

Fatty Acylation Promotes Fusion of Transport Vesicles with Golgi Cisternae

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Abstract. Two different methods, stimulation of transport by fatty acyl-coenzyme A (CoA) and inhibition of transport by a nonhydrolyzable analogue of palmitoyl-CoA, reveal that fatty acylation is required to promote fusion of transport vesicles with Golgi cisternae. Specifically, fatty acyl-CoA is needed after the attach-

ment of coated vesicles and subsequent uncoating of the vesicles, and after the binding of the NEM-sensitive fusion protein (NSF) to the membranes, but before the actual fusion event. We therefore suggest that an acylated transport component participates, directly or indirectly, in membrane fusion.

SPECIFIC fusion of biological membranes is a central requirement for many cellular processes. Steps such as transport between subcellular compartments, secretion, endocytosis, and cell division require strongly regulated and coordinated fusion events (Palade, 1975; Warren, 1985; Pfeffer and Rothman, 1987). The best studied examples of membrane fusion mediated by proteins are those by viral membrane glycoproteins (White et al., 1983; Stegmann et al., 1989). Several but not all, viral glycoproteins contain a hydrophobic sequence that apparently serves as "fusion peptide" inducing the steps that lead to perturbation and fusion of lipid bilayers. A number of fusogenic viral glycoproteins are covalently modified by a fatty acid such as palmitate (Towler et al., 1988; Schultz et al., 1988). The fusion process is often activated by a pH-induced conformational change of the glycoprotein leading to exposure of fusogenic sequences (Stegmann et al., 1989). As a result, the fusion occurs only after endocytosis of the virus into an acidic cellular compartment.

The numerous spatially restricted fusion events occurring in a common cytoplasm in living cells, however, are surely not mediated by such a simple mechanism. Rather, a multi-subunit fusion complex seems to be assembled at the junction between transport vesicle and a specific acceptor membrane that triggers the fusion of the bilayers (Block et al., 1988; Malhotra et al., 1988; Orci et al., 1989; Weidman et al., 1989). Recently, the first component required in this fusion process was purified on the basis of a fusion assay involving transport vesicles originating in *cis*-Golgi and targeted to *medial* Golgi cisternae. This 76-kD, NEM-sensitive protein (NSF)¹ (Block et al., 1988) was also found to be es-

sential for a variety of cellular fusion processes, such as vesicular transport from endoplasmic reticulum to *cis*-Golgi, transport to *trans*-Golgi, and fusion of endocytic vesicles (Rothman, 1987; Wilson et al., 1989; Beckers et al., 1989; Diaz et al., 1989) suggesting that the mechanisms unravelled in the cell-free transport from *cis*- to *medial* Golgi are valid for many vesicular transport processes.

From a combined biochemical and morphological dissection of transport from *cis*- to *medial* Golgi, we established the following details of vesicular transfer (Fig. 1) (Balch et al., 1984b; Orci et al., 1986, 1989; Wattenberg and Rothman, 1986; Wattenberg et al., 1986; Melançon et al., 1987; Block et al., 1988; Malhotra et al., 1988; Weidman et al., 1989). Incubating "donor" Golgi stacks (housing the vesicular stomatitis virus-encoded glycoprotein [VSV-G protein]) in the presence of cytosol and ATP produces vesicles that carry the G protein and that are coated with nonclathrin proteins (Malhotra et al., 1989). These vesicles are targeted to and attach to the "acceptor" Golgi stacks. Then the bound vesicles are uncoated. A step prerequisite for uncoating is blocked by GTP γ S, suggesting the involvement of a GTP-binding protein. Then, the junction between vesicle and acceptor cisterna matures through a series of prefusion complexes. First, the presence of NSF is required. Then, a soluble 25-kD component, termed factor B, is needed for further maturation. In a final step that involves ATP, but which is apparently independent of further cytosolic components, the vesicle fuses with the acceptor cisterna and releases its content to it. By measuring the incorporation of [³H]GlcNAc (*N*-acetylglucosamine) into VSV-G protein upon delivery to the *medial* cisternae in which this glycosylation promptly occurs (Balch et al., 1984b), transport from the *cis* compartment of donor stacks (that lack GlcNAc transferase I in their *medial* compartment) to the *medial* compartment of acceptor stacks is conveniently monitored.

