We characterized the \emph{in vitro} fusion of endosomal compartments from \emph{Dictyostelium discoideum}. Fusion activity was restricted to early compartments, was dependent on cytosolic proteins, and was activated by GTP and guanosine 5'-O-(3-thio)triphosphate (GTP\textsubscript{S}). This stimulation suggests the involvement of a small G protein, which we propose to be Rab7 on the basis of the strong inhibitory effect of anti-Rab7 antibodies. It is noteworthy that in the presence of GTP\textsubscript{S}, the concentration of ATP-Mg\textsuperscript{2+} could be reduced to less than 1 mM without loss of fusion activity. Under these conditions, competing residual ATP with adenosine 5'-O-(3-thio)triphosphate-Mg\textsuperscript{2+} also failed to inhibit endosome fusion. The presence of an ATP-depleting system alone blocked fusion probably because endogenous ATP was removed by coupling through NDP kinase. Moreover, whether ATP was present or not, GTP\textsubscript{S}-activated fusion was equally sensitive to anti-Rab7 antibodies or N-ethylmaleimide and was restricted to early compartments. These results show that soluble ATP-Mg\textsuperscript{2+} is not needed for endosome fusion. Since homotypic fusion of endosomes in \emph{D. discoideum} has been shown to depend on the ATPase N-ethylmaleimide-sensitive factor (Lenhard, J. M., Mayorga, L., and Stahl, P. D. (1992) \textit{J. Biol. Chem.} 267, 1806–1903), the nucleotide exchange on the N-ethylmaleimide sensitive factor must take place before GTP\textsubscript{S} activation in this system.

Endocytosis is the process by which eukaryotic cells internalize, sort, and digest extracellular molecules (pinocytosis and receptor-mediated endocytosis) or particles (phagocytosis). In wild-type \emph{Dictyostelium discoideum}, living on soil bacteria, phagocytosis is very active, but fluid phase uptake is almost undetectable. In contrast, axenic mutant cell lines such as Ax-2 exhibit intense pinocytosis in parallel with normal phagocytic behavior. Pinocytosis in \emph{d. discoideum} Ax-2 strain should therefore be considered as a deregulated phagocytosis, whose defect is as yet unknown. Although initial findings argued in favor of clathrin-coated vesicle-mediated internalization (1), it has recently been shown that most of the fluid ingested by Ax-2 is contained in intracellular structures similar to phagosomes (2). Thus, \emph{D. discoideum} is a very attractive organism to study the intracellular fate of phagosomes, because of the ease of biochemical experiments, the existence of a whole series of endocytosis mutants, and the possibility of genetic engineering.

In mammalian cells, phagosomes become acidic and progressively acquire hydrolytic enzymes from primary lysosomes to eventually constitute phagolysosomes (3). In \emph{D. discoideum}, the internalized material similarly passes into an acidic, hydrolase-rich compartment, but, contrary to mammalian lysosomes (which can retain the ingested material for expanded time periods (4)), undigested material transits through a less acidic postlysosomal compartment before egestion (5, 6). An understanding of how the material is carried along these successive compartments could be brought about by the \emph{in vitro} study of the different steps of the phagocytic pathway and by the identification of the proteins responsible for membrane recognition and fusion.

Of central importance in this respect has been the discovery by Rothman and co-workers of the N-ethylmaleimide-sensitive factor (NSF).\(^1\) First isolated on the basis of its capability to restore intra-Golgi transport after treatment by N-ethylmaleimide (NEM), this ATPase has now been shown to participate in almost all intracellular vesicle fusion events (7). In its ATP-bound form, NSF binds to membranes via soluble NSF attachment proteins (SNAPs) and acts upon a complex made by integral membrane proteins specific for the donor and acceptor compartments called v- and t-SNAREs (SNAP receptors). It is currently proposed that hydrolysis of ATP by NSF drives the rearrangement of SNAREs, which actually permits membrane fusion and the dissociation of the SNARE complex (8). The binding of ATP by NSF is therefore a prerequisite for membrane fusion.

