The EMBO Journal Vol.18 No.8 pp.2074–2083, 1999

Cytosolic ATPases, p97 and NSF, are sufficient to mediate rapid membrane fusion

Marlies Otter-Nilsson[1,2], Rob Hendriks[3], Eve-Isabelle Pecheur-Huet[2], Dick Hoekstra[2,4] and Tommy Nilsson[1,4]

[1]EMBL, Cell Biology and Biophysics Programme, Meyerhofstrasse, 69117 Heidelberg, Germany, [2]Department of Physiological Chemistry, Medical Faculty, University of Groningen, 9713 AV, The Netherlands and [3]ZMBH, 69120 Heidelberg, Germany

[4]Corresponding authors
e-mails: nilsson@embl-heidelberg.de and d.hoekstra@med.rug.nl

D.Hoekstra and T.Nilsson contributed equally to this work

Much recent work has focussed on the role of membrane-bound components in fusion. We show here that p97 and NSF are sufficient to mediate rapid membrane fusion. Fractionation of cytosol revealed that p97 and its co-factor, p47, constitutes the major fusion activity. This was confirmed by depleting p97 from the cytosol, which resulted in an 80% decrease in fusion. Using purified protein, p97 or NSF was found to be sufficient to mediate rapid fusion in an ATP-dependent manner. A regulatory role was observed for their corresponding co-factors, p47 and α-SNAP. When present at a molar ratio half of that of the ATPase, both co-factors increased fusion activity significantly. Intriguingly, at this ratio the ATPase activity of the complex measured in solution was at its lowest, suggesting that the co-factor stabilizes the ATP state. The fusion event involved mixing of both leaflets of the opposing membranes and contents of liposomes. We conclude from these data that p97, NSF and perhaps other related ATPases catalyse rapid and complete fusion between lipid bilayers on opposing membranes. This highlights a new role for p97 and NSF and prompts a re-evaluation of current fusion models.
Keywords: ATPases/membrane fusion/NSF/p97

Introduction

Both cytosolic and membrane proteins have been implicated in cellular fusion events. Fusion of transport vesicles to acceptor membranes requires the action of cytosolic ATPases such as *N*-ethylmaleimide-sensitive fusion protein (NSF), which binds to membrane-bound SNAP receptors (SNAREs) via its co-factor α-SNAP (Rothman, 1994). Different SNAREs have been identified, both on vesicle and target membranes, and recent evidence suggests a role for v- and t-SNAREs in fusion (Weber *et al*., 1998). Indeed, the SNARE hypothesis put forward by Rothman and colleagues postulates that v- and t-SNAREs mediate docking and fusion of two opposing membranes, and that correct pairing of v- and t-SNAREs accounts for the specificity required for homo- and heterotypic fusion (Sollner *et al*., 1993). To initiate fusion, the ATPase NSF and its co-factor α-SNAP are thought to break up preformed complexes of v- and t-SNAREs on the same membrane, thus allowing the SNAREs to perform fusion (Barnard *et al.*, 1997; Hanson *et al.*, 1997; Otto *et al*., 1997). The NSF and α-SNAP induced break up of SNAREs can occur already on undocked membranes suggesting that fusion can be primed at an early stage, even before docking between membranes has occurred (Swanton *et al.*, 1998).

In a similar fashion to NSF, the abundant cytosolic ATPase, p97, has been shown to be required for fusion and reassembly of both the nuclear envelope (Peters *et al.*, 1990; Latterich *et al.*, 1995; Patel *et al.*, 1998) and the Golgi apparatus (Acharya *et al.*, 1995; Rabouille *et al.*, 1995). Recently, Latterich and co-workers demonstrated a direct interaction between the yeast homologue of p97, cdc48p, and its t-SNARE, Ufe-1, and that this interaction was required for fusion of endoplasmic reticulum (ER) membranes (Patel *et al.*, 1998). In mammalian cells, this ATPase is thought to bind to the t-SNARE, syntaxin 5, in much the same way as NSF: through its co-factor, p47 (Kondo *et al.*, 1997; Patel *et al*., 1998). In fact, both p47 and α-SNAP compete for binding to the same t-SNARE, syntaxin 5. The binding of NSF–α-SNAP and p97–p47 to syntaxin 5 suggests that they interact with a common fusion machinery: the SNAREs. Alternatively, SNAREs may constitute a common platform for the recruitment of a cytosolic fusion machinery such as p97 and NSF. That SNAREs indeed mediate a necessary or facilitated bridging between two opposing membranes was shown by homotypic fusion of vacuolar membranes (Nichols *et al*., 1997). Only when placing a v- and a t-SNARE on the opposing membranes was efficient fusion observed, showing the need for correct SNARE pairing. To demonstrate that SNAREs themselves mediate fusion a liposome–liposome fusion assay was used incorporating v- and t-SNAREs on the opposing membranes (Weber *et al.*, 1998). This minimal fusion assay provided some evidence which suggested that v- and t-SNAREs could suffice in fusion. However, kinetics were slow and extent of fusion was low. Strikingly, no major effect of NSF or its corresponding co-factors (SNAPs) could be demonstrated. This warrants further study.

In this study, we have examined fusion of liposomes to Golgi membranes and fusion between liposomes. We show that surprisingly, cytosol alone suffices to mediate fusion and that p97 constitutes the major cytosolic fusion activity. Indeed, p97 alone was able to mediate fusion between opposing membranes in an ATP-dependent manner. Similarly, using recombinant protein, we show that NSF also induced fusion in an ATP-dependent manner. Furthermore, we reveal a regulatory function for their corresponding co-factors, p47 and α-SNAP, in what appears a

stabilization of the ATP state of the fusion complex. Both ATPases were found to catalyse complete fusion, as shown by using both lipid- and content-mixing assays. The implications of these findings are discussed.

Results

Rationale of the fusion assay

Two dequenching assays were deployed in this study to examine either fusion between donor liposomes and rat liver Golgi membranes (L–G) or fusion between donor and acceptor liposomes (L–L). Donor liposomes were made up by phospholipids plus 0.8% *N*-(7-nitro-2,1,3,-benzoxadiazole-4-yl)-phosphatidyl ethanolamine (NBD-PE) and 0.8% *N*-(lissamine rhodamine B sulfonyl) (rhodamine-PE). Prior to fusion, NBD-PE is quenched by rhodamine-PE through efficient energy transfer (Struck *et al.*, 1981; Hoekstra and Duzgunes, 1993). Upon fusion, energy transfer is decreased in proportion to dilution and a concomitant increase in NBD fluorescence is then monitored over time as a measure of fusion (see Figure 1A, control, for a typical fusion curve). Unilamellar liposomes (50–70 nm) were made of mixtures of dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylserine (DOPS) and/or dioleoylphosphatidylcholine (DOPC). These are the three most abundant phospholipids in the cytosolic leaflet of Golgi membranes (Kleinsmith and Kish, 1995). Upon examining different mixtures of phospholipids, we found that incorporation of DOPE into donor liposomes gave rise to optimal fusion kinetics (see Table I, top six rows, for direct comparison in fusion kinetics using different phospholipids). Fusion was also observed when the liposomes were made entirely of DOPC or in combination with DOPE and/or DOPS. However, with DOPC alone, both the fusion kinetics and final extent of fusion were almost seven times lower. The facilitated membrane fusion observed with DOPE was reminiscent of previous studies where peptide-induced fusion and liposome fusion to endocytic vesicles or viruses were examined (Epand *et al.*, 1993; Vidal and Hoekstra, 1995; Pecheur *et al.*, 1998). These studies revealed that the negative curvature-promoting properties of DOPE, which is related to its cone-shaped structure, stimulate fusion. This stimulative effect could be overcome by the incorporation of the inverted cone-shaped and positive curvature-stimulating lipid, lysophosphatidylcholine (lysoPC) (Chernomordik *et al.*, 1997) or by the addition of a tri-peptide, ZfFG. The latter interferes directly with the negative curvature transition of DOPE (Epand *et al.*, 1995). We tested both lysoPC and ZfFG in the L–G and L–L fusion assays and as can be seen, fusion was inhibited or abolished completely in the presence of lysoPC or ZfFG. This shows that negative curvature favoured fusion in our system as well (Table I). In passing, we note that neither lysoPC nor ZfFG alone caused a change in NBD fluorescence as a consequence of a direct transfer of lipid probes between membranes. We also examined the role of pH and found that fusion kinetics were optimal between pH 6 and 7, both in the L–G fusion assay and the subsequent L–L minimal fusion assay using defined components. Therefore, fusion was carried out using this pH range throughout this study. A typical assay was performed at 37°C and monitored for 5 min; with further incubations as long as 1 h, little if any further fusion was observed. Extent of membrane fusion is expressed as a percentage of maximal fluorescence obtained upon addition of detergent measured at conditions that were otherwise identical to those in the experiment.