Long chain fatty acyl-coenzyme A (CoA) (e.g., palmitoyl-CoA), stimulated vesicular transport in the Golgi, especially under conditions where the amount of NSF in the assay was limiting (Glick and Rothman, 1987; Rothman, 1987), sug-

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1. *Abbreviations used in this paper:* CoA, coenzyme A; GlcNAc, *N*-acetylglucosamine; LCRI, low cytosol-requiring intermediate; NEM, *N*-ethylmaleimide; NSF, NEM-sensitive fusion protein; SNAP, soluble NSF attachment protein; VSV, vesicular stomatitis virus; VSV-G, VSV-encoded glycoprotein.

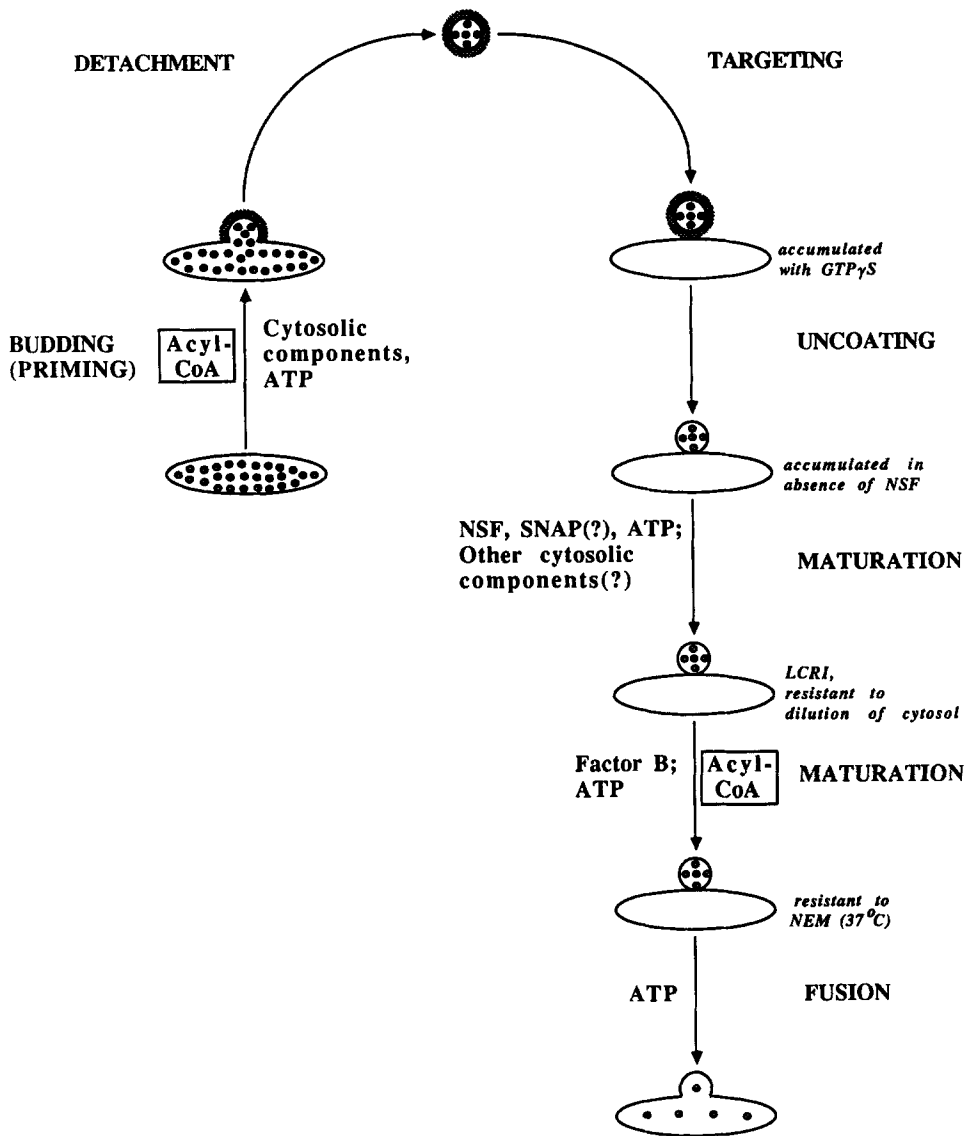


Figure 1. Working model of vesicular transport from *cis*- to *medial* Golgi as suggested by Balch et al. (1984*b*), Wattenberg and Rothman (1986), Wattenberg et al. (1986), Orci et al. (1986, 1989), Melançon et al. (1987), Block et al. (1988), Malhotra et al. (1988), Weidman et al. (1989), Pfanner et al. (1989), and this study. This model represents an extension of models shown in Malhotra et al. (1988) and Orci et al. (1989). At present, it cannot be decided unambiguously if the transport intermediate independent of acyl CoA is the NEM (37°C) resistant intermediate (as diagrammed) or the preceding step. The dots in the lumen of cisternae and vesicles shall represent transported proteins (the VSV-G protein of which the transport is measured in our cell-free system is a membrane protein). *SNAP*, soluble NSF attachment protein.