The small GTP-binding proteins of the Rab subfamily also play a crucial role in the fusion process. The most widely held model states that for each intracellular membrane fusion event, activation of a specific Rab protein is needed (9). Several reports indicate that Rab proteins regulate the rate of formation of the SNARE complexes (10–12). In mammalian cells, Rab4, Rab5, Rab7, and Rab 9 are known to be involved in the endocytic pathway and regulate the shuttling of recycling vesicles, early endosome fusion, early endosome to lysosome transport, and lysosome to Golgi transit (13). In \emph{D. discoideum}, only

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\(^1\) The abbreviations used are: NSF, N-ethylmaleimide-sensitive factor; NEM, N-ethylmaleimide; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; HRP, horseradish peroxidase; b-HRP, biotinylated horseradish peroxidase; GDP\textsubscript{S}, guanosine 5'-O-(2-thio)triphosphate; GTP\textsubscript{S}, guanosine 5'-O-(3-thio)triphosphate; ATP\textsubscript{S}, adenosine 5'-O-(3-thio)triphosphate; A-485, 4-acetamido-4'-maleimidystilbene-2,2'-disulfonic acid; DTT, dithiothreitol; PNS, postnuclear supernatant; PBS, phosphate-buffered saline.

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Endosome Fusion in D. discoideum

Rab7 and to a lesser extent Rab4 have been shown to be present in the endocytic pathway (14, 15). Like mammalian endosomes, D. discoideum endosomes are able to fuse in vitro, reproducing two characteristic features of mammalian endosome fusion, namely sensitivity to GTPγS and inhibition by NEM (16). Furthermore, the addition of mammalian NSF could reverse this inhibition, which showed that the general fusion machinery described above is conserved in the D. discoideum endocytic pathway. In this study, we established an in vitro fusion assay using partially purified D. discoideum endocytic vesicles and cytosol and determined the nucleotide requirements of early endosome fusion.

EXPERIMENTAL PROCEDURES

Reagents, Cell Culture, and General Procedures—Horseradish peroxidase (HRP), avidin, GTP, GDP, GTPγS, ATP, ATPγS, creatinine phosphate, and creatine phosphate kinase were from Boehringer Mannheim. 4-Acetamido-4′-maleimidylstibene-2,2′-disulfonic acid (A-485) was from Molecular Probes. Other biochemical reagents and chemicals were from Sigma and Prolabo. Biotinylated horseradish peroxidase (b-HRP) was synthesized by coupling biotinamidocaproate with 3 mg of HRP. Biotinylated horseradish peroxidase, and creatine phosphate kinase were from Boehringer Mannheim. Protein concentrations were determined by the BCA assay (Pierce) in a Beckman SW41 rotor (100,000 rpm, 120,000 g, 1 h). The supernatant was 95% purity) were mixed (1:1) with 70% glycine buffer (100 μl total reaction volume of 120 μl, RCI was omitted, and MgCl2 was raised to 3 mM.

After 60 min of incubation at 21 °C, the assay was stopped by the addition of one volume of ice-cold 2% Triton X-100 and incubated at 4 °C for 30 min. The samples were then loaded into the wells of a protein-avidin-coated immunosorbent assay plate. The b-HRP-avidin complexes were allowed to bind for 2 h at 21 °C or overnight at 4 °C, and the wells were washed twice in PBS-Tween. To measure the amount of immobilized b-HRP, 100 μl of HRP substrate (0.1 mg/ml 1-tetramethylbenzidine, 0.6% H2O2 in 0.05 M sodium citrate, pH 5.0) was added to each well. The reaction was stopped by the addition of 20 μl of 3 mM H2SO4, and the optical density was measured at 450 nm. Fusion efficiency was defined as the ratio of the amount of b-HRP immobilized to the amount of potentially immobilizable b-HRP, obtained in a separate reaction where biotinylated insulin was omitted.

