-1} before and 2.0 μl^{-1} after dilution.

Fig. 3. Determination of the apparent vesicle-to-Golgi ratio. Transport incubations were carried out with a fixed amount of Golgi membranes and the indicated amounts of a vesicle preparation. The vesicle concentration and the maximum glycosylation signal were determined by curve-fitting of the model to the data. In the experiment shown, the apparent vesicle concentration is 3.8 μl^{-1} .

The dilution sensitivity of the assay predicts how many vesicles inactivate during the incubation

Having shown that c_v^{app} can be estimated for a typical vesicle preparation, it was important to test vesicle stability under the exact conditions of the *in vitro* incubation. The fraction of functional vesicles that could possibly inactivate during the incubation instead of binding and fusing with Golgi membranes can be determined using the kinetic model. The apparent vesicle concentration was measured again after adding an equal volume of all the assay components except for membranes (Figure 4). A 2-fold dilution reduces the binding speed by half, but inactivation should not be affected. Therefore, if without dilution, half of all vesicles bound and half inactivated, then after dilution, 1/3 should bind and 2/3 should inactivate. This would result in a reduction of c_v^{app} by 2/3. More generally, f_b , which is the fraction of the total vesicles that bind rather than inactivate can be calculated from the measured ratio $r = c_v^{\text{app}} / c_v^{\text{app}}$ by $f_b = 2 - r$.

At high concentration, c_v^{app} was 1.41 ± 0.083 (mean $\pm$ SD, $n = 4$) times larger than after 2-fold dilution. This corresponds to $59 \pm 8.2\%$ of vesicles binding before they inactivate at high concentration, or an inactivation rate that is $\sim 2/3$ of the binding rate at the higher concentration (5 μl of Golgi membranes per 30 μl assay), or 1/3 larger than binding after 2-fold dilution. It appears that the vesicles are slowly inactivated *in vitro*, possibly by the action of cytosolic enzymes.

The vesicle half-life is determined by a stimulation experiment

In addition to concentration and stability, vesicles may also differ in their kinetic parameters. Initial data suggested that two rate constants can describe the overall assay kinetics: one for the overall consumption of vesicles, which are inactivation and binding, and one for fusion. However, extracting two rate

vesicle volume and c_v^{app} is the apparent vesicle concentration. An example is shown in Figure 3. We observe that such a curve can be closely fitted to the experimental measurements (black dots). In this particular experiment, c_v^{app} was determined to be $3.9 \pm 0.2 \mu\text{l}^{-1}$ (mean $\pm$ SD, $n = 4$). In other words, when 0.25 μl of the vesicle preparation were added to the incubation the signal was 63% of the maximum assay signal. This is the case according to the kinetic model, when on average one vesicle fusion event occurs per Golgi membrane.

Fig. 5. Time course of vesicle consumption. At the indicated times we determined the factor by which the assay signal increased when an equal volume of a reaction mixture containing Golgi membranes, but no vesicles, was added (black dots). The curve shows the decrease predicted by the model. n and k_v were determined by curve-fitting of the equation to the data. In this experiment, n is 4.5 and k_v is 0.058 min^{-1} .

Fig. 6. Kinetics of the complete reaction. At the indicated times, an aliquot of the reaction was transferred on ice (black dots). The curve is the result of fitting the data to the kinetic model. k_f was determined by optimizing agreement between the data and the kinetic model, using values for n and k_v that were determined from other experiments. In the experiment shown, n is 1, $k_v + k_i$ is 0.064 min^{-1} and k_f is 0.019 min^{-1} .

constants from the same experiment is not very reliable. Therefore, the vesicle consumption rate was determined independently. The experiment described here measures how long vesicles remain available for docking and fusion with newly added Golgi membranes. In other words, this experiment measures how long it takes for the vesicles to become committed to a specific membrane.

A reaction with a set amount of vesicles and Golgi membranes was added to an equal volume of a reaction mixture that contained all components including Golgi membranes, but no vesicles. When the addition is performed at the beginning of the incubation and vesicles are in excess, stimulation will be 2-fold. If the number of vesicles is limiting, the stimulation will be minimal. When the addition is performed during the incubation, it will depend on the fraction of vesicles that remain available for fusion with newly added Golgi membranes. This depends on the rate with which vesicles are consumed. The model predicts that the stimulation can be expressed as:

$$F(t) = \frac{2 - \exp\left[-\frac{n}{2} \cdot \exp(-k_v \cdot t)\right] - \exp\left\{-\frac{n}{2} \cdot [2 - \exp(-k_v \cdot t)]\right\}}{1 - \exp(-n)}$$

where n is the vesicle-to-Golgi ratio and k_v is the combined rate constant for vesicle consumption, which includes both binding and inactivation. Figure 5 shows an experiment of this type. As can be seen, the curve can be fitted to the experimental results with good precision. k_v was determined to be $0.0635 \pm 0.0089 \text{ min}^{-1}$ (mean $\pm$ SD, $n = 6$), which corresponds to a vesicle half-life of $11 \pm 1.5 \text{ min}$.

The vesicle fusion rate can be measured from the overall reaction kinetics

The experiments described so far allow us to calculate the apparent vesicle-to-Golgi ratio for a given preparation of

vesicles and Golgi membranes, the fraction of vesicles that inactivates prior to binding, and the kinetics with which vesicles are consumed by binding to Golgi membranes or inactivation. The remaining kinetic parameter to be determined is the fusion of bound vesicles.

Figure 6 shows an example of the overall reaction kinetics of the assay using amounts of vesicles and Golgi membranes that on average will yield one vesicle fusion event per Golgi compartment. At the Golgi concentration of this experiment, approximately half the number of vesicles inactivate and half bind and fuse. The vesicle consumption rate was determined to be 0.0635 min^{-1} . The reaction speed first slowly increases, then levels off into a steady state, and finally, decreases as the reaction approaches its end point. This kinetic behavior is typical for a reaction that proceeds through one intermediate, such as Golgi membranes with bound but not fused vesicles. As no improvement in the quality of the curve fit could be made by assuming more than one intermediate in the reaction, all other intermediates (other than vesicles bound but not fused) must be too short lived to have a measurable effect on the kinetics.

The kinetic model combines all steps following vesicle docking in a single rate constant, k_f , which is proportional to the number of vesicles bound per Golgi membrane. Optimal agreement between the kinetics predicted by the model and the measured data (Figure 6) was found when k_f equaled 0.019 min^{-1} . This corresponds to a half-life of 35 min, which is the time until half of all bound vesicles have fused. It can be calculated that if vesicles distribute stochastically over all Golgi membranes, and that on average one vesicle fusion event occurs per Golgi compartment, 37% of the membranes would not be hit at all, and 37, 18, 6.1 and 1.5% would have received 1, 2, 3 and 4 vesicles, respectively. At this vesicle-to-Golgi ratio, Golgi compartments undergoing fusion receive 1.6 vesicles. Therefore, the average rate constant at which Golgi membranes with bound vesicles are converted to Golgi membranes with at least one

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Fig. 7. Kinetics of progression past the BAPTA-sensitive step. As in Figure 6, except that 5 mM BAPTA was added at the indicated times and the incubation was continued at 37°C. In the experiment shown, n is 1, $k_b + k_i$ is 0.064 min^{-1} and k_f is 0.035 min^{-1} .

fused vesicle is 1.6 times greater than the rate constant for the vesicle fusion reaction, or 0.030 min^{-1} , or a half-life of 23 min.

For comparison, the reaction was interrupted by adding BAPTA, a Ca^{2+} chelator, at different times, followed by further incubation to complete glycosylation. Under these conditions, k_f was found to be 0.035 min^{-1} (Figure 7), which corresponds to a half-life of 20 min for an individual vesicle, or 0.056 min^{-1} and 12 min for the average Golgi membrane. This suggests that the reaction progresses slightly faster past the BAPTA-sensitive step, with the reaction having a half-life of ~10 min. This is in good agreement with the time course of glycosylation under these conditions (Hiebsch and Wattenberg, 1992), which suggests that BAPTA inhibits a step late in the fusion reaction.

DISCUSSION

The original promise of the *in vitro* complementation assay was to allow the rigor of biochemistry to be applied to a problem of cell biology (Fries and Rothman, 1980). This required a reliable quantitative tool, which was established by Balch *et al.* (1984). Building on these accomplishments we have shown that the predominant contribution to the assay signal is transport of wild-type Golgi enzymes to the mutant Golgi membranes (Love *et al.*, 1998). In this study, I show that a mathematical model closely mimics the behavior of the transport assay. Of course, this quantitative model is only a first approximation of the much higher complexity *in vivo*, and the model will have to be refined as more data become available. The ability to quantitatively predict the behavior of a given system, such as this assay, provides not only more accurate knowledge but also an increased resolution about the individual reaction steps taking place.

Specifically, the assay signal's dependency on membrane concentrations and kinetic parameters is quantitatively explained. While the exact numerical values are not very important, it is of the utmost importance that they can be measured with known precision. Only now is it possible to compare functional properties of different vesicle preparations. Differences in the amount of

functional vesicles can be clearly separated from differences in their inactivation, binding or fusion kinetics. Similarly, it is now possible to evaluate the role of cytosolic factors in vesicle binding and fusion.

For the time being, detailed studies have focused by necessity on the fusion of Golgi enzyme-containing vesicles. In only one study was COP1-dependent transport of secretory cargo between Golgi membranes reconstituted (Ostermann *et al.*, 1993). However, the low yield made this system impractical for routine use. With Golgi enzyme-containing vesicles, even a single vesicle fusion per cisterna is sufficient for full glycosylation of its cisternal contents. This signal amplification is crucial in the light of the observation that inactivation is as fast as or faster than binding. A better understanding and prevention of the observed inactivation pathway will be necessary before a functional role of anterograde cargo-containing COP1 vesicles can be evaluated further.

The rapid inactivation of vesicles stresses once more the distinctiveness of fusion competence. Only transport vesicles, but not Golgi membranes, fuse under the conditions of this assay (Lin *et al.*, 1999). It is not yet understood which properties of the vesicles make this possible. The mathematical model presented here will greatly simplify future studies to measure quantitatively how vesicles fuse, and how their fusion competence correlates with the presence or modification of specific proteins and lipids. Most importantly, this stoichiometric and kinetic information can now be obtained with minimal manipulation of the assay itself, dramatically reducing the complexity of the experiments. Now, this complexity is safely constrained by mathematics. In a general sense, the approach taken here illustrates the power of mathematical modeling to describe a complex system and thus, stimulate the use of mathematics in cell biology.

METHODS

***In vitro* complementation assay.** Vesicles were isolated as described in Lanoix *et al.* (1999). Infection of lec1 Golgi membranes with VSV was essentially carried out as described in Balch *et al.* (1984), with the modifications described in Colombo *et al.* (1991). Before homogenization and isolation of Golgi membranes, cells were incubated for 2–3 h at 15°C. *In vitro* complementation with ^{35}S -labeled VSV-G was carried out as described in Lin *et al.* (1999). Cytosol was prepared from lec1 cell homogenate and was desalted on PD10 columns. The final cytosol concentration was 20% of the assay volume.

Data analysis. The equations used to explain the assay signal in different experiments were derived from the experimental model that is included as supplementary data to this paper (available at *EMBO reports* Online). Curve-fitting of these equations to experimental data was done using MacCurveFit (Kevin Raner Software). Kinetic analysis of the complete reaction was done using Berkeley Madonna (Robert I. Macey and George F. Oster).

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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