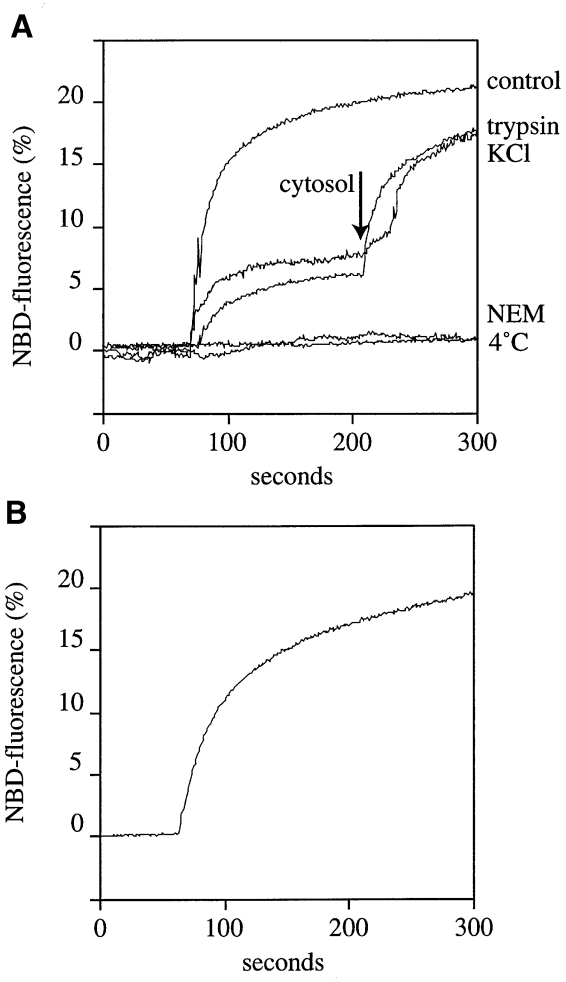

Fig. 1. Cytosol is sufficient for fusion. (**A**) Fluorescently labelled DOPE/DOPS liposomes were incubated with Golgi membranes at 37°C or 4°C. Golgi membranes were pre-incubated on ice for 30 min with either buffer as a control, trypsin (10 μg/ml), KCl (2 M) or NEM (2 mM). Treated membranes were spun down at 13 000 r.p.m. in an eppendorf table centrifuge for 15 min at 4°C to remove trypsin or KCl before being added to the liposomes. Cytosol (0.3 mg) was added after the reaction between liposomes and treated membranes had stabilized (arrow). DTT (3 mM) was added after the NEM reaction to quench excess NEM. (**B**) Rat liver cytosol (0.3 mg) was added to fluorescently labelled DOPE/DOPS (1:1) donor and a 5-fold excess of unlabelled DOPE/DOPC (1:1) acceptor liposomes. Extent of membrane fusion is expressed as the percentage of the maximal NBD-fluorescence signal that was obtained upon addition of 0.1% Triton X-100 to liposome/membrane mixtures in parallel but identical experiments.

Fusion of liposomes to purified Golgi acceptor membranes

To determine the need for membrane-bound and cytosolic components, we first examined fusion using the L–G fusion assay. The diameter of liposomes was chosen to reflect that of ER to Golgi and intra Golgi transport vesicles (Malhotra *et al.*, 1989). Unilamellar donor liposomes (50–70 nm in diameter) were incubated for ~1 min at 37°C with buffer in the reaction vessel to establish a baseline. Golgi membranes were then added to the reaction. As can be seen in Figure 1A, a rapid and efficient fusion

Table I. Effect of lipid composition on membrane fusion

Lipid composition	L–G	L–L
DOPE/DOPS	100	100
DOPE/DOPS/DOPC	61.3	100
DOPE/DOPC	56.0	72.4
DOPS	38.7	56.5
DOPC/DOPS	17.2	52.2
DOPC	13.9	40.5
DOPE/DOPS + LPC-1	37.9	nd
DOPE/DOPS + LPC-2	7.4	0.4
DOPE/DOPS + ZfFG	9.6	11.7
DOPE/DOPS + ZGGA	99.0	100

Donor liposomes of different lipid compositions at equal molar ratios (100 μM final concentration) were either mixed with rat liver Golgi membranes (L–G) or with 5-fold excess of unlabelled acceptor liposomes (DOPE/DOPS, L–L), the latter in the presence of purified p97–p47 complex in the presence of 1 mM ATP. Relative fusion rates are expressed as a percentage of the maximal fusion rate (100%) obtained with the DOPE/DOPS donor liposomes. The top six rows reflect influence of individual lipids on fusion with respect to their propensity to form negative curvature. The bottom left four rows show the effect of external modulators on membrane fusion: here, DOPE/DOPS donor liposomes were mixed with rat liver Golgi membranes pre-incubated with lysoPC [50 μM final concentration, LPC-1 (palmitoyl)] or with peptides (20 μM final concentration, ZfFG or ZGGA). Note that the control peptide, ZGGA, showed no inhibition of fusion. Alternatively, donor liposomes were pre-incubated with lysoPC [10 μM final concentration and mixed with Golgi membranes (LPC-2)]. Donor liposomes were also mixed with acceptor liposomes pre-incubated with either lysoPC or with tri-peptides in the presence of purified p97–p47 complex (bottom right three rows).
nd, not determined.

of liposomes with Golgi membranes was observed: a typical reaction was usually completed within 1 or 2 min with a relative fusion efficiency of 20% (control). Pre-incubation of Golgi membranes with *N*-ethyl maleimide (NEM) (2 mM), an alkylating agent, for 30 min on ice, followed by addition of 3 mM dithiothreitol (DTT) to quench any remaining NEM prior to adding the membranes to the liposomes, abolished fusion completely (Figure 1A). Pre-treatment of Golgi membranes with either trypsin (10 μg/ml) or KCl (2 M), which would strip them of membrane-associated but not -embedded proteins, produced an inhibition of 50–60% (Figure 1A). These results showed a requirement of peripheral proteins in fusion. This was further supported by the ability of cytosol to almost completely restore fusion following trypsin or KCl pre-treatment (Figure 1A, arrow). In contrast, NEM-treated membranes could only be partially restored upon addition of cytosol (25%; not shown), suggesting that once inactivated but still bound to the membrane, fusion components could not be replenished by the cytosol. Fusion of liposomes with Golgi membranes was also shown to be temperature dependent, as no fusion was observed at 4°C (Figure 1A). As trypsin or KCl treatment did not completely reduce fusion (compare with NEM), it was possible that membrane proteins such as SNAREs did mediate the observed fusion, and that this was regulated or stimulated by cytosolic factors such as p97 and NSF. Alternatively, the trypsin or KCl treatment may have only partially removed cytosolic fusion factors. To distinguish between the two possibilities, we first examined whether or not cytosol alone would suffice in mediating fusion using a L–L fusion assay.

Identification of an ATPase, p97–p47, as a major cytosolic fusion complex

Prior to testing cytosol in fusion, we performed control experiments to verify the reliability of monitoring lipid mixing in the L–L fusion assay. First, addition of cytosol to donor liposomes (DOPE/DOPS) containing NBD-PE and rhodamine-PE caused no effect on fluorescence. Secondly, no alterations in fluorescence were observed when mixing donor with acceptor liposomes in the absence of cytosol. Having tested the reliability of the assay, we added cytosol to the L–L assay. As can be seen in Figure 1B, rapid and efficient fusion was observed, perhaps only slightly slower than that seen in Figure 1A. Taken together, this shows that cytosol alone is sufficient to promote fusion between two opposing membranes. This allowed us to fractionate the cytosol to determine which factor(s) was involved. We devised a three-step fractionation procedure where cytosol was first subjected to protein precipitation using ammonium sulfate (AS), followed by two chromatography steps using a Resource Q and a Mono Q column. To monitor the fusion activity of the fractions (V_{Fus}), we expressed the initial difference in NBD fluorescence (Δ) over time (minutes) per amount protein (microgrammes) using the L–L fusion assay. For the cytosol, the calculated V_{Fus} was low as a consequence of the multitude of cytosolic proteins. Upon fractionation, the fusion factor(s) became enriched over other proteins and the value of V_{Fus} increased as a consequence. Figure 2A shows the result of the entire fractionation experiment. The fusion activity was first pelleted by the addition of AS. We tested several concentrations, including higher ones, and found that 20% AS was satisfactory to pellet most of the fusion activity. Precipitated material was then resuspended, dialysed and applied to a Resource Q column (Figure 2A, middle). A peak was observed around fractions 11 and 12, and this material was pooled and applied to the Mono Q column (Figure 2A, right). A sharp increase in V_{Fus} was observed in fraction 5 from the Mono Q eluate. Subjecting fractions to SDS–PAGE followed by Coomassie staining revealed a single protein band in fraction 5 migrating at the same position as the 97 kDa mol. wt marker (Figure 2B, shown are representative lanes of the fractionation experiment). The identity of this protein was confirmed by Western blotting and immunodetection using antibodies against p47 and p97. The 97 kDa protein corresponded to the abundant cytosolic ATPase, p97 (Figure 2B, right lane, WB). To show that the fractionation had correctly revealed the major fusion factor, we depleted p97 from the cytosol using a recombinant and His-tagged version of the co-factor, p47. The co-factor was adsorbed to a solid support and p97 was removed from the cytosol (Figure 2C). This resulted in a 7-fold reduction in the initial fusion rate, showing that p97 constitutes the major fusion activity in cytosol. That p97 indeed co-fractionated with the fusion activity throughout the fractionation experiment is shown in Figure 2D. Furthermore, p47 appeared to co-fractionate with p97, suggesting that p97–p47 exists as a stable complex. To show that the depleted cytosol could be rescued in terms of fusion activity, we added purified p97–p47 to the depleted cytosol at the original concentration present in the cytosol (Figure 2E). We also monitored the functionally and structurally related NSF. Rather than peaking in