gesting a possible linkage between the action of NSF and a fatty acylation process. Thus, fatty acyl-CoA may, like NSF, be of importance for many cellular fusion processes. It was, therefore, to our surprise when we found that fatty acyl-CoA is required for budding of transport vesicles from donor membranes, a step that is independent of NSF (Pfanner et al., 1989) (Fig. 1).

To resolve this apparent contradiction, we have examined the fusion pathway in greater detail to find out whether any additional steps might depend on fatty acyl-CoA. In fact, we have found that fusion also requires fatty acyl-CoA and is inhibited by a nonhydrolyzable analogue of palmitoyl-CoA. Fatty acylation is essential for maturation of the vesicle-acceptor cisterna junction and occurs only after NSF has bound to the membranes; i.e., binding of NSF to the membranes is a prerequisite for this fatty acylation step. However, fatty acylation occurs before the actual fusion event.

Materials and Methods

Assay components were prepared and the standard incubations were performed as described previously (Balch et al., 1984*a*; Balch and Rothman,

1985; Glick and Rothman, 1987; Melançon et al., 1987; Block et al., 1988; Malhotra et al., 1988; Pfanner et al., 1989). Particular incubation conditions are given in the figure legends. The nonhydrolyzable analogue of palmitoyl-CoA (heptadecan-2-onyldethio-CoA) (Ciardelli et al., 1981) was a kind gift of Professor T. Wieland.

Results

Addition of fatty acyl-CoA (e.g., palmitoyl-CoA) was found to stimulate vesicular transport in the cell-free system (Glick and Rothman, 1987). It was excluded that the acylation of the VSV-G protein itself is needed for transport (for a detailed discussion, see Glick and Rothman, 1987; Pfanner et al., 1989). Furthermore, the trivial possibility that transport stimulation by fatty acyl-CoA is due to a bulk detergent effect can be excluded (Glick and Rothman, 1987; Pfanner et al., 1989; and this study). Two main methods have been developed to study the role of fatty acyl-CoA in the cell-free system. (a) Use of a nonhydrolyzable analogue of palmitoyl-CoA (Ciardelli et al., 1981) that competitively inhibits transport (Pfanner et al., 1989). The analogue contains a methylene group in place of the sulfur atom. (b) Use of select

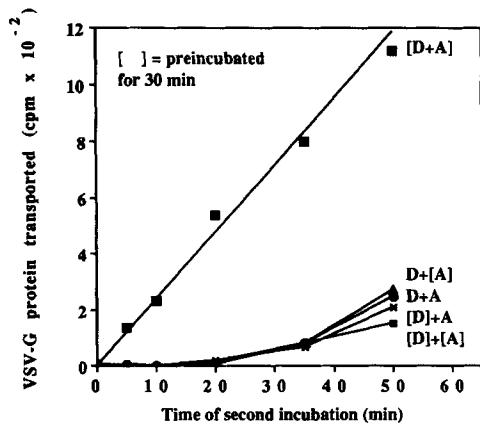


Figure 2. Generation of a transport intermediate resistant to a nonhydrolyzable analogue of palmitoyl-CoA requires coincubation of donor and acceptor. Golgi membranes (*D*, donor; *A*, acceptor) are incubated either separately (*crosses*, only donor; *triangles*, only acceptor; *small open squares*, donor and acceptor separately; *circles*, no preincubation) or together (*closed squares*) in the presence of cytosol and ATP under standard assay conditions for 30 min at 37°C. Then the membranes were mixed, 12 μ M of the nonhydrolyzable analogue of palmitoyl-CoA and UDP-[³H]GlcNAc was added and a second incubation at 37°C was performed. Transport was measured by incorporation of [³H]GlcNAc into the VSV-G protein.

conditions under which transport *in vitro* depends strongly (e.g., fivefold) upon the addition of fatty acyl-CoA (Pfanner et al., 1989). This is achieved by adding solvents like (~2%) ethanol (controls show that transport in the presence of solvents and palmitoyl-CoA exhibits all criteria of specific vesicular transport) or by adding very low concentrations of detergents (that were found to deplete membrane pools of fatty acyl-CoA). Addition of palmitoyl-CoA restores transport inhibited by solvents or low concentrations of detergent (Pfanner et al., 1989).

Transfer of Vesicles to Acceptor Cisternae Is Required for Generation of a Transport Intermediate Independent of Fatty Acyl-CoA

In a previous paper, we reported that the budding of vesicles from donor Golgi cisternae required a fatty acylation process (Pfanner et al., 1989). Is this the only step requiring fatty acyl CoA? If so, then preincubation of donor membranes in the presence of cytosol, ATP, and palmitoyl-CoA (generation of "primed" donor [Balch et al., 1984b]) should be sufficient to generate a transport intermediate that is independent of fatty acyl-CoA. Thus, neither nonhydrolyzable analogue of palmitoyl-CoA nor ethanol should inhibit consumption of the primed donor containing already formed coated vesicles. This is manifestly not the case.

We preincubated donor membranes for 30 min at 37°C, then acceptor was added to consume primed donor in the presence of the nonhydrolyzable analogue (Fig. 2) or ethanol (Fig. 3) in a second stage of incubation at 37°C. Preincubation of donor membranes in the presence of (endogenous) fatty acyl-CoA did not lead to generation of a transport intermediate that was independent of fatty acyl-CoA for completion of transport. This means that there must be other step(s) after budding that require fatty acyl-CoA.

Indeed, when donor and acceptor membranes were prein-

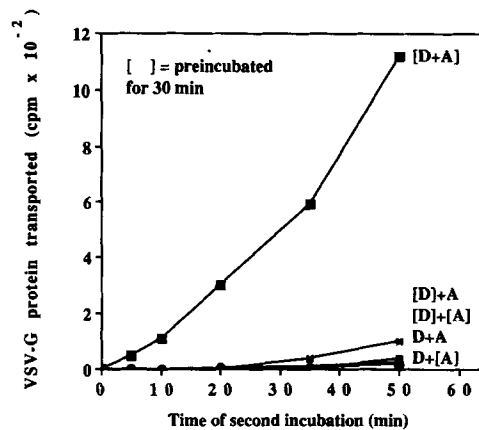


Figure 3. Coincubation of donor and acceptor is required for generation of an ethanol-resistant transport intermediate. The experiment was performed as described in the legend of Fig. 2 except that ethanol (2% [wt/vol]) was added instead of the analogue. The curve for *D* + [*A*] (*triangles*) is exactly overlaid by the curve for *D* + *A* (*circles*).

cubated together, an intermediate whose consumption was resistant to the nonhydrolyzable analogue of palmitoyl-CoA (Fig. 2) and to ethanol (Fig. 3) was formed. Thus, coincubation of donor and acceptor, permitting transfer of coated vesicles from the cisternae of donor stacks to the cisternae of acceptor stacks is required for transport to proceed past the last acyl-CoA-requiring step. This suggests that at least one later transport step, in addition to the budding of vesicles, depends on fatty acylation. Moreover, since preincubation of acceptor membranes does not lead to formation of a resistant intermediate (Figs. 2 and 3), it is clear that the acceptor cisternae cannot be preactivated with fatty acyl-CoA. Attachment of transport vesicles to the acceptor membranes seems to be a prerequisite for this acylation step to take

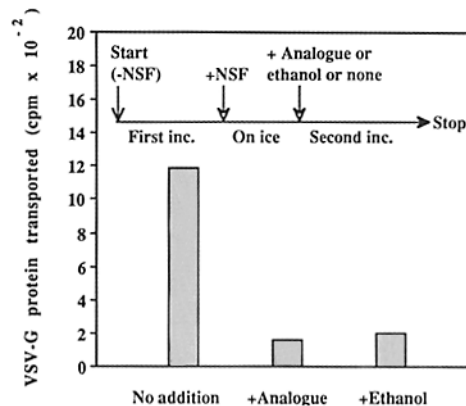


Figure 4. Fatty acylation is needed after binding of NSF to the membranes. Donor, acceptor, cytosol, UDP-[³H]GlcNAc, and ATP were incubated under NSF-depleted conditions (pretreatment of the membranes with NEM at 0°C [Glick and Rothman, 1987; Block et al., 1988]) for 25 min at 37°C. Then 60 ng purified NSF was added (Block et al., 1988; Weidman et al., 1989) and after 3 min at 0°C either the nonhydrolyzable analogue of palmitoyl-CoA (12 μ M) or ethanol (2%), or neither were added. Then an incubation for 60 min at 37°C was performed. For each condition, the background obtained without adding NSF (254–297 cpm) was subtracted.

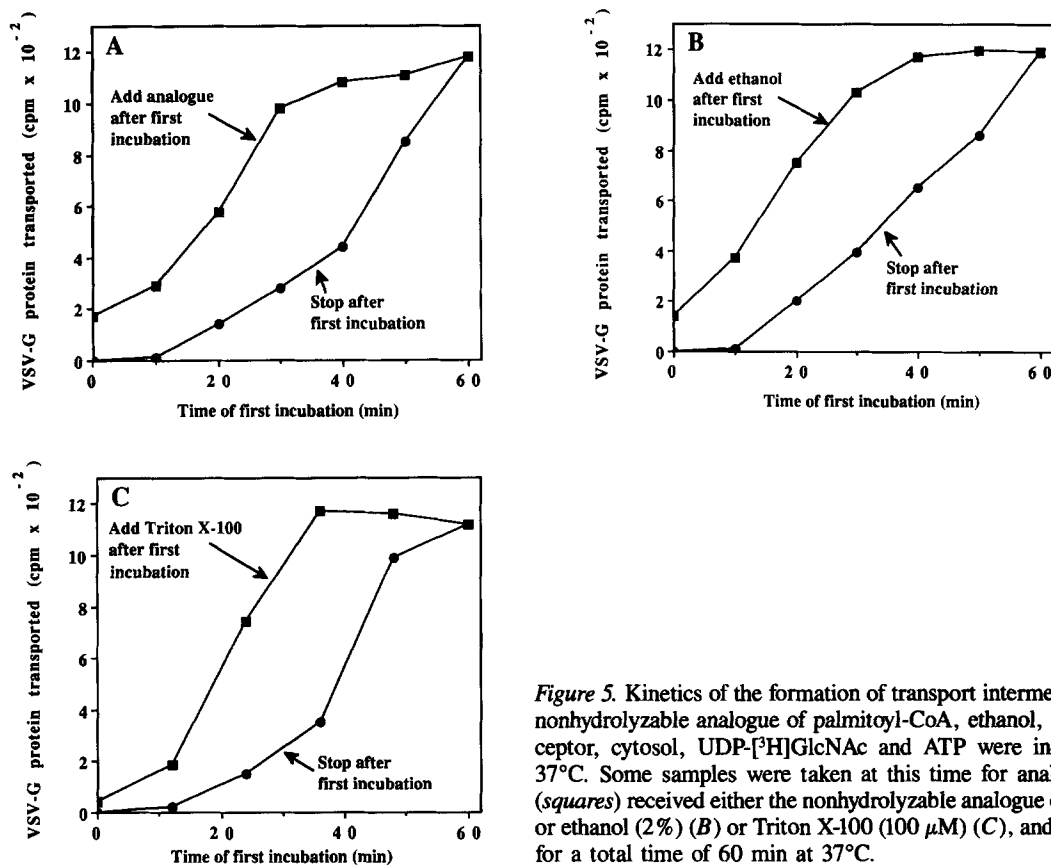


Figure 5. Kinetics of the formation of transport intermediates that are resistant to the nonhydrolyzable analogue of palmitoyl-CoA, ethanol, and Triton X-100. Donor, acceptor, cytosol, UDP-[³H]GlcNAc and ATP were incubated for various times at 37°C. Some samples were taken at this time for analysis (circles). Other aliquots (squares) received either the nonhydrolyzable analogue of palmitoyl-CoA (12 μM) (A) or ethanol (2%) (B) or Triton X-100 (100 μM) (C), and the incubation was continued for a total time of 60 min at 37°C.

place. This suggests that acylation is required at one or more stages in the fusion process.

The experiments shown in Figs. 2 and 3 and evidence shown below (Figs. 5 and 6) also exclude the trivial possibility that either the nonhydrolyzable analogue or ethanol interfere with the uptake of sugar-nucleotides or the actual glycosylation of VSV-G protein, since both processes are unaffected in the reactions where donor and acceptor membranes were preincubated together.

Fatty Acyl-CoA Is Required at a Step after Binding of NSF to the Membranes

The purified protein NSF can be withheld until coated vesicles have budded, transferred, and uncoated at the acceptor membrane; but NSF is needed for subsequent fusion (Block et al., 1988; Malhotra et al., 1988; Orci et al., 1989). The experiments just described suggest that acyl-CoA is needed for fusion also. Is fatty acyl-CoA needed before or after NSF in the fusion pathway?