Determination of the Free ATP Concentration in Endosome Fusion Assays—After 2 min of incubation at 21 °C, a portion of an endosome fusion assay was clarified by centrifugation (100,000 × g, 15 min, 4 °C), and free ATP in the supernatant was separated from protein-bound ATP by ultrafiltration on a 10-kDa cut-off Ultrafree Millipore filter. The concentration of cytosolic or membrane-bound proteins was 0.8 mg/ml, and the optical density was measured at 450 nm. Fusion efficiency was defined as the ratio of the amount of b-HRP immobilized to the amount of potentially immobilizable b-HRP, obtained in a separate reaction where biotinylated insulin was omitted.

Preparation of Avidin- or b-HRP-loaded D. discoideum Endosomes—Avidin- or b-HRP-loaded endosomes were prepared in parallel from the same batch of cells. Ameobae (1 × 109/ml) were incubated for 5 min at 21 °C in axenic medium containing either b-HRP or avidin (1 mg/ml). Internalization of the markers was stopped by the addition of 5 volumes of ice-cold washing buffer (200 mM sucrose, 0.5 mM EGTA-KOH, 10 mM HEPES-KOH, pH 7.4).

Cells were washed twice in washing buffer and resuspended in breaking buffer (1 mM dithiothreitol (DTT), 5 mM MgCl2, 10% glycerol, pH 7.0). When fusion between whole endocytic compartments was assayed, PNSs at this stage were used.

To prepare early endosomes, 3 ml of PNS was loaded onto a discontinuous sucrose gradient by layering 1 ml of 54%, 4 ml of 40%, 2.8 ml of 30%, and 2 ml of 20% sucrose, 1 mM DTT, 10 mM HEPES-KOH, pH 7.4. Avidin and b-HRP-loaded D. discoideum endosomes (5 μl each) were mixed in a 50-μl total volume with biotinylated insulin (0.1 mg/ml), an ATP-regenerating buffer (200 μM ATP-Mg2+, 10 mM creatine phosphate, 85 μM GTPγS, creatinine phosphate kinase, pH 7.4) or ATP-depleting (10 mM glucose, 2.2 mM Na2ATP, 10 units/ml 5′-nucleotidase) system, and D. discoideum cytosol (10 μl). Except where otherwise stated, the final concentration of cytosolic or membrane-bound proteins was 0.8 mg/ml or 0.4–0.8 mg/ml, respectively, and the ATP-depleting system consisted of hexokinase and glucose. Fusions between whole endosomal compartments contained in PNS were conducted in the same way except that 40 μl of both b-HRP- and avidin-loaded D. discoideum PNS were mixed in a total reaction volume of 120 μl, RCI was omitted, and MgCl2 was raised to 3 mM.

Endosome Fusion Assay—Fusions between purified endosomes were conducted in 10 mM KCl, 2 mM MgCl2, 0.5 mM EGTA-KOH, 200 mM sucrose, 1 mM DTT, 10 mM HEPES-KOH, pH 7.4. Avidin and b-HRP-loaded D. discoideum endosomes (5 μl each) were mixed in a 50-μl total volume with biotinylated insulin (0.1 mg/ml), an ATP-regenerating buffer (200 μM ATP-Mg2+, 10 mM creatine phosphate, 85 μM GTPγS, creatinine phosphate kinase, pH 7.4) or ATP-depleting (10 mM glucose, 2.2 mM Na2ATP, 10 units/ml 5′-nucleotidase) system, and D. discoideum cytosol (10 μl). Except where otherwise stated, the final concentration of cytosolic or membrane-bound proteins was 0.8 mg/ml or 0.4–0.8 mg/ml, respectively, and the ATP-depleting system consisted of hexokinase and glucose. Fusions between whole endosomal compartments contained in PNS were conducted in the same way except that 40 μl of both b-HRP- and avidin-loaded D. discoideum PNS were mixed in a total reaction volume of 120 μl, RCI was omitted, and MgCl2 was raised to 3 mM.

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Preparation of His6-Rab7 Recombinant Protein—D. discoideum rab7 was amplified from a vegetative cell library (kindly given by Dr. Herb Ennis, New York) using Taq DNA polymerase. The oligonucleotide primers were designed to contain a BamHI site on the 5′-end. The PCR product was cloned into pGEM-T (Promega), and sequenced and used for protein expression.

Cells expressing His6 Rab7 were grown at 37 °C to an optical density of A600 = 0.8 in LB medium containing 200 μg/ml ampicillin and 12.5 μg/ml tetracycline and induced for 4 h at 37 °C with 2 mM isopropyl β-D-thiogalactoside. Cells were lysed by centrifugation, resuspended in lysis buffer (150 mM KCl, 1 mM MgCl2, 1 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM HEPES, pH 7.0), and disrupted by a French press. Cell debris were removed by centrifugation (9000 × g, 15 min). The supernatant was clarified by centrifugation (250,000 × g, 1 h) and loaded onto a 1-ml nickel-nitriolactric acid-agarose (Qiagen) column. The column was washed with 10 ml of lysis buffer, followed by 10 ml of 50 mM imidazole, 120 mM KCl, 1 mM MgCl2, 1 mM β-mercaptoethanol, 10% glycerol, pH 7.0. His6-Rab7 was eluted with 10 ml of 250 mM imidazole, 120 mM KCl, 1 mM MgCl2, 1 mM β-mercaptoethanol, 10% glycerol, pH 7.0. The His6-Rab7-containing fractions (95% purity) were mixed (1:1) with 70% glycerol, 120 mM KCl, 1 mM MgCl2, 1 mM β-mercaptoethanol and stored at −20 °C until use. His6-Rab7 proteins were then desalted into PBS containing 1 mM MgCl2 through a small gel filtration column (Hitrap, Pharmacia Biotech, Inc.).

Preparation and Affinity Purification of Anti-Rab7 Antibodies—To raise antibodies against Rab7, two peptides from the effector (amino acids 37–51) and the C-terminal hypervariable domains (amino acids 176–191) were coupled to rabbit serum albumin and cojugated in rabbits (Elevage Scientifique des Dombes, Romans, France). An affinity column was prepared by coupling the above peptides to glutaraldehyde-activated Affi-Gel 102 beads (Bio-Rad) and used to purify anti-Rab7 antibodies from rabbit serum as described in Ref. 20. The antibody fractions eluted in acidic or alkaline conditions were immediately neutralized, dialyzed twice against PBS, concentrated by ultrafiltration (Centricon, Amicon), and stored at 4 °C. Both pools of anti-Rab7 antibodies inhibited the endosome fusion assay equally.

RESULTS

An in vitro assay for homotypic fusions between fluid phase loaded D. discoideum endocytic compartments was derived
Endosome Fusion in D. discoideum

**FIG. 1.** **Fusion between D. discoideum endocytic compartments is restricted to the early ones.** A. amoebas were pulsed in parallel with avidin or b-HRP at 21 °C for 5 min and chased for the indicated times. Preparations of PNS from avidin- or b-HRP-loaded cells of the same chase time points were allowed to fuse in the presence of an ATP-regenerating system. Fusion efficiency is defined under “Experimental Procedures.” The solid line is the best exponential fit of the data. B. amoebas were incubated with avidin or b-HRP at 21 °C for 5 min, and PNS were prepared and fractionated in parallel on discontinuous sucrose gradients as described under “Experimental Procedures.” After centrifugation, the light 30–40% and dense 40–54% interfaces were recovered (fractions 6 and 2 in Fig. 2), and two of them were combined in a fusion assay performed in the presence of an ATP-regenerating system with (+) or without (−) 50 μM GTP S. Avidin and b-HRP refer to avidin- or b-HRP-loaded endosomes, and L and D stand for the light and dense fractions.

from the one based on the formation of a complex between b-HRP and avidin described for mammalian cells (17). In preliminary experiments, the uptake rate, intracellular transit time, and exit rate of b-HRP and avidin were found similar to those of fluorescein isothiocyanate-dextran, a fluid phase marker in D. discoideum (21). In various mammalian cells like macrophages and hepatocytes, HRP has been shown to enter the cells by a mannose receptor pathway, characterized by macrophages and hepatocytes, HRP has been shown to enter the cells by a mannose receptor pathway, characterized by mannose receptors on the cell surface. In contrast, HRP uptake by D. discoideum did not exhibit any saturation from 0.05 mg ml⁻¹ up to the maximum concentration tested of 5 mg ml⁻¹. Avidin uptake was also linear with concentration in the same range. It is therefore likely that these markers are internalized in the fluid phase and not bound to a receptor. This validates the use of b-HRP and avidin as fluid phase markers in D. discoideum.

**Kinetic Characterization and Partial Purification of Fusogenic Endosomal Compartments in D. discoideum—Avidin and b-HRP were internalized by D. discoideum cells for 5 min and chased for various times. PNS were prepared, and those having the same chase time were combined in a fusion assay. All PNS preparations contained the same amount of internalized markers; however, the efficiency of the fusion reaction decreased exponentially with chase duration (t½ = 5 min, Fig. 1A). Furthermore, markers contained in PNS with 0-min chase time were unable to fuse with markers contained in PNS with 15-min chase time (data not shown), which shows that only homotypic and no heterotypic fusion of early endocytic compartments occurs in D. discoideum.

To separate the fusogenic endosomes from other membranes and from the cytosol, a PNS preparation from D. discoideum cells loaded with b-HRP for 5 min was fractionated by centrifugation through a discontinuous sucrose gradient. Cytosolic components were retained on the upper 3 ml of the gradient, as indicated by the high protein concentration, whereas the dense lysosomes, assessed by the presence of cathepsin B, acid phosphatase, α-mannosidase, and β-glucosidase activities, sedimented to the bottom 40–54% sucrose interface. Most of the alkaline phosphatase activity, a marker of the plasma membrane and the contractile vacuolar system in D. discoideum, was present at the 8–30% interface, as expected (23). Little HRP activity was found at the top of the gradient, indicating that most of the endosomal compartments remained sealed during the purification procedure. Half of the HRP activity was recovered at the 30–40% interface, and half sedimented to the 40–54% interface (Fig. 2). When fluid phase markers were chased for 15 min, the whole HRP activity was found in a single peak at the 40–54% interface, which indicated that the light compartments were occupied before the dense ones by the markers and that the transit time from the lighter to the denser compartment was less than 15 min. Identical results were obtained with fluorescein isothiocyanate-dextran as a fluid phase marker.

After a 5-min pulse, when endocytic compartments recovered at each of the interfaces were tested for homotypic fusion ac-
activating effect of GDP, but not GDP for GTP and GDP, while GDP showed heterotypic fusion activity (Fig. 1 at the 30–40% interface and at the 40–54% interface, despite the presence or absence of guanine and adenine nucleotides be self-fusogenic, regardless of the cytosol concentration and compartments present at the 40–50% interface were never found to with that obtained with PNS. The high density endocytic com-

ments formed at the indicated concentrations of GTP·S (.), GTP (●), or GDP·S (▲) in the presence of an ATP-regenerating (A) or -depleting (B) system. For clarity, the effect of GDP, which is identical to that of GTP, was omitted. Identical batches of early endosomes and cytosol (protein concentration 0.8 mg·mL⁻¹) were used for both experiments.

GTP and Nonhydrolyzable Analogue activate early endosome fusion. In vitro fusion of partially purified early endosomes was performed at the indicated concentrations of GTP·S (.), GTP (●), or GDP·S (▲) in the presence of an ATP-regenerating (A) or -depleting (B) system. For clarity, the effect of GDP, which is identical to that of GTP, was omitted. Identical batches of early endosomes and cytosol (protein concentration 0.8 mg·mL⁻¹) were used for both experiments.

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DISCUSSION

In this study, we show that only early endosomal compartments of *D. discoideum* are capable of homotypic fusion *in vitro*. In addition, no heterotypic fusion between early endosomal and lysosomal or postlysosomal compartments is observed. The early endosomes probably correspond to the prelysosomal vesicles (30) and are separated from the lysosomes and other dense organelles on a sucrose density gradient. In additional experiments, we observed that these dense compartments inhibited the fusion reaction of early endosomes (data not shown). Conversely, the addition of early endosomes to a fusion reaction conducted with the dense compartments did not promote lysosome fusion. The ability of light endosomes to fuse is therefore an intrinsic property of these membranes, and the
endosome fusions performed in the absence of ATP. Four fusion assays were prepared with partially purified early endosomes and an ATP-depleting system as described under “Experimental Procedures,” except that no DTT was added. Samples a and b were also supplemented with 50 μM GTPγS. After 20 min of incubation at 21 °C, all samples were set on ice. Samples b and d were treated with 1 mM A-485 for 10 min followed by 2 mM DTT, and samples a and c were mock-treated by reversing the order of A-485 and DTT additions. Furthermore, samples c and d were supplemented with 50 μM GTPγS. All samples were then returned to 21 °C for 40 min, and the amount of b-HRP-avidin complexes was thereafter quantified as described under “Experimental Procedures.”

**Fig. 7.** Early endosome fusion performed in the absence of ATP is specific to early compartments and sensitive to anti-Rab7 antibodies and to the NEM analogue A-485. A, fusion assays were conducted exactly as in Fig. 1B, except that the ATP-regenerating system was replaced by an ATP-depleting system. B, fusion assays were conducted exactly as in Fig. 5B, except that the ATP-regenerating system was replaced by an ATP-depleting system. C, partially purified endosomes prepared in the absence of DTT were treated with 1 mM A-485 for 10 min on ice, and then 2 mM DTT was added (A-485 treated). As a control, the order of A-485 and DTT additions was reversed (mock treated). These endosomal membranes were then assayed for fusion activity in the presence of 50 μM GTPγS and an ATP-regenerating (+) or -depleting system (−). Use of a membrane-impermeable NEM analogue was necessary, because the NEM treatment inhibits the formation of b-HRP-avidin complexes.

The effect of dense compartments results either from the release of a soluble inhibitor of the fusion reaction or from the removal of a soluble limiting factor.

Our results show that GTP and GTP analogues activate endosome fusion. The presence of an activated G protein, but not its deactivation, is therefore required for the fusion process. This stimulatory G protein probably does not belong to the Arf subfamily, because impairment of GTP hydrolysis on Arf proteins is known to block homotypic or heterotypic fusions (31–33). In contrast, impairment of GTP hydrolysis on Rab5 protein activates early endosome fusion in mammalian cells (29, 33, 34). Rab proteins are therefore good candidates to account for the activating effect of GTP and GTPγS. Furthermore, affinity-purified anti-Rab7 antibodies inhibit early endosome fusion, the inhibition being reverted by purified recombinant Rab7. Rab7 is indeed present on magnetically purified endocytic compartments corresponding to early endosomes and lysosomes but not postlysosomes. It has recently been proposed that Rab7 is involved in the recycling of lysosomal enzymes from the postlysosomal compartment back to the lysosomes (35). Based on our results, we propose to extend this hypothesis and assume that Rab7, perhaps along with other Rab proteins, also controls the fusion between these recycling vesicles and incoming material. In this context, homotypic endosome fusion would appear as a side effect of the physiologically relevant fusion between endosomes and recycling vesicles. The hypothesis is further supported by the phenotype of an overexpressed mutant rab7 (35). Rab7Q223C, defective in GTP binding, exhibited a highly reduced rate of fluid phase entry and acidification, the opposite effect being observed with Rab7Q67L, a mutant protein impaired in GTP hydrolysis. Rab7 could therefore couple both ends of the endocytic pathway in *D. discoideum*.

An unexpected feature of the in vitro fusion of *D. discoideum* early endosomes is the absence of a requirement for free ATP-Mg2+, provided that GTPγS is present. Similarly, fusion assays performed with whole PNS were also insensitive to ATP depletion in the presence of GTