Kinetics of Fusion between Endoplasmic Reticulum Vesicles in Vitro*

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The endoplasmic reticulum (ER) is a highly dynamic organelle, continuously undergoing membrane fusion and fission. We have measured homotypic fusion between ER vesicles isolated from Chinese hamster ovary cells kinetically in vitro, using an assay based on the metabolic incorporation of pyrene-labeled fatty acids into the phospholipids of cellular membranes. An increase in pyrene-monomer fluorescence was observed after mixing labeled and unlabeled ER vesicles in the presence of ATP and GTP. The protein, temperature, and nucleotide dependence of the increase indicated that it was caused by membrane fusion rather than molecular transfer of labeled lipids to unlabeled membranes. This assay allowed the first kinetic measurements with virtually nonexchangeable probes of a homotypic membrane fusion event. At 37 °C, fusion started off immediately at a rate of 1.14 ± 0.29%/min and reached a half-maximal level after 56 min. In the presence of guanosine 5′-O-(3-thiotriphosphate) (GTPγS) or after treatment of the membranes with N-ethylmaleimide, fusion was reduced but not completely inhibited. Addition of GTP during a fusion reaction immediately accelerated, and GTPγS immediately slowed down the fusion reaction. Thus, these kinetic measurements indicate that G-proteins might act to rapidly enhance fusion beyond a basic level.

The molecular mechanism of membrane fusion is best known for fusion induced by the hemagglutinin protein of influenza virus. This is an exoplasmic fusion event, in which the initial contact takes place between membrane leaflets that face the extracellular environment of cells (1, 2). Endoplasmic fusion, with initial contact between cytoplasmic leaflets, differs from exoplasmic fusion, because it occurs in a controlled environment which contains factors that could be used to drive a fusion reaction (2). Thus, whereas exoplasmic fusion is mostly induced by a single integral membrane protein present on one of the membranes, like hemagglutinin, endoplasmic fusion was found to involve complexes of soluble, peripheral, and integral membrane proteins and requires factors such as ATP and GTP (3, 4).

In recent years, many proteins involved in endoplasmic fusion have been identified. With few exceptions (5), heterotypic fusion, between vesicles derived from one organelle fusing with the next organelle on the endocytic or exocytic pathway, appears to involve proteins such as N-ethylmaleimide (NEM)1-sensitive fusion protein (NSF), soluble NSF attachment proteins (SNAPs), and SNAP receptors or SNAREs (3) in mammalian cells and homologues of these proteins in yeast. Homotypic (self) fusion between mammalian Golgi vesicles and fragments, leading to Golgi reassembly (6, 7), requires the NSF homologue p97 in addition to NSF. Homotypic fusion between ER membranes in yeast requires the yeast homologue of p97, the cell cycle protein Cdc48p (8). Despite the progress in identifying these proteins, the mechanism of endoplasmic fusion remains an mystery.

Studies of the mechanism of hemagglutinin-induced fusion have been greatly facilitated by the development of quantitative and kinetic, fluorescence-based assays for fusion. The assays are mostly based on fluorescent phospholipid analogues present in artificial membranes. Fusion of these membranes with unlabelled viral membranes leads to a dilution of the probes in the membrane, resulting in a relief of fluorescence quenching or of self-quenching, and the resulting change in fluorescence can be directly correlated with fusion. Phospholipid analogues are reliable probes for the measurement of fusion, because the molecular exchange of phospholipids between labeled and unlabeled membranes, which would lead to a false-positive fusion signal, is unlikely (9, 10). However, phospholipids are not easily taken up by biological membranes, limiting their use in the measurement of fusion between biological membranes.

Here, we have studied homotypic fusion between vesicles produced from the ER. The ER is a highly dynamic organelle. Elongation, branching and fusion of ER tubules takes place continuously in vivo (11, 12), and this process has been reconstituted in vitro (13). Moreover, during mitosis, the ER fragments, and these fragments rapidly fuse to re-form a functional organelle in the daughter cells (14, 15). We have isolated ER vesicles from CHO cells labeled with pyrene phospholipids through the metabolic incorporation of pyrene fatty acids. Fusion of these vesicles with unlabeled ER vesicles was measured by an assay based on the concentration-dependent formation of pyrene excimers. The assay, which was used previously to measure virus-induced membrane fusion (16, 17), allowed fusion to be followed in real time. It was found that fusion was affected by a number of factors known to play a role in microsome fusion, such as GTP (18–20), ATP (20), and also by NEM. Addition of GTP or guanosine 5′-O-(3-thiotriphosphate) 

1 The abbreviations used are: NEM, N-ethylmaleimide; NSF, NEM-sensitive fusion protein; C45E4, octaethylene glycol mono n-dodecyl ether; CLAP, chymostatin, leupeptin, antipain, and pepstatin A; FB, fusion buffer; GTPγS, guanosine 5′-O-(3-thiotriphosphate); SB, sucrose buffer; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; DTT, dithiothreitol; CHO, Chinese hamster ovary; ER, endoplasmic reticulum.
Lipids were extracted from the vesicles according to Folch (26) and up in phosphate-buffered saline, washed in SB, resuspended in 1 ml of provided by Dr. J.-M. Peters.

Cell Culture—CHO K1 cells were grown in minimal essential medium-a (Life Technologies, Inc., Basel, Switzerland) supplemented with 7.5% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO₂.

Preparation and Characterization of ER Vesicles—Suspension-grown CHO K1 cells in log phase were harvested by centrifugation in the presence of 1 mM EDTA and washed with sucrose buffer (SB: 10 mM Hepes/KOH, 250 mM sucrose, pH 7.4). Cells were resuspended in a 4-fold volume of sucrose buffer containing 1 μg/ml each of chymotrypsin, leupeptin, antipain, and pepstatin A (SB/CLAP) and homogenized by 11 passes through a “cell cracker” (21) with a clearance of 18 μm. The homogenate was spun at 715 × g for 5 min, the supernatant was saved, and the pellet was washed once with SB/CLAP. The pooled supernatants were loaded on a two-step gradient (0.25 ml of 2 mM sucrose and 2 ml of 0.63 mM sucrose, both in 10 mM Hepes/KOH, pH 7.4) and centrifuged for 1 h at 225,000 × g SR. Fractions were collected as follows: 0.5 ml from the top (fraction 1), the next 1.5 ml containing the uppermost interface (fraction 2), the following 1.7 ml until the second interface (fraction 3), and the material banding on the 2 mM sucrose cushion (fraction 4). The protein concentration in the fractions was determined according to Bradford (22). The activity of organelle-specific marker enzymes was measured to assess the purity of the ER vesicles by this method. Alkaline phosphatase, a marker for plasma membranes, was assayed by measuring the dephosphorylation of 4-nitrophenyl phosphate according to Van Belle (23), with minor changes. Briefly, samples were prepared for determination and NAO assay at 450 and 390 nm. After addition of the reaction mixture, the reaction was terminated by the addition of NaOH to 250 mM. The monomer fluorescence of pyrene was measured around 475 nm (Fig. 2, monomer peaks, at 377 and 397 nm, and an excimer peak at around 475 nm). The fluorescence scale was calibrated by setting the fluorescence immediately after injection of the probes' concentration. Dilution of pyrene-labeled phospholipids can be used to measure fusion because excited pyrene molecules can form excimers (dimers) with pyrene molecules in the ground state in a concentration-dependent fashion. Excimers emit fluorescence at a higher wavelength than monomers, and therefore the ratio of excimer to monomer fluorescence is a measure of the probes’ concentration. Dilution of pyrene-labeled phospholipids upon fusion of labeled with unlabeled membranes causes an increase in monomer fluorescence and a decrease in excimer fluorescence (16, 17).

CHO K1 cells were grown in the presence of the pyrene-labeled fatty acid 1-pyrenehexadecanoic acid, and after a 1-h chase in fresh medium without label to remove remaining free fatty acids, ER vesicles were isolated as described under “Experimental Procedures.” The purity of the vesicles was assessed by the analysis of marker enzymes (Fig. 1). TLC analysis of lipid extracts from the vesicles showed that pyrene was incorporated efficiently into the phospholipids, similar to what was reported for enveloped viruses (16). 44.7% ± 4.0% of the pyrene was found in phosphatidylcholine and 34.0% ± 12.4% in phosphatidylethanolamine, whereas 11.9% ± 3.7% was still in fatty acids (averages of five labelings). A fluorescence emission spectrum of the vesicles, with excitation at 330 nm, showed two monomer peaks, at 377 and 397 nm, and a broad excimer peak around 475 nm (Fig. 2, spectrum a). The excimer to monomer ratio (measured at 475 and 377 nm, respectively) was always between 0.20 and 0.30, meaning that pyrene was present in the...
membranes at concentrations sufficient for fusion measurements. After incubation of labeled with unlabeled vesicles (1:10 ratio) in the presence of GTP, ATP, an ATP-regenerating system, and cytosol at 37 °C for 30 min, a different spectrum was recorded (Fig. 2, spectrum b). The excimer peak had decreased by 23%, whereas the intensity of the monomer peak at 377 nm was 23% higher. These changes could be the result of dilution of the pyrene-labeled lipids into the unlabeled membranes after fusion between labeled and unlabeled vesicles. Addition of detergent lysed the membranes, dispersing individual probe molecules into separate micelles. As a result, the excimer peak disappeared and the monomer fluorescence increased considerably (Fig. 2, spectrum c). The monomer fluorescence after addition of detergent was used as a measure of the fluorescence at infinite dilution of the probe.

To measure the kinetics of the interaction between labeled and unlabeled vesicles, the fluorescence at 377 nm was monitored continuously, with excitation at 330 nm. Upon the addition of a mixture of labeled and unlabeled ER vesicles (1:10 ratio) to a cuvette containing GTP, ATP, an ATP-regenerating system, and cytosol, at pH 7.4, 37 °C, an increase in fluorescence was observed (Fig. 3, curve a). The initial rate of fluorescence increase, determined as described under “Experimental Procedures,” was 1.14 ± 0.29%/min (average of 23 preparations). The final level of fluorescence increase, measured after 11 h, was 20.7% and a half-maximal increase was obtained after 56 min. Cytosol could be omitted from the mixture without having an effect on the rate or efficiency of the reaction (not shown). In the absence of added GTP, ATP, an ATP-regenerating system, and cytosol, an increase in fluorescence was observed with an initial rate of 0.43 ± 0.13%/min (Fig. 3, curve b), reaching a final level of 22.8%. The addition of cytosol alone did not affect the reaction (not shown). Incubation of labeled vesicles alone in the presence or absence of GTP, ATP, an ATP-regenerating system, and cytosol gave rise to a slight decrease in fluorescence, at least at protein concentrations of labeled vesicles at and above 57 μg/ml (Fig. 3, curve c). Below that concentration, the decrease in fluorescence was more pronounced. It was found that the decrease was not due to photobleaching or oxygen quenching of pyrenes (not shown), but probably involved adsorption of vesicles to the cuvette wall or the Teflon stirrer. Thus, all subsequent experiments were performed at labeled vesicle protein concentrations of 57 μg/ml.

With this assay, an increase in fluorescence could be caused by membrane fusion or by the molecular transfer of pyrene-labeled free fatty acids or phospholipids from labeled to unlabeled membranes. Spontaneous exchange of phospholipids between membranes is not very efficient and was not seen using pyrene-labeled phospholipids in two other systems involving biological membranes (16, 17). Since fusion, but not necessarily molecular lipid transfer, most likely depends on specific proteins, the protein dependence and specificity of the reaction were tested. When increasing concentrations of a protease mix containing Pronase and subtilisin were added to the cuvette, the fluorescence increase in the presence of nucleotides and cytosol was reduced by 90–95% at the highest concentration (16.3 μg/ml each) (Fig. 4). To investigate whether the inhibition was caused by the proteolytic action of the proteases or resulted from other interactions (e.g. steric inhibition) between the preparation and the enzymes, the reaction between labeled and unlabeled vesicles was also measured in the presence of both trypsin and trypsin inhibitor. Inhibition was observed when the vesicles were digested with trypsin for 20 min on ice, followed by the addition of trypsin inhibitor (Fig. 4, curve d). However, if the vesicles were preincubated on ice with FB without trypsin, the presence of these reagents in the cuvette did not inhibit the increase in fluorescence (Fig. 4, curve c). If labeled membranes were mixed with liposomes, either prepared from commercially obtained pure lipids (not shown) or from lipids extracted from unlabeled ER vesicles (Fig. 4, curve c), only a slight increase in fluorescence was observed, which could be caused by some unspecific fusion or by lipid exchange. Thus, the increase in fluorescence seen upon mixing labeled and unlabeled ER vesicles is almost entirely protein-dependent, indicating that it is caused by fusion and not by the molecular transfer of lipids between the vesicles.

Moreover, if the fluorescence increase would be caused by the transfer of individual probe molecules between the membranes, the initial rate of fluorescence increase should be rate-limited by diffusion and thus vary according to the absolute temperature. The temperature dependence of the initial rate of fluorescence increase, measured as the tangent to the steepest part of the curve as described under “Experimental Procedures,” is shown in Fig. 5. The initial rate of fluorescence increase was optimal at 37 °C. At 10 °C, the rate was reduced by 75% compared with 37 °C, for an absolute temperature difference of 9%. Furthermore, although the initial rate at 10 °C was 25% of that measured at 37 °C, the fluorescence increase leveled off very fast at low temperatures, and the extent of fluorescence increase after 25 min at 10 °C was only 14% of that seen at 37 °C (not shown). Moreover, at 0 °C no increase in fluorescence was observed. Taken together, the above data indicate that the increase in fluorescence is caused by membrane fusion.
In mammalian cells, heterotypic endoplasmic fusion appears to almost universally involve the NEM-sensitive fusion protein NSF (3), and other NEM-sensitive proteins were found to be involved in Golgi-Golgi homotypic fusion (6, 7). Therefore, we investigated the influence of NEM on fusion. Labeled and unlabeled ER vesicles were both treated separately with NEM at 0°C, the NEM activity was quenched with a 2-fold concentration of DTT, and fusion was measured. It was found that treatment with millimolar concentrations of NEM caused an inhibition of fusion. Preincubation of vesicles with 1 mM NEM affected fusion by about 55% (Fig. 6, curve a compared with curve c). After treatment with 2 or 5 mM NEM, fusion was reduced more (Fig. 6, curve b). These data suggest that a membrane-associated protein was inactivated by NEM. NSF could be membrane associated, but there is also a pool of NSF in the cytosol (3). However, inhibition of fusion by NEM could not be overcome by the addition of a 4-fold concentration (500 μg/ml) of cytosol, nor by cytosol that had been isolated in the presence of 1 mM each of ATP and DTT in order to stabilize ATP-binding proteins like NSF (29) (not shown). These data suggest that cytosolic factors were not needed for fusion or, after NEM treatment of membranes, could not be supplied by cytosol. In fact, the addition or omission of cytosol did not affect any of the reactions shown in this paper.

Recently, p97, an NEM-sensitive homologue of NSF, was reported to mediate homotypic fusion between Golgi membranes from mammalian cells (6, 7, 30). We found that the addition of 20 μg/ml Xenopus p97 did not overcome the inhibition resulting from the treatment of membranes with NEM (not shown).

It was reported that a 1 M KCl extract of Golgi membranes provided NSF and SNAPs for Golgi-Golgi fusion (6). When labeled and unlabeled vesicles were extracted separately with 1 M KCl and then incubated with nucleotides, cytosol, and an ATP-regenerating system, the initial rate of fusion was reduced to 56.2% compared with unextracted vesicles. In order to verify that the extraction did not just remove soluble proteins, fusion was measured with vesicles extracted with 0.25 M KCl. The initial rate of fusion in this case was reduced to 72.5 ± 3.8% (data from two preparations). Thus, extraction with higher salt concentrations inhibited fusion more strongly, indicating that peripheral membrane proteins were required for fusion. Adding back a 1 M KCl extract (made in the presence of 1 mM ATP to stabilize ATP-binding proteins) to the extracted vesicles partially restored fusion, indicating that a fusion-active compound(s) was contained in the extract. We then tested whether the 1 M KCl extract could provide factors that had been inactivated by NEM. However, addition of the extract to preparations containing NEM-treated vesicles, even in the presence of cytosol (isolated in the presence of ATP), nucleotides and p97 did not restore fusion. Together, these data suggest that additional NEM-sensitive factors, which cannot be extracted from ER membranes by high salt, are required for fusion between ER vesicles.

In order to investigate whether fusion required ATP, ATP was omitted from an incubation which contained labeled and unlabeled vesicles, GTP, cytosol, and an ATP-regenerating system. For different preparations, the extent of fusion in the absence of ATP varied, but it was reduced by 10–40% compared with fusion in the presence of ATP (not shown). For a given preparation, the presence or absence of cytosol or a regenerating system did not affect the reaction (data not shown), indicating that ATP was not provided in significant amounts by cytosol or by conversion of GTP into ATP. These observations suggest that some ATP was still present, in varying amounts, in the vesicle preparations. Indeed, when labeled and unlabeled vesicles were pretreated separately with APase at 4°C to deplete residual ATP and ADP and then incubated in the presence of GTP, with or without cytosol, fusion was reduced more (Fig. 7, curve a). Variation of the ATP concentration in the presence of an ATP-regenerating system, cytosol and an excess of GTP, revealed that the extent, but not the initial rate of fusion depended on the addition of ATP (Fig. 7, curves b–c). In
general, addition of more than 100 \(\mu M\) ATP did not further increase fusion. Thus, in the first minutes of the reaction, additional ATP was not required, suggesting that ATP in the vesicle preparations is present in a form that allows fusion to start at the maximal rate provided GTP is present.

GTP has been reported to be required for, or to stimulate, fusion between microsomes in vitro (18–20). We found that in the presence of ATP, an ATP-regenerating system, and cytosol, the addition of GTP stimulated fusion (Fig. 8, curves a and b). GTP had an effect on the extent as well as the initial rate of fusion. If both GTP\(\gamma S\) and GTP were added (Fig. 8, curve c), fusion was inhibited, but not quite to the level observed in the absence of GTP (Fig. 8, curve a). Therefore, hydrolysis of GTP stimulates fusion, confirming previous observations (18–20), but there clearly is fusion in the absence of GTP. Titration of GTP revealed a clear dependence of the initial rate of fusion on the GTP concentration (Fig. 9). Fusion was not further stimulated at GTP concentrations above 50 \(\mu M\). AlF\(_4\), which affects the activity of heterotrimeric G-proteins (Kahn, 1991, 1562), had no influence on fusion (not shown). Thus, fusion between ER vesicles is stimulated by GTP hydrolysis and probably involves small monomeric, not large heterotrimeric GTP-binding proteins.

G-proteins have been found to function as molecular switches in many different processes in cells (31). A family of monomeric G-proteins, the Rab proteins, is known to be involved in fusion (32). To measure the effect of GTP on fusion kinetically, GTP was added at various time points after the onset of fusion in the absence of GTP. It was found that the addition of GTP at 100, 300, or 1000 s after the start of a reaction resulted in an immediate stimulation of fusion (Fig. 10A). Comparison with a control reaction that contained GTP from the start revealed that the rate of fluorescence increase shortly after injection of GTP was about as high as in the control at the time point where fusion had proceeded to the same extent. For example, when GTP was added after 300 s, where the extent of fusion was 3.0%, the rate, measured as the tangent to the fusion curve at that point, immediately after injection, was 1.07%/min. In the control, the rate was also 1.07%/min when the fluorescence increase had proceeded to 3.0%, namely after 185 s. For fusion that was already taking place in the presence of GTP (70 \(\mu M\)), ATP, cytosol, and a regenerating system, addition of more GTP after 1300 s did not further stimulate fusion (data not shown), indicating that GTP was not significantly depleted within the time frame of our measurements. If GTP\(\gamma S\) was injected at various times after the start of fusion in the presence of GTP, fusion was slowed down immediately and proceeded with kinetics that were apparently similar to those seen in a control in which GTP\(\gamma S\) had been present from the onset (Fig. 10B). These data most likely indicate that injection of GTP immediately increases the num-

**Fig. 7. ATP dependence of fusion.** Labeled and unlabeled vesicles were incubated at 37°C with 0 (curves a and b), 2 (curve c), 50 (curve d), or 800 (curve e) \(\mu M\) ATP, 175 \(\mu g\) of cytosol, 10 mM creatine phosphate, 50 \(\mu g/ml\) creatine kinase, and 800 \(\mu M\) GTP, and the monomer fluorescence increase was recorded as described for Fig. 3. For curve a, ATP and ADP were depleted from labeled and unlabeled vesicles by incubating them separately with 20 units/ml of apyrase on ice for 20 min.

**Fig. 8. Fusion between ER vesicles is stimulated by the hydrolysis of GTP.** Fusion of labeled and unlabeled vesicles was measured in the presence of 200 \(\mu M\) ATP, 175 \(\mu g\) of cytosolic protein, 10 mM creatine phosphate, 50 \(\mu g/ml\) creatine kinase (curve a) and, additionally, 70 \(\mu M\) GTP (curve b) or 70 \(\mu M\) GTP and 400 \(\mu M\) GTP\(\gamma S\) (curve c), as described for Fig. 3.

**Fig. 9. GTP dependence of fusion.** Fusion between ER vesicles was measured in the presence of 175 \(\mu g\) of cytosol, 10 mM creatine phosphate, 50 \(\mu g/ml\) creatine kinase, 800 \(\mu M\) ATP and GTP at the concentrations indicated at 37°C as described for Fig. 3. The initial rates of fluorescence increase were determined as described under “Experimental Procedures” and normalized to the rate observed with 800 \(\mu M\) GTP.

**Fig. 10. Effect of the addition of GTP or GTP\(\gamma S\) during the reaction.** A, fusion between labeled and unlabeled ER vesicles in the presence of 175 \(\mu g\) of cytosol, 10 mM creatine phosphate, 50 \(\mu g/ml\) creatine kinase, and 200 \(\mu M\) ATP was measured as described for Fig. 3. Fusion was measured in the absence of GTP (curve a) or 70 \(\mu M\) GTP was added at the times indicated by the arrows (curves b–d). B, fusion between labeled and unlabeled ER vesicles was measured as described above, in the presence of 70 \(\mu M\) GTP (curve a). At the times indicated by the arrows, 200 \(\mu M\) GTP\(\gamma S\) was added (curves b–d).
ber of vesicles that fuse per unit time, whereas GTP\textsubscript{yS} promptly decreases this number. Thus, G-proteins could act as fusion switches, but a basic amount of fusion is maintained in the absence of GTP.

**DISCUSSION**

We have measured fusion between ER vesicles in vitro using a novel assay, based on the fluorescent labeling of cellular membrane phospholipids with pyrene (16, 17). Upon fusion between ER vesicles isolated from labeled and unlabeled CHO cells, lipid mixing due to fusion gave rise to an increase in pyrene monomer fluorescence. While GTP and ATP were not required for fusion, they enhanced its rate and efficiency. The hydrolysis of GTP was required for this stimulation, and we found that the addition of GTP or GTP\textsubscript{yS} had an immediate effect on the reaction, suggesting that G-proteins might be involved in this process. Fusion was inhibited after treatment of the membranes with NEM, but the inhibition could not be overcome by adding preparations of some of the NEM-sensitive proteins known to play a role in fusion, suggesting that novel proteins could be involved.

Inhibition of fusion by GTP\textsubscript{yS} was never complete. Although the reported GTP dependence of mammalian microsome fusion varies widely (see below), some reports indicate that GTP is required (18) or that micromolar concentrations of GTP\textsubscript{yS} completely inhibit fusion (20). Therefore, we have considered the possibility that the increase in fluorescence in the absence of nucleotides might, at least in part, arise from the molecular transfer of labeled lipids to unlabeled membranes. Such a phenomenon was demonstrated for fluorescent probes like octadecylrhodamine (17, 33), particularly when fusion of vesicles derived from organelles, including ER vesicles, was investigated (34, 35). However, here we have used an assay based on pyrene-labeled membrane lipids. Phospholipids do virtually not exchange spontaneously between membranes. We found that the increase in fluorescence was strictly protein-dependent (Fig. 4) and did not occur at 0 °C, excluding a diffusion-controlled, protein-independent lipid transfer process. Lipids can also be exchanged between membranes by lipid exchange proteins, which are present in the cytosol. These enzymes can catalyze lipid transfer to pure lipid membranes and do not require nucleotides for their activity (36). However, only a slight increase in fluorescence was observed if labeled ER vesicles were mixed with unlabeled liposomes prepared from extracted ER lipids (Fig. 4), and this was independent of the addition of cytosol. Also, cytosol did not enhance the increase in fluorescence resulting from the interaction between labeled and unlabeled ER vesicles. Therefore, the increase in fluorescence was caused by membrane fusion, and differences between the GTP dependence reported here and elsewhere are most likely due to differences in experimental set-up and species. Previously published kinetic measurements of the fusion between rat liver microsomes were performed with a resonance energy transfer assay based on octadecylrhodamine and 5-(N-octadecanoyl)aminofluorescein (19). However, in this case, considering the nature of the probes used, contributions to the measured increase in fluorescence by the molecular probe between membranes cannot be excluded.

GTPases often function as molecular switches. The development of a kinetic assay for fusion allowed us to establish the effect of GTP kinetically. GTP was found to immediately enhance fusion, corroborating results obtained with rat liver microsomes (19), and GTP\textsubscript{yS} rapidly slowed down fusion. These results are compatible with a switch function of GTPases, but it appears that to some extent, fusion can take place if the switches are in their "off" position.

In contrast, the addition of ATP did not stimulate the initial rate of fusion, but increased the final extent, indicating that membranes probably have enough bound ATP for a maximal fusion rate initially. ATP is most likely bound to the NEM-sensitive protein(s) that were involved in ER vesicle fusion. One NEM-sensitive protein, NSF, was found to be involved in most intracellular fusion events (3), but homotypic fusion between ER membranes in yeast requires a different, related NEM-sensitive protein, Cdc48p (8), and an NEM-sensitive Cdc48p homologue, p97 (30), is involved in homotypic fusion between mammalian Golgi membranes, in addition to NSF (6, 7). Pools of p97 and Cdc48p are found in the cytosol and nucleus (8, 30), but Cdc48p was also found on nuclear envelopes and peripheral ER (8). Membrane-bound Cdc48p could not be removed from microsomes by extraction with 2 M KCl or 3 M urea (37). In our hands, *Xenopus* p97 could not restore the NEM inhibition of fusion, whether or not it was combined with cytosol, isolated in the presence of ATP, and a 1 M KCl extract of ER membranes. Cytosol isolated in the presence of ATP most likely contained functional p97 (30), and a KCl extract from Golgi membranes was found to supply sufficient NSF and SNAPs to mediate Golgi fusion (6). Therefore, it seems that other NEM-sensitive factors than NSF or p97 are required for mammalian ER vesicle fusion, which could either be tightly associated peripheral membrane proteins, like Cdc48p, or integral membrane proteins. Fusion independent of cytosolic factors was also found for yeast ER vesicles (37). In this respect, ER vesicle fusion seems comparable with nuclear envelope fusion, which required the addition of nucleotides but not of cytosol (38), except that the ER vesicle membranes apparently contained proteins in their ATP-bound form. Whether such novel proteins could function alone or would require any of the already known NEM-sensitive proteins, remains to be determined.

The kinetics of fusion reported by the pyrene assay are best compared with those reported by Watkins et al. (20) for the fusion of microsomes from myeloma cells, measured by the formation of antibody. They found half-maximal fusion after about 40 min, which is in line with the \( t_{1/2} \) of 56 min that we observed. But in contrast to their observations, we did not see a slow onset of fusion leading to a sigmoidal time dependence of fusion (20), indicating an additional slow step in the fusion reaction. Although in our hands fusion continued at a very low rate for a long time, it reached a final level of 40.7% after several hours. Assuming that all vesicles have the same size, and that one labeled vesicle fuses only once with an unlabeled vesicle (one round of fusion), this would mean that 81% of the labeled vesicles fused. In the case of labeled vesicles fusing with a large number of unlabeled vesicles, the amount of labeled vesicles that fused would be closer to 41%. This is in the range of 25–50% that Watkins et al. (20) found for the efficiency of fusion between purified microsomes and a post-nuclear supernatant, respectively. These data indicate that even with this rather crude preparation of ER vesicles, most of the fusion must have been induced by material stemming from the ER.

The use of kinetic assays for membrane fusion should facilitate the study of the mechanisms of intracellular fusion, particularly if membrane fusion events are reconstituted in vitro using purified components. The assay used in this paper is potentially widely applicable, as all cellular membranes are labeled with pyrene.

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