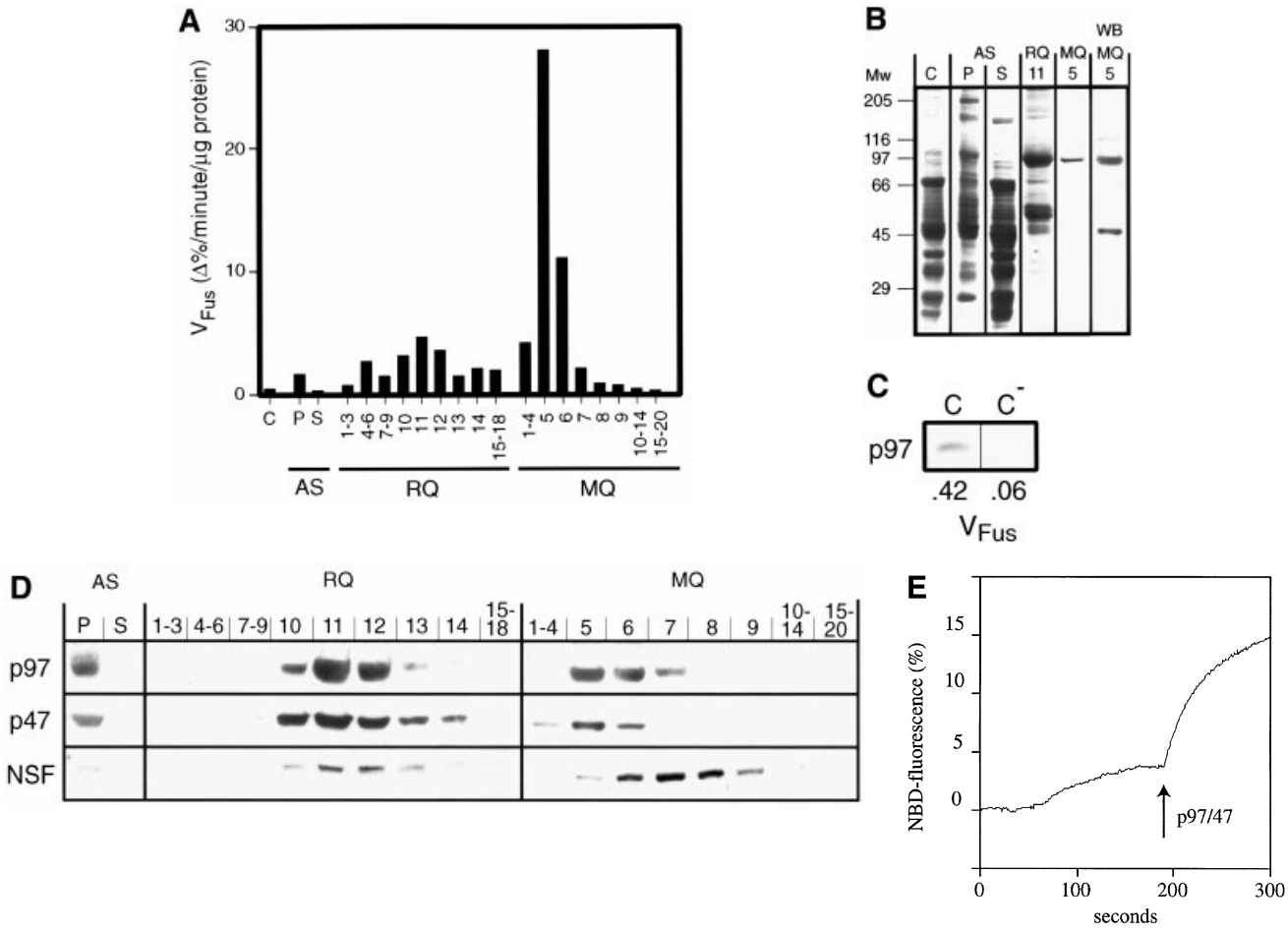

Fig. 2. Fractionation of rat liver cytosol reveals p97 as the major fusion factor. (**A**) Rat liver cytosol (C) was precipitated with 20% AS. Pellet (P) and supernatant (S) were dialysed and the former was applied to a Resource Q column (RQ). Fractions were analysed for protein amount and initial fusion rate (as in Figure 1B). Peak fractions 11 and 12 were pooled and applied to Mono Q column (MQ). (**B**) Ten microlitres of selected fraction was subjected to SDS–PAGE followed by Coomassie staining and Western blot analysis to reveal p97 and p47. Apparent mol. wt (Mw) was determined by mol. wt standards and expressed in kilodaltons. (**C**) Rat liver cytosol was incubated with p47 attached to Ni-Sepharose to deplete p97. (**D**) Ten microlitres of all fractions were subjected to SDS–PAGE followed by Western blot analysis to reveal p97, p47 and NSF. (**E**) Depleted cytosol [from (C)] was incubated with donor and acceptor liposomes to monitor fusion. Purified complex p97–p47 (3 μg/ml final concentration) was added (arrow) to restore fusion.

fraction 5, this ATPase peaked in fraction 7 and 8 of the Mono Q eluate, well away from the peak fusion activity. In conclusion, the data showed that cytosol alone suffices to mediate fusion between donor and acceptor liposomes and that the p97–p47 complex constitutes the major fusion activity.

Purified p97–p47 complex catalyses rapid membrane fusion

Is the p97–p47 complex enough to induce membrane fusion? First, anti-p47 antibodies were added to rat liver Golgi membranes (Figure 3A). This resulted in a >50% inhibition of fusion compared with control antibodies using the L–G fusion assay. Cytosol (Figure 3A, arrow) then restored fusion to normal values. In contrast, re-addition of p97-depleted cytosol failed to restore fusion, indicating that p97 had been the target in the antibody inhibition experiment. Indeed, addition of p97–p47 in the form of a purified complex restored fusion (not shown).

To test whether the p97–p47 complex adequately promotes fusion, we monitored fusion using the L–L fusion assay. Purified p97–p47 complex (10 nM) derived from rat liver cytosol was mixed with donor and acceptor liposomes. This resulted in a rapid fusion event (Figure 3B). To approach the question of whether the fusion event was an ATP- driven process, we utilized the fact that p97 has a pH optima for ATP hydrolysis between 8 and 9 (Zhang *et al.*, 1994). The p97–p47 complex was subjected to a pH shift to 9.0 for 30 min at 4°C, and then shifted back to neutral pH and subsequently tested for fusion using the L–L fusion assay. Figure 3B shows that subjecting the complex to high pH and returning this to neutral pH completely inactivated the ability to mediate fusion. Strikingly, addition of 1 mM Mg-ATP (Figure 3B, arrow) restored fusion almost completely. Presumably, due to a conformational change in the complex, the higher pH had allowed p97 to hydrolyse its ATP more rapidly leaving the complex in an ADP state (Hanson *et al.*, 1997). Re-addition of ATP then restored the ability of the complex to mediate fusion. This suggested that bound ATP was a prerequisite for the p97–p47 complex mediated fusion event.

To examine the role of ATP more directly, we performed a series of experiments using p97 alone or in combination with p47. Highly purified fractions of p97 were obtained

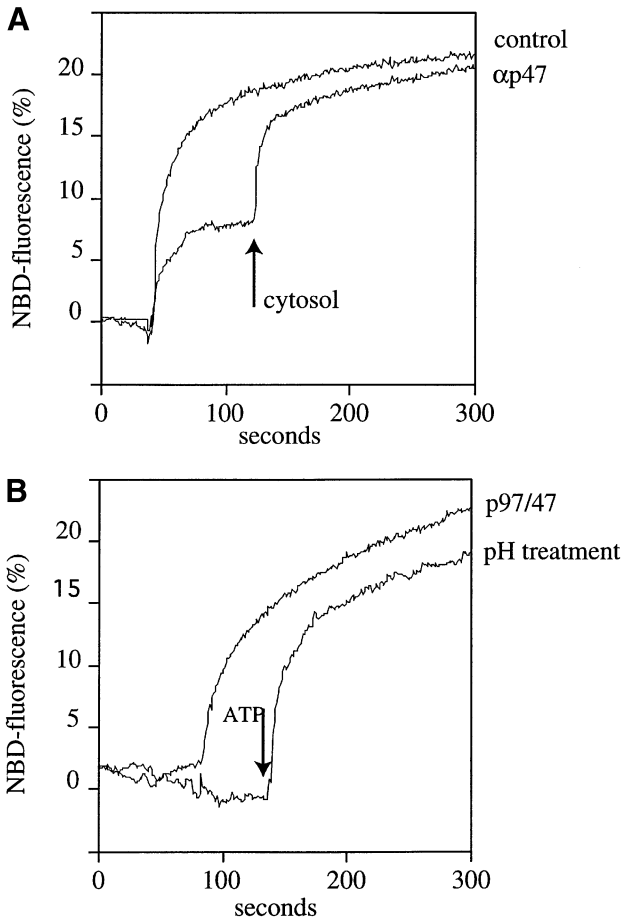

Fig. 3. p97–p47 is sufficient for membrane fusion. (**A**) Rat liver Golgi membranes were pre-incubated with a rabbit polyclonal control antibody or anti-p47 antibody (1:50) on ice for 30 min and then added to fluorescently labelled DOPE/DOPS liposomes as described in Figure 1A. (**B**) A 5-fold excess unlabelled DOPE/DOPC liposomes were mixed with fluorescently labelled DOPE/DOPS liposomes as described in Figure 1B. Purified p97–p47 complex (final concentration 10 nM) from rat liver cytosol was added to the mixture. Alternatively, the complex was pre-incubated on ice at pH 9 for 30 min and then returned to pH 6.0 and transferred to the cuvette. After 2 min, 1 mM of Mg-ATP (final concentration) was added to the mixture (arrow).

Table II. Influence of co-factors p47 and α-SNAP on ATPase activity and fusion

A		p97	NSF
	–	2.3	1.5
	ATP	5.9	2.9
	ATPγS	2.5	1.7
	HD	2.5	1.7
	DS	2.4	1.5
	RG	10.8	5.2
20:1	ATP	5.3	2.7
2:1	ATP	18.3	11.1
1:5	ATP	2.6	1.7
2:1	ATPγS	2.7	2.3
2:1	ATP→DS	16.9	10.0
20:1	RG	10.5	4.5
2:1	RG	35.7	19.0
1:5	RG	4.3	2.9
:CF		V_{Fus}	V_{Fus}

B	p97	NSF
–	9.1	5.5
20:1	8.0	5.2
2:1	1.9	1.0
1:5	4.5	3.3
:CF	V_{Pi}	V_{Pi}

(**A**) Purified p97 (2 μg/ml final concentration) and NSF (5 μg/ml final concentration) were tested in terms of fusion activity with or without ATP (1 mM final concentration), ATPγS, ATP DS or an ATP RG. p97 and NSF were also subjected to heat-inactivation for 5 min at 95°C (HD). Both ATPases were also tested together at different ratios with their corresponding co-factors (:CF), p47 and α-SNAP. p97 and NSF were pre-incubated with ATP and their co-factors for 30 min on ice and then in the presence of an ATP DS (ATP→DS). Fusion activity is expressed as the initial difference in NBD fluorescence (Δ) over time (minutes) per amount protein (μg). (**B**) ATPase activity (V_{Pi}) was determined (Ames, 1966) and is expressed as nmol Pi/h/μg of ATPase.

from bovine liver and frog cytosol (kind gifts from Drs Latterich and Robinson, Salk Institute, San Diego, CA). These fractions contained very little or no additional proteins, as judged by SDS–PAGE followed by silver staining (not shown). Using purified p97, we either omitted or included ATP. As can be seen in Table IIA, addition of 1 mM ATP resulted in an ~2-fold stimulation of fusion. In contrast, addition of ATPγS, a non-hydrolysable analogue of ATP, reduced fusion to values comparable to p97 in the absence of ATP. To rule out the possibility that p97 caused fusion non-specifically, perhaps by being partially unfolded or denatured, we heat-denatured p97. Subjecting p97 to heat denaturation resulted in an inactivation of the observed fusion activity, showing that this was not the case. A similar decrease in fusion activity was also observed upon addition of an ATP depletion system (DS). Taken together, these demonstrate that ATP hydrolysis was required for the p97-mediated fusion event. However, the observed overall fusion kinetics with purified p97 were lower than expected (compare with fraction 5 of the Mono Q-eluate in Figure 2). A reasonable explanation for this was found in elegant structural work revealing that p97 and NSF form hexameric ring structures (Peters *et al.*, 1992; Kessel *et al.*, 1995; Hanson *et al.*, 1997; Kondo *et al.*, 1997; Rabouille *et al.*, 1998). For NSF, it was shown that even in the presence of 1 mM ATP, this ATPase preferred to be in an ADP state (Hanson *et al.*, 1997). Thus, addition of 1 mM ATP to NSF would result in an initial and rapid conversion to ADP. Since ADP is the preferred state, this would then result in a less active ATPase. As p97 and NSF are similar in both structure and function, it was feasible that p97 also preferred ADP over ATP. To test this, an ATP regenerating system (RG) was included. This would ensure that a buildup of ADP was avoided over the course of the incubation. As can be seen, this boosted fusion ~2-fold (Table IIA, RG). However, kinetics were still lower than for the enriched p97–p47 complex in the fractionation experiment (Figure 2A). The probable explanation for this was that p47 was also required. To test this, purified p47 was mixed together with p97 and 1 mM ATP for 30 min on ice and then added to the L–L fusion assay. Several ratios were tested, and Table IIA shows the values obtained for three of these (p97:p47; 20:1, 2:1 and 1:5). Strikingly, at a ratio of 2:1, fusion kinetics more than doubled compared with p97 or ATP alone. In contrast, excess of p47 appeared to inhibit fusion altogether. Furthermore, as p47 alone exerted neither ATPase nor fusion activity (not shown), the increased fusion activity at the 2:1 ratio suggested that p47 modulated

p97, perhaps by promoting exchange of ADP to ATP. To examine further whether p47 contributed to fusion in a p97- and ATP-dependent manner, we pre-incubated p97 and p47 at the optimal 2:1 ratio in the presence of ATPγS. This reduced fusion to the values obtained with p97 in the presence of ATPγS or in the absence of ATP, showing that p47 acted through p97-driven ATP hydrolysis. We next tested the ATPase activity (V_{Pi}) of p97 and the p97–p47 complex. Surprisingly, at the ratio of 2:1 (optimal for fusion) the ATPase activity of the complex was at its lowest (Table IIB). This argues that p47 promotes an exchange of ADP to ATP by stabilizing the fusion complex in the ATP state. This finding has important implications in terms of a regulatory mechanism and may explain why a role for ATPases in fusion has previously been placed upstream of the actual fusion event. An explanation for this is based on the observation that fusion is insensitive to the action of hexokinase following rearrangement of the SNARE complex. In light of above findings, it is reasonable to assume that p47 'protects' the ATP bound to p97. To test this, the p97–p47–ATP complex was pre-assembled on ice and then incubated in the presence of an ATP DS. As can be seen in Table II, addition of an ATP DS had little if any effect on the ability of the complex to mediate fusion showing that, once formed, the p97–p47–ATP complex is resistant to ATP depletion by hexokinase. However, depletion of ATP from the preformed complex was possible during incubation at pH 9, suggesting that p47 now failed to protect the ATPase–ATP complex. Finally, we tested the co-factor in the presence of an RG. This promoted fusion even further, ~2-fold, presumably by removing possibly competing ADP. Taken together, the above data show that the p97–p47–ATP complex suffices to mediate efficient and rapid membrane fusion. This requires hydrolysis of ATP. Fusion is promoted greatly by removal of competing ADP. The role of the co-factor, p47, when present at a ratio of 2:1 (p97:p47) appears to be to stabilize the ATP state of p97 and provides regulatory means for the fusion complex.

Purified NSF–α-SNAP complex catalyses rapid membrane fusion

As p97 and NSF are structurally and functionally similar, it was now important to examine NSF in membrane fusion. The fractionation experiment presented in Figure 2 shows that NSF did not co-fractionate with the peak fusion activity, suggesting that NSF was not a major fusion factor. However, the amount of p97 in the cytosol is at least 10 times higher than that of NSF (Dr G.Warren, personal communication). It was therefore likely that the fractionation experiment had favoured p97 over NSF, and perhaps other ATPases. Also, within the limit of detection using an anti-bovine α-SNAP antibody, α-SNAP did not appear to co-fractionate with NSF, but rather peaked in fraction 5 of the Mono Q eluate (not shown), further explaining why NSF had not been revealed in the fractionation experiment. To test directly whether NSF mediates membrane fusion, we used recombinant and highly purified NSF (Sollner *et al.*, 1993) and α-SNAP (Whiteheart *et al.*, 1993) (kind gifts from Drs H.McBride and M.Zerial, EMBL, Heidelberg, Germany). Table II shows that NSF mediates fusion in the same manner as that observed for p97. Consistently, the fusion kinetics were ~50% compared with p97, which might have been the consequence of using recombinant material. Fusion required ATP and was blocked by the addition of ATPγS, showing that ATP hydrolysis was a prerequisite for NSF-mediated fusion. In a similar manner to p47, the co-factor α-SNAP exhibited a modulating role and the optimal ratio for fusion was again 2:1 (NSF:α-SNAP). As with p97–p47, the ATPase activity of the complex was at this ratio at its lowest. Furthermore, assembly of NSF and α-SNAP in the presence of ATPγS blocked fusion, and α-SNAP was able to stabilize and protect the NSF complex from hexokinase. As a control, we also tested α-SNAP alone. No ATPase or fusion activity was detected with this co-factor (not shown).

The similarity between the two ATPases, p97 and NSF and their respective co-factors p47 and α-SNAP, was striking, and raises the interesting possibility that other structurally related ATPases may catalyse fusion as well.

Lipid and content mixing assays demonstrates complete fusion

Having established that p97 and NSF efficiently promote rapid membrane fusion, it was important to examine whether fusion had mixed both leaflets and contents of the liposomes. Lipid mixing of the inner leaflet was examined by extinguishing NBD fluorescence specifically in the outer leaflet by pre-incubating NBD-labelled donor liposomes with sodium dithionite (Hoekstra *et al.*, 1993). This left NBD intact in the inner leaflet. Upon fusion with acceptor liposomes (DOPE/DOPC) in the presence of the purified p97–p47 complex, similar fusion kinetics were observed compared to non-treated liposomes. The total fluorescence signal was ~40% of that of the control (Figure 4). This can be ascribed to the smaller surface area of the inner relative to the outer leaflet (44:56) in the liposomes used in this study (50–70 nm in diameter). A content mixing assay was also deployed mixing donor liposomes containing amino-naphthalenetrisulphonic acid (ANTS) with acceptor liposomes containing *p*-xylylene bis(pyridium)bromide (DPX) (Duzgunes and Wilschut, 1993). Upon fusion and content mixing, ANTS becomes quenched by DPX and this can be monitored as a decrease in relative fluorescence of ANTS. As can be seen in Figure 4, mixing of ANTS and DPX initially occurred with kinetics similar to previous fusion experiments. The total extent of fusion was somewhat lower. However, as the initial fusion rate was similar to fusion experiments using NBD dequenching, these data are explicative for a complete fusion event as mediated by the p97–p47 complex. In addition, the apparent similarity of the initial kinetics of lipid and contents implies that the p97–p47-induced fusion event was essentially a non-leaky process. Indeed, when monitoring directly the leakage of the ANTS–DPX complex, as reflected by an increase of ANTS fluorescence (Ellens *et al.*, 1984), essentially no fluorescence development is seen up to 2–3 min after addition of p97–p47. Even after extended incubation periods (10 min), leakage was still barely detectable (<5%). In conclusion, we have demonstrated that ATPases such as p97 and NSF catalyse rapid and complete membrane fusion between two opposing membranes. As p97 and NSF are cytosolic, it is likely they interact with membranes and partially embed themselves in

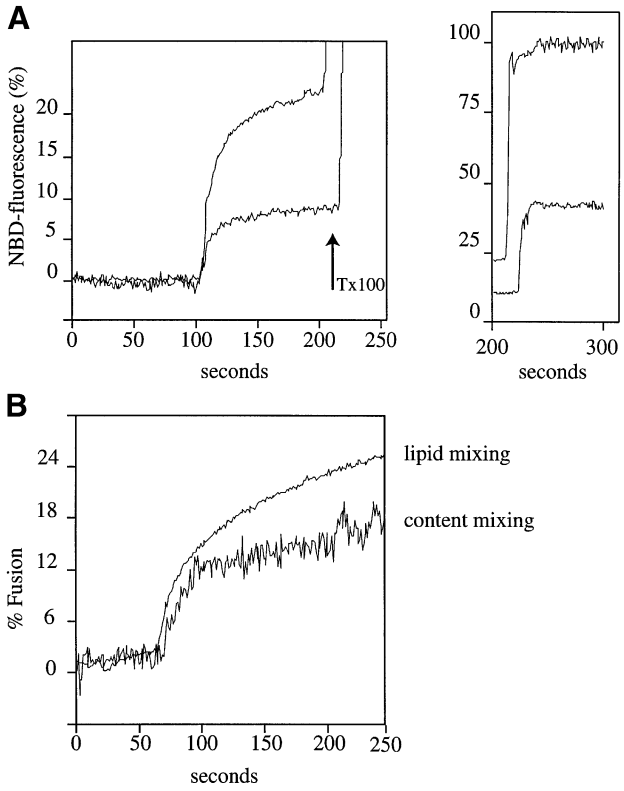

Fig. 4. The p97–p47 complex mediates mixing of both leaflets and liposomal contents. (**A**) Purified p97–p47 complex (10 nM) was added to untreated (upper curve) or sodium dithionite treated (lower curve) donor liposomes (DOPE/DOPS) and acceptor liposomes (DOPE/DOPC). Following fusion, 0.1% Triton X-100 was added (arrow) to determine maximal fluorescence signal. (**B**) Donor liposomes containing 25 mM ANTS were mixed with acceptor liposomes containing 90 mM DPX. Upon addition of purified p97–p47 (10 nM), fusion was monitored as a decrease in ANTS. To enable a direct comparison with the lipid mixing assay, the curve displayed in Figure 3B is shown as a reference. However, the extent of fusion is here directly expressed as the percentage of total fusion. The 5-fold excess of acceptor liposomes results in that a 100% fusion signal corresponds to 83.5% of obtained NBD fluorescence. Hence, the reference curve here shows a maximum extent of 24% fusion. The data for the content mixing curve have been inverted and is expressed as percent fusion. Note the similar initial kinetics of the two assays.

the lipid bilayer. Presumably, this allows them to modulate locally the properties of the membrane to promote fusion.

Discussion

The fusion event

The suggestion that p97 and NSF modulate membrane properties to promote or catalyse fusion is consistent with postulates of the stalk–pore fusion model (Chernomordik and Zimmerberg, 1995). First, cytoplasmic leaflets fuse to form a stalk across cytoplasmic space. This is followed by fusion of the extra-cytoplasmic leaflets which are brought in close proximity to the stalk to form a fusion pore. The pore then extends laterally to complete the process. In this model, formation of a stalk depends on a negative curvature in the cytoplasmic leaflets, enabling these to extend outwards. Such dependency has been demonstrated previously for viral fusion proteins (Epand *et al.*, 1995; Chernomordik *et al.*, 1997). Our data showing an enhancing effect of DOPE both in the L–G and the L–L fusion assay are also consistent with this model, in that negative curvature was favourable in the ATPase-mediated fusion event. The use of DOPE, DOPS and DOPC approximates the physiological situation in that these represent the most abundant phosholipids in the outer leaflet of Golgi membranes. That the ATPase-mediated fusion event is in support of the stalk–pore fusion model is also suggested by the ability of lysoPC and the tri-peptide ZfFG to inhibit fusion in both the L–G and the L–L assay. Both lysoPC and ZfFG oppose negative curvature, and consequently inhibited the ability of the ATPases to catalyse fusion.

To determine whether p97 and NSF catalysed complete rather than hemifusion or worse, depletion of fluorescent lipid and/or content was tested directly. First, liposomes and incorporated fluorescence were stable over long periods of time, well exceeding that of the time course used in this study. Secondly, very little leakage of content was observed in the presence of purified p97–p47 and ATP. Thirdly, mixing of the inner leaflets and between the contents of the donor and acceptor liposomes was observed. Together, these show that fusion was complete, and that pore formation and dilation took place, permitting contents to mix. The similarity of the initial kinetics and extent of fusion in the different fusion assays used in this study is further support for this conclusion.

In addition, we rule out the possibility that p97 and NSF could mediate fusion by being presented as denatured proteins. Three lines of evidence reason against this. First, removal of the abundant p97 from cytosol resulted in an 80% decrease in fusion, showing that it was needed for the fusion even in the presence of a multitude of cytosolic proteins, each potentially capable of mediating non-specific fusion. Secondly, heat denaturation of both p97 and NSF inhibited fusion, further emphasising that the fusion assay was measuring an active event mediated by p97 or NSF. Thirdly, the fusion event required ATP hydrolysis.

The role of ATPases and their co-factors in fusion

In light of our findings, it is not difficult to reconcile previous observations showing the importance of p97 and NSF in organelle reassembly and vesicular transport. However, both were invariably viewed as accessory proteins rather than primary fusion proteins. The surprising finding that p97 and NSF are primary fusion components raises several interesting questions: given that they are cytosolic and abundant, how are they targeted and regulated? What is the role of interacting SNAREs?

Clues to how p97 and NSF are targeted and regulated can be derived from structural work (Peters *et al.*, 1992; Kessel *et al.*, 1995; Hanson *et al.*, 1997; Kondo *et al.*, 1997; Rabouille *et al.*, 1998). These studies show that NSF and p97 exist in the form of barrel-shaped hexameric ring structures. Each barrel has an outer diameter of 13–17 nm and a typical height of 9–10 nm, with a small channel at its centre. Two different states could be distinguished. By adding the non-hydrolysable ATP analogue, ATPγS, the NSF hexamer was locked in an 'ATP' state, which displayed characteristic features such as globular 'feet' extending outward from the base of the barrel. In contrast, when observed in an 'ADP' state, these feet had contracted inward (Hanson *et al.*, 1997). This

argues that a structural conformation of NSF takes place upon ATP hydrolysis. As the data presented in this study shows that ATP hydrolysis by NSF or p97 is required for fusion, we propose that it is this conformational change which causes the first fusion intermediate, the stalk, to form. Why this would not occur at random is based in part on the observation that the hexameric NSF prefers to be in an ADP state even in the presence of 1 mM ATP and that the stability of the NSF oligomer is very much dependent on nucleotide binding and ATP hydrolysis (Whiteheart *et al*., 1994; Hanson *et al.*, 1997; Matveeva *et al*., 1997). In the absence of co-factor, we argue that hexamers of NSF (and p97) spend most of their time in an ADP state. This alone would guard against promiscuous fusion. In addition, as the protruding feet are observed only in the ATP state and these correspond to the N-domain (essential for co-factor binding and hence SNARE binding), targeting of the ADP hexamer to the membrane would not occur.

We further predict that it is upon binding of the co-factor that the hexamer of the ATPase is stabilized in the ATP state. We observed that with addition of co-factors at a particular ratio (2:1, ATPase:co-factor), fusion was stimulated greatly. At this ratio, the ATPase activity of the ATPase–co-factor complex was greatly reduced in solution. That addition of the co-factor resulted in less ATPase activity in solution is consistent with previous observations for p97 and p47 (Meyer *et al*., 1998), but not with NSF and α-SNAP. In the case of NSF, addition of α-SNAP appears to stimulate ATPase activity (Sudlow *et al*., 1996; Steel and Morgan, 1998). However, in those studies, a prerequisite for stimulation was that α-SNAP had to be immobilized on a plastic support prior to addition of NSF, providing a possible explanation for the observed difference. We also observed that addition of an ATP regenerating system boosted the p97- and NSF-mediated fusion event. This effect was striking, and presumably a consequence of having minimized the increase of ADP levels as a consequence of ATPase activity. One would infer from this that the ATPase would become less active in terms of fusion at elevated ADP levels, perhaps as a consequence of more readily assuming an inactive but preferred ADP state. In summary, we suggest from our findings and those of others that the preferred ADP state of p97 and NSF provides one way to ensure that they do not catalyse fusion at random. Upon binding to corresponding co-factors, ADP is exchanged with ATP, priming the complex for fusion.

The role of SNAREs in p97 and NSF mediated fusion

Having shown that p97 and NSF suffice for fusion, we suggest that the role of SNAREs is to provide specificity and regulation. It is known that recruitment of the ATPase complex to membranes occurs through the binding of the co-factor to SNAREs (Sollner *et al.*, 1993; Kondo *et al.*, 1997; Rabouille *et al*., 1998). This would ensure targeting of the fusion complex to the correct membrane. Upon interaction with SNAREs, the co-factor would then be released from the now membrane-bound ATPase, which is now ready to catalyse fusion. This would place SNAREs upstream of the actual fusion event consistent with recent findings by Ungermann *et al*. (1998a,b), who suggest that SNARE pairing *in trans* precedes fusion, as this *trans* pairing can be disassembled without affecting fusion rates. However, SNARE pairing or re-arrangement has been shown to be the direct consequence of ATP hydrolysis by NSF (Sec18 in yeast). This has been interpreted as a prerequisite for ATP hydrolysis by Sec18 (NSF) prior to fusion (Mayer *et al*., 1996; Sato and Wickner, 1998; Ungermann *et al*., 1998a,b). A subsequent role for Sec17 and Sec18 would according to those data not be required for fusion. In addition, dissociation of Sec17 occurs upon ATP hydrolysis of Sec18 but well before fusion. These differences are not easy to reconcile and will require further work. For example, using our liposome-based fusion assay, it should be possible to examine fusion in the context of SNAREs and other components to determine the relative role exerted by each component. As both the p97- and NSF-mediated fusion event requires ATP hydrolysis, it should also be possible to trap fusion intermediates which would yield a detailed elucidation of membrane fusion by cellular proteins such as p97 and NSF.

Materials and methods

Materials

Lipids were purchased from Avanti Polar (Alabaster, AL). Sodium-dithionite was from Fluka (Deisenhofen, Germany), and ANTS and DPX from Molecular Probes (Eugene, OR). Peptides, ZfFG (*N*-CBZ-D-phenylalanine-phenylalanine-glycine) and ZGGA (*N*-CBZ-glycine-glycine-alanine) were from Sigma (Deisenhofen, Germany). Murine monoclonal antibody against p97 was from Progen (Heidelberg, Germany).

Preparation of the rat liver Golgi membranes

Rat liver Golgi membranes were prepared according to Slusarewics *et al.* (1994). Briefly, four to six rats were starved for 24 h prior to sacrifice. Livers were quickly removed and immersed in 200 ml of ice-cold buffer I (0.5 M sucrose, 5 mM $MgCl_2$, 100 mM K_2HPO_4/KH_2PO_4, pH 6.7). Livers were rinsed, minced into small pieces and passed through a 150 μm-mesh steel laboratory sieve. The homogenate was then collected and brought to a final volume of 80 ml with the addition of buffer I. Six centrifuge tubes containing 7.5 ml of buffer II (1.3 M sucrose, 5 mM $MgCl_2$, 100 mM K_2HPO_4/KH_2PO_4, pH 6.7) with a layer of 13 ml of buffer III (0.86 M sucrose, 5 mM $MgCl_2$, 100 mM K_2HPO_4/KH_2PO_4, pH 6.7) on top of it were prepared. The homogenate (13 ml) was layered on top and overlaid with 2 ml of buffer IV (0.25 M sucrose, 5 mM $MgCl_2$, 100 mM K_2HPO_4/KH_2PO_4, pH 6.7). The gradients were spun for 1 h at 28 000 r.p.m. in a Beckman ultracentrifuge L_{70}, with a fixed angle rotor, type SW28 at 4°C. The Golgi fraction was collected at the interface of buffers I and III, and diluted to 8–9% w/w sucrose with buffer V (5 mM $MgCl_2$, 100 mM K_2HPO_4/KH_2PO_4, pH 6.7). The tubes were topped up with buffer IV and underlaid with 100 μl of buffer II to form a sucrose cushion. The gradients were spun for 30 min at 7000 r.p.m. in a Beckman ultracentrifuge L_{70}, with a fixed angle rotor, type SW28 at 4°C. The pellets were resuspended in 2 ml of buffer IV and 38 ml of buffer IV was added to the Golgi fraction. The gradients were then spun once more on a 100 μl sucrose cushion of buffer V, for 30 min at 7000 r.p.m. in a Beckman ultracentrifuge L_{70}, with a fixed angle rotor, type SW28 at 4°C. Finally, pellets were resuspended in 4–5 ml of buffer IV, frozen in liquid nitrogen and stored at –80°C.

Cytosol fractionation and depletion of p97

Proteins of rat liver cytosol were precipitated using 20% AS. The pellet and supernatant were dialysed against HEPES buffer (10 mM, 140 mM NaCl, pH 7.4) and tested for fusion activity. The material from the pellet, containing the majority of the fusion activity, was applied to a Resource Q column. Fractions (0.5 ml/fraction) were collected and analysed for protein amount and initial fusion rate. The latter using the L–L fusion assay with DOPE/DOPS (1:1) donor and a 5-fold excess unlabelled DOPE/DOPC (1:1) acceptor liposomes (a maximal final lipid concentration of 500 μM). Peak fractions 11 and 12 were pooled and applied to Mono Q column. Fractions (0.5 ml/fraction) were then

analysed as above. Aliquots of all fractions and the original cytosol (C), p97–p47-depleted cytosol (C^-) and the 20% AS pellet supernatant fractions were also subjected to SDS–PAGE, followed by Coomassie staining and Western blot analysis using antibodies to p97, p47, NSF and α-SNAP. Depletion of p97 from the cytosol was performed using recombinant and His-tagged p47 attached to Ni-Sepharose (Qiagen, Hilden, Germany). Approximately 10 mg of p47 were used to deplete 10 ml rat liver cytosol (0.3 mg/ml).

Preparation of the liposomes

Liposomes, 50–70 nm in diameter, labelled with 0.8% rhodamine-PE and 0.8% NBD-PE were made up essentially as described previously (Struck *et al.*, 1981). Briefly, 0.8% of total lipid rhodamine-PE and 0.8% of total lipid NBD-PE were added to DOPE/DOPS (molar ratio 1:1). Unlabelled liposomes consisted of DOPE/DOPC (molar ratio 1:1), DOPE/DOPC/DOPS (molar ratio 1:1:1), DOPC/DOPS (molar ratio 1:1), pure DOPC or DOPS. Lipid solutions were dried under nitrogen and kept under vacuum for 3 h. After drying, 500 µl of fusion buffer (10 mM HEPES, 140 mM NaCl, 10 mM KOAc, 1 mM $MgCl_2$, pH 6.5) was added and the mixture was vortexed vigourously and quickly freeze–thawed 10 times. The lipid mixture was then transferred to a hand-held extruder device (Liposofast) from Milsch Equipment (Laudenbach, Germany). The mixture was passed 30–50 times through a 50 nm pore-size polycarbonate filter from Millipore (Eschborn, Germany). Vesicle size was checked by a Nicomb particle analyser. Lipid analysis was carried out according to Bartlett (1959).

Fusion assay

Lipid mixing. The fusion assay is essentially as described by Struck *et al.* (1981) and Hoekstra and Duzgunes (1993). Typically, fluorescently labelled liposomes (final lipid concentration 100 µM) were added to 500 µl of buffer (10 mM HEPES, 140 mM NaCl, 10 mM KOAc, 1 mM $MgCl_2$, pH 6.5) at 37 °C in a thermostatted cuvette. At these concentrations, it is known that neither Na^+ nor Mg^{2+} causes fusion (Duzgunes *et al.*, 1987). Rat liver Golgi membranes (50 µg/ml final protein concentration) were injected into the cuvette through a small hole in the lid of the fluorimeter (SLM Aminco Bowman Series 2). In case of the L–L fusion assay, the labelled and unlabelled liposomes (1:5 ratio, final lipid concentration 500 µM) were mixed and added to the cuvette with fusion buffer. The ATPases and other compounds were added by injection. The fusion reaction was monitored as an increase in NBD fluorescence as a function of time with 1 s resolution at excitation and emission wavelengths of 465 and 530 nm, respectively. For calibration, 0% was taken as the intrinsic fluorescence intensity of NBD/rhodamine-labelled liposomes and the 100% as the maximal fluorescence obtained after addition of 0.1% Triton X-100 (final concentration) corrected for detergent quenching of NBD fluorescence. For the preparation of the inner leaflet labelled liposomes, NBD/rhodamine-labelled liposomes were incubated with 20 mM sodium dithionite for 30 min on ice to extinguish NBD fluorescence in the outer leaflet. Excess sodium dithionite was removed on a Sepharose G50 column. Initial rates of velocity of fusion were calculated by drawing a tangent at the steepest (initial) part of the curve (Hoekstra and Duzgunes, 1993).

Content mixing. Liposomes for the contents mixing assay were made essentially as described by Struck *et al.* (1981) and Duzgunes and Wilschut (1993). After drying, the lipids were dissolved in fusion buffer supplemented with either 25 mM ANTS or 90 mM DPX. Unincorporated ANTS and DPX was removed beforehand using a Sepharose G50 column. Fusion was monitored in time with 1 s resolution as a decrease in ANTS fluorescence at excitation and emission wavelenghths of 360 and 530 nm, respectively. The maximal signal (100%), is represented by non-quenched ANTS fluorescence. The minimal signal (0%) is represented by a population of liposomes containing pre-mixed ANTS/DPX (25 mM/90 mM), which yields maximum quenching of ANTS fluorescence. To enable direct comparison with the lipid mixing assay, the content mixing curve has been inverted and is expressed as percent fusion as is the lipid mixing curve. The 5-fold excess of acceptor liposomes results in a 100% fusion signal that corresponds to 83.5% of obtained NBD fluorescence. Hence, the reference curve shows a maximum of 24% fusion.

ATP regenerating and depletion system

ATP RS was made according to Traub *et al.* (1993). Stock solutions of ATP (300 mM in fusion buffer, pH 6.5), creatine phosphate (300 mM in fusion buffer) and creatine kinase (1200 U/ml in fusion buffer with 50% glycerol) were made. ATP solution (0.535 ml) was added to 2.3 ml of creatine phosphate solution and 0.666 ml of creatinine kinase solution. Fusion buffer (3.43 ml) was then added to the mixture. Aliquots were flash-frozen in liquid nitrogen and used after thawing as 10× concentrated ATP RS. ATP depletion was done by incubating samples with 150 U of hexokinase in the presence of 1 mM glucose for 5 min at room temperature. To block further hexokinase activity, glucose-6-phosphate (2 mM final concentration) was added.

Acknowledgements

We are indebted to the following people for providing invaluable reagents: Drs Latterich and Roberts (Salk Institute, San Diego, CA) for purified p97 from bovine brain and frog cytosol; Drs Hisao Kondo and Graham Warren (ICRF, London, UK) for anti-p47 antibody, purified p97–p47 and recombinant p47; Drs Heidi McBride and Marino Zerial (EMBL, Heidelberg, Germany) for recombinant NSF, α-SNAP, anti-NSF and anti-α-SNAP antibody; Professor Brunner (ETHZ, Zurich, Switzerland) for help with initial experiments; and Drs Brian Storrie (Blacksburg, VA) and Chris J. Echeverri (EMBL) for helpful discussions and comments.

References

Acharya,U., Jacobs,K., Peter,J.M., Watson,N., Farquhar,M.G. and Malhotra,V. (1995) The formation of Golgi stacks from vesiculated Golgi membranes requires two distinct fusion events. *Cell*, **82**, 895–904.

Ames,B.N. (1966) Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.*, **8**, 115–118.

Barnard,R.J.O., Morgan,A. and Burgoyne,R.D. (1997) Stimulation of NSF ATPase activity by α-SNAP is required for SNARE complex disassembly and exocytosis. *J. Cell Biol.*, **139**, 875–883.

Bartlett,G.R. (1959) Phosphorus assay in column chromatography. *J. Biol. Chem.*, **234**, 466–468.

Chernomordik,L.V. and Zimmerberg,J. (1995) Bending membranes to the task: structural intermediates in bilayer fusion. *Curr. Opin. Struct. Biol.*, **5**, 541–547.

Chernomordik,L.V., Leikina,E., Frolov,V., Bronk,P. and Zimmerberg,J. (1997) An early stage of membrane fusion mediated by the low pH conformation of influenza hemagglutinin depends upon membrane lipids. *J. Cell Biol.*, **136**, 81–93.

Duzgunes,N., Allen,T.M., Fedor,J. and Papahadjopoulos,D. (1987) Lipid mixing during membrane aggregation and fusion: why fusion assays disagree. *Biochemistry*, **26**, 8435–8442.

Duzgunes,N. and Wilschut,J. (1993) Fusion assays monitoring intermixing of aqueous contents. *Methods Enzymol.*, **220**, 3–14.

Ellens,H., Bentz,J. and Szoka,F.C. (1984) pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact. *Biochemistry*, **23**, 1532–1538.

Epand,R.M., Epand,R.F., Richardson,C.D. and Yeagle,P.L. (1993) Structural requirements for inhibition of membrane fusion by carbobenzoxy-D-Phe-Phe-Gly. *Biochim. Biophys. Acta*, **1152**, 128–134.

Epand,R.M., Shai,Y., Segrest,J.P. and Anantharamaiah,G.M. (1995) Mechanisms for modulation of membrane bilayer properties by amphipathic helical peptides. *Biopolymers*, **37**, 319–338.

Hanson,P.I., Roth,R., Morisaki,H., Jahn,R. and Heuser,J.E. (1997) Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell*, **90**, 523–535.

Hoekstra,D. and Duzgunes,N. (1993) Lipid mixing assays to determine fusion in liposome systems. *Methods Enzymol.*, **220**, 15–32.

Hoekstra,D., Buist-Arkema,R., Klappe,K. and Reutelingsperger,C.P.M. (1993) Interaction of annexins with membranes: The N-terminus as a governing parameter as revealed with a chimeric annexin. *Biochemistry*, **32**, 14194–14202.

Kessel,M., Maurixi,M.R., Kim,B., Kocsis,E. Trus,B.L., Singh,S.K. and Steven,A.C. (1995) Homology in structural organization between *E.coli* ClpAP protease and the eukaryotic 26S proteasome. *J. Mol. Biol.*, **250**, 587–594.

Kondo,H., Rabouille,C., Newman,R., Levine,T.P., Pappin,D., Freemont,P. and Warren,G. (1997) p47 is a cofactor for p97-mediated membrane fusion. *Nature*, **388**, 75–78.

Kleinsmith,L.J. and Kish,V.M. (1995) In McLaughlin,S., Trost,K. and MacElree (eds), *Principles of Cell Biology*. Harper-Collins College Publishers, New York, NY, pp. 155–193.

Latterich,M., Frohlich,K.U. and Schekman,R. (1995) Membrane fusion

and the cell cycle: Cdc48p participates in the fusion of ER membranes. *Cell*, **82**, 885–893.
Malhotra,V., Serafini,T., Orci,L., Shepherd,J.C. and Rothman,J.E. (1989) Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell*, **58**, 329–336.
Matveeva,E.A., He,P. and Whiteheart,S.W. (1997) *N*-Ethylmaleimide-sensitive fusion protein contains high and low affinity ATP-binding sites that are functionally distinct. *J. Biol. Chem.*, **272**, 26413–26418.
Mayer,A., Wickner,W. and Haas,A. (1996) Sec18p (NSF)-driven release of Sec17p (α-SNAP) can precede docking and fusion of yeast vacuoles. *Cell*, **85**, 83–94.
Membranes and membrane transport. In Kleinsmith,L.J. and Kish,V.M (eds), *Principles of Cell and Molecular Biology*. 2nd edn. Harper Collins College Publishers. pp. 155–193.
Meyer,H.H., Kondo,H. and Warren,G. (1998) The p47 co-factor regulates the ATPase activity of the membrane fusion protein, p97. *FEBS Lett.*, **437**, 255–257.
Nichols,B.J., Ungermann,C., Pelham,H.R., Wickner,W.T. and Haas,A. (1997) Homotypic fusion mediated by t- and v-SNAREs. *Nature*, **387**, 199–202.
Otto,H., Hanson,P.I. and Jahn,R. (1997) Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin and SNAP-25 in the membrane of synaptic vesicles. *Proc. Natl Acad. Sci. USA*, **94**, 6197–6201.
Patel,S.K., Indig,F.E., Olivieri,N., Levine,N.D. and Latterich,M. (1998) Organelle membrane fusion: a novel function for the syntaxin homolog Ufe1p in ER membrane fusion. *Cell*, **92**, 611–620.
Pecheur,E.I., Martin,I., Ruysschaert,J.M., Bienvenue,A. and Hoekstra,D. (1998) Membrane induced by 11-mer anionic and cationic peptides. A structure–function study. *Biochemistry*, **37**, 2361–2371.
Peters,J.-M., Walsh,M.J. and Franke,W.W. (1990) An abundant and ubiquitous homo-oligomeric ring-shaped ATPase particle related to the putative vesicle fusion proteins Sec18p and NSF. *EMBO J.*, **9**, 1757–1767.
Peters,J.M., Harris,J.R., Lustig,A., Muller,S., Engel,A., Volker,S. and Franke,W.W. (1992) Ubiquitous soluble Mg^{2+}-ATPase complex. A structural study. *J. Mol. Biol.*, **223**, 557–571.
Rabouille,C., Levine,T.P., Peters,J.-M. and Warren,G. (1995) An NSF-like ATPase, p97 and NSF mediate cisternal regrowth from mitotic Golgi fragments. *Cell*, **82**, 905–914.
Rabouille,C., Kondo,H., Newman,R., Hui,N., Freemont,P. and Warren,G. (1998) Syntaxin 5 is a common component of the NSF- and p97-mediated reassembly pathways of Golgi cisternae from mitotic Golgi fragments *in vitro*. *Cell*, **93**, 603–610.
Rothman,J.E. (1994) Mechanisms of intracellular protein transport. *Nature*, **372**, 55–63.
Sato,K. and Wickner,W. (1998) Functional reconstitution of Ypt7p GTPase and a purified vacuole SNARE complex. *Science*, **281**, 700–702.
Slusarewicz,P., Nilsson,T., Hui,N., Watson,R. and Warren,G. (1994) Isolation of a matrix that binds medial Golgi enzymes. *J. Cell Biol.*, **124**, 405–413.
Sollner,T., Whiteheart,S.W., Brunner,M., Erdjument-Bromage,H., Geromans,S., Tempst,P. and Rothman,J.E. (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature*, **362**, 318–324.
Steel,G.J. and Morgan,A. (1998) Selective stimulation of the D1 ATPase domain of *N*-ethylmaleimide-sensitive fusion protein (NSF) by soluble NSF attachment proteins. *FEBS Lett.*, **423**, 113–116.
Struck,D.K., Hoekstra,D. and Pagano,R.E. (1981) Use of resonance energy transfer to monitor membrane fusion. *Biochemistry*, **20**, 4093–4099.
Sudlow,A.W., McFerran,B.W., Bodill,H., Barnard,R.J., Morgan,A. and Burgoyne,R.D. (1996) Similar effects of α- and β-SNAP on Ca^{2+}-regulated exocytosis. *FEBS Lett.*, **393**, 185–188.
Swanton,E., Sheehan,J., Bishop,N., High,S. and Woodman,P. (1998) Formation and turnover of NSF- and SNAP-containing 'fusion' complexes occur on undocked, clathrin-coated vesicle-derived membranes. *Mol. Biol. Cell*, **9**, 1633–1647.
Traub,L.M., Ostrom,J.A. and Kornfeld,S. (1993) Biochemical dissection of AP-1 recruitment onto Golgi membranes. *J. Cell Biol.*, **123**, 561–573.
Ungermann,C., Nichols,B.J., Pelham,H.R. and Wickner,W. (1998a) A vacuolar v–t-SNARE complex, the predominant form *in vivo* and on isolated vacuoles, is disassembled and activated for docking and fusion. *J. Cell Biol.*, **140**, 61–69.
Ungermann,C., Sato,K. and Wickner,W. (1998b) Defining the functions of *trans*-SNARE pairs. *Nature*, **396**, 543–548.
Vidal,M. and Hoekstra,D. (1995) *In vitro* fusion of reticulocyte endocytic vesicles with liposomes. *J. Biol. Chem.*, **270**, 17823–17829.
Weber,T., Zemelman,B.V., McNew,J.A., Westermann,B., Gmachi,M., Parlati,F., Sollner,T. and Rothman,J.E. (1998) SNAREpins: minimal machinery for membrane fusion. *Cell*, **92**, 759–772.
Whiteheart,S.W., Griff,I.C., Brunner,M., Clary,D.O., Mayer,T., Buhrow,S.A. and Rothman,J.E. (1993) SNAP family of NSF attachment proteins includes a brain-specific isoform. *Nature*, **362**, 353–355.
Whiteheart,S.W., Rossnagel,K., Buhrow,S.A., Brunner,M., Jaenicke,R. and Rothman,J.E. (1994) *N*-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis of ATP is required for membrane fusion. *J. Cell Biol.*, **126**, 945–954.
Zhang,L., Ashendel,C.L., Becker,G.W. and Morre,D.J. (1994) Isolation and characterization of the principal ATPase associated with transitional endoplasmic reticulum of rat liver. *J. Cell Biol.*, **127**, 1871–1883.

Received November 5, 1998; revised February 8, 1999; accepted February 24, 1999