As one way to ascertain this, donor, acceptor, cytosol, and ATP were preincubated in the absence of NSF (using membranes pretreated with NEM at 0°C; Glick and Rothman, 1987; Block et al., 1988) for 25 min at 37°C (Fig. 4). At this stage, completion of transport requires the addition of NSF (and a crude cytosol fraction) and further incubation at 37°C (Malhotra et al., 1988; Orci et al., 1989). We added purified NSF to these incubations and incubated further for 3 min at 0°C; recent studies showed that functional binding of NSF to the Golgi membranes is complete in less than 1 min at 0°C (Weidman et al., 1989). Then, either the nonhydrolyzable

analogue of palmitoyl-CoA or ethanol were added and a second incubation at 37°C was performed. Fig. 4 shows that both the analogue and ethanol strongly inhibited the completion of transport after NSF had bound to the membranes. This suggests that fatty acylation is needed for the maturation of the vesicle-cisterna junction, and that the last acyl-CoA-requiring step occurs after NSF binds.

Fatty Acylation Is Required before Actual Fusion of Vesicle with Cisterna

Where in the fusion pathway is acylation needed? We can map the fatty acyl-CoA-independent intermediate with respect to two previously identified and successive prefusion complexes that are situated after the step involving the binding of NSF (Fig. 1; Malhotra et al., 1988) but before fusion per se. Wattenberg et al. (1986) found that fusion becomes resistant to a fivefold dilution of the cytosol with a half-time of ~5 min from the start of transport. This "dilution-resistant" intermediate requires about five times lower cytosol concentration for its consumption than for its production, and is thus termed the low cytosol-requiring intermediate (LCRI) (Malhotra et al., 1988). A second prefusion transport intermediate that follows LCRI was revealed according to its resistance to NEM at 37°C, a harsher condition than the 0°C treatment that selectively inhibits NSF. This NEM (37°C) resistant intermediate is generated with a half-time of 25–30 min from the start of transport and occurs ~5 min before the actual fusion event (Balch et al., 1984b; Wattenberg et al., 1986). Both the LCRI and the subsequent NEM (37°C) resistant intermediate require coinci-

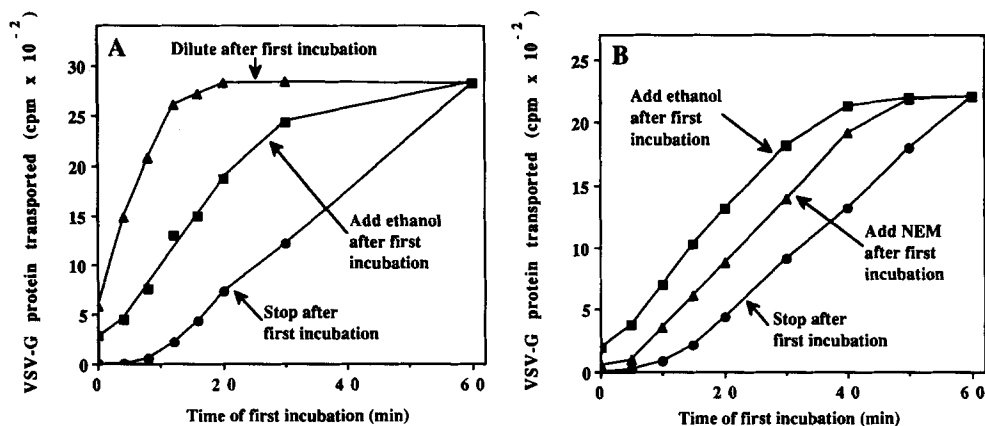


Figure 6. The ethanol-resistant intermediate forms after the low cytosol-requiring intermediate and before the NEM (37°C) resistant intermediate. Time course (circles) and ethanol-resistant intermediate (squares) were determined, as described in the legend of Fig. 5 B. (A) To measure dilution resistant intermediate (triangles), aliquots were diluted fivefold at indicated times with assay buffer containing ATP and UDP-[³H]GlcNAc but not cytosol or membranes, as described (Wattenberg et al., 1986), and the incubation was continued for a total

time of 60 min at 37°C. (B) To measure the NEM (37°C) resistant intermediate (triangles), aliquots received NEM (1 mM) at stated times, and the incubation was continued for a total time of 60 min.

bation of donor with acceptor to form, as they occur after transfer of vesicles and uncoating.

To measure the time course of production of nonhydrolyzable analogue resistant fusion intermediates, transport reactions were started under standard conditions (donor, acceptor, cytosol, and ATP at 37°C), and after various times the nonhydrolyzable analogue of palmitoyl-CoA was added (Fig. 5 A, squares). The incubation was then continued for a total time of 60 min to allow completion of a round of transport. In addition, aliquots were stopped at the time of analogue addition to determine how much transport had been completed at this time (Fig. 5 A, circles). As expected, a considerable amount of transport is completed following the addition of the nonhydrolyzable analogue, indicating the formation of an analogue resistant intermediate. Transport intermediates resistant to ethanol (Fig. 5 B), low concentrations of Triton X-100 (Fig. 5 C), and analogue are all formed with very similar kinetics, with half-times of 15–25 min. This suggests that the formation of a prefusion complex whose consumption is independent of fatty acyl-CoA occurs after generation of the LCRI (half-time ~5 min) but before the actual fusion event.

To confirm this directly, we compared the kinetics of formation of the ethanol-resistant intermediate to the kinetics of formation of the LCRI (Fig. 6 A) and of the NEM (37°C) resistant intermediate (Fig. 6 B) in the same experiment. For this purpose, aliquots were diluted fivefold after an incubation under standard conditions, and the incubations were continued for a total time of 60 min to observe the generation of the LCRI. As would be expected, the production of the ethanol-resistant intermediate occurs 3–4 times more slowly than the production of the LCRI (Fig. 6 A). The transport intermediate resistant to a treatment with 1 mM NEM at 37°C (Balch et al., 1984b; Wattenberg et al., 1986) was not generated before the ethanol-resistant intermediate (Fig. 6 B). This demonstrates that the last transport step that leads to fusion of the membranes (ATP-dependent consumption of the NEM (37°C) resistant intermediate) is independent of fatty acyl-CoA.

Since the transport reaction can become resistant to the nonhydrolyzable analogue of palmitoyl-CoA, to ethanol, and to low concentrations of Triton X-100 without interfering

with the actual fusion event, possible nonspecific damaging effects of these substances on the membranes in the transport reaction can be ruled out. Such concerns are further excluded by electron microscopy studies, and especially by the reversibility of inhibition by addition of palmitoyl-CoA (Pfanner et al., 1989; and see below).

Our results predict that fatty acyl CoA should still be able to stimulate transport after LCRFI forms, but should cease to stimulate with the same kinetics with which the ethanol resistant intermediate forms. To test this, complete transport reactions were incubated for increasing times before either ethanol alone or both ethanol and palmitoyl-CoA were added. The incubations were continued for a total time of 60 min (Fig. 7). We found that palmitoyl-CoA could stimulate as late as the inhibition of transport by ethanol occurs, well after the formation of LCRFI.

In conclusion, we infer that a fatty acylation step is required in the fusion process. The step takes place after binding of NSF to the membranes and after the generation of the LCRI, but at or before the formation of the NEM (37°C) resistant intermediate and before the actual fusion event.

Discussion

We describe here a novel and previously unsuspected mechanism in the complex process of fusion of intracellular membranes. Using several different methods, we demonstrate that fatty acyl CoA is required in an essential step after attachment and uncoating of vesicles at the acceptor membrane. Fatty acyl CoA is presumably required for an acyl transfer reaction, as a nonhydrolyzable analogue does not substitute for acyl CoA and in fact inhibits. However, it is clear that fatty acyl-CoA itself is not required for bilayer fusion per se (e.g., consumption of the NEM (37°C) resistant intermediate). This excludes a direct physical involvement of fatty acyl-CoA in promoting lipid bilayer fusion. A detailed mapping of this fatty acylation step with respect to other maturation steps of the vesicle-cisterna junction is summarized in Fig. 1 (Balch et al., 1984b; Wattenberg and Rothman, 1986; Wattenberg et al., 1986; Melancon et al., 1987; Block et al., 1988; Malhotra et al., 1988; Orci et al., 1989; Weidman et al., 1989).

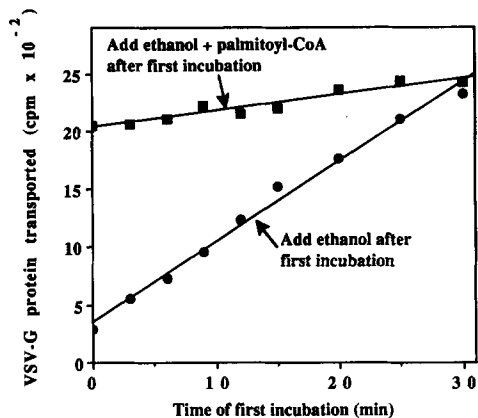


Figure 7. Stimulation of transport by palmitoyl-CoA can occur as late as the inhibition by ethanol occurs. The experiment is performed as described in the legend of Fig. 5 B except that either ethanol (2%; circles) or both ethanol (2%) and palmitoyl-CoA (12 μ M) (squares) were added at the indicated times and the incubation was continued for a total time of 60 min at 37°C.

We conclude that a transport component(s) has to be modified by a fatty acid to function in mediating membrane fusion. The nature of the relevant acyl chain recipient(s) are unknown at present. Inhibition by the nonhydrolyzable analogue of palmitoyl-CoA shows that palmitate can be the modifying fatty acid (however, the specificity for acyl chain length remains to be determined). Several polypeptides of the Golgi membranes are labeled upon addition of [³H]palmitoyl-CoA under assay conditions. NSF does not appear to be one of these labeled polypeptides. As expected, the labeling is competitively inhibited by the nonhydrolyzable analogue (data not shown). Factor B, a partially purified component whose native molecular mass is \sim 25 kD, is needed for maturation from the LCRI to the NEM (37°C) resistant intermediate (Wattenberg and Rothman, 1986; Wattenberg et al., 1986). If acyl-CoA, as seems likely, is needed for the transition from LCRI to the NEM (37°C) prefusion intermediate, then factor B may well be related to the acylation reaction. Factor B could be the acylated transport component or a catalyst or cofactor of acylation. The GTP-binding protein inhibited by GTP γ S to accumulate coated vesicles (Melançon et al., 1987; Orci et al., 1989) is obviously not the acylated component needed in fusion as described here, since the transport block by GTP γ S occurs before the binding of NSF to the membranes.

Our present working model envisions that at the junction between vesicle and acceptor cisterna a fusion machinery is assembled (or its assembly is completed) from several subunits; among them appear to be NSF (Malhotra et al., 1988), soluble NSF attachment protein (SNAP), an NSF receptor in the membranes (Weidman et al., 1989), and possibly factor B and other components, at least one of which becomes acylated during assembly. After assembly, the fusion of vesicle with the cisterna occurs in an ATP-dependent reaction (Balch et al., 1984b), and the fusion machinery must be disassembled to enable its components to recycle for later rounds of transport (Block et al., 1988; Malhotra et al., 1988). The data reported here, and this model of a multi-subunit fusion machinery that includes NSF and an acylated

component, are consistent with our original finding that fatty acyl-CoA stimulates transport when endogenous NSF is depleted (Glick and Rothman, 1987). This can now be explained because the acylation reaction would require, as a prerequisite, that NSF be bound to the membranes, strongly reinforcing the important role of NSF for the acylation reaction, although NSF itself is not acylated (unpublished data). Thus, when NSF is limiting, this "block" can be overcome by increasing the concentration of fatty acyl-CoA. Indeed, when NSF is limiting higher concentrations of any of the other partners for a multi-subunit fusion complex, in addition to the acylated component, it would be expected to increase the efficiency of fusion.

There are at least three mechanisms by which an acylated transport component could promote membrane fusion. (a) The hydrophobic lipid moiety of the component could directly participate in fusion by perturbing the lipid bilayers. This would resemble the function of hydrophobic fusion sequences of viral glycoproteins (Stegmann et al., 1989). (b) The acylated component could serve passively as a membrane anchor to promote stable assembly of the acylated component and possibly other components on the membrane. After the fusion event, deacylation would allow an easy and coordinated disassembly of the machinery. (c) The bound fatty acid could exert an allosteric effect on other components of the fusion apparatus. In all of the cases, acylation and deacylation would offer an important means to regulate the fusion process. Upon acylation, the component would be activated for its function in vesicle-cisterna maturation and fusion. Upon deacylation, it would be inactivated and set free for new rounds. Note that in practically all examples studied so far, palmitate is bound to a protein via thioester or oxyester linkages that can be easily hydrolyzed by specific esterases; in contrast, amide linkages are almost exclusively with myristate and are very stable (Schultz et al., 1988; Towler et al., 1988).

In light of our previous finding that budding of coated vesicles requires fatty acyl-CoA (Pfanner et al., 1989; Fig. 1) it would seem that proteins with a covalently bound hydrophobic moiety may play crucial roles in both budding and in triggering fusion. As the mechanisms unravelled in the cell-free system measuring transport from *cis*- to *medial* Golgi likely apply to many vesicular transport processes (Rothman, 1987; Wilson et al., 1989; Beckers et al., 1989; Diaz et al., 1989), we imagine that the acylated components may generally be employed in transport processes that require separation or fusion of lipid bilayers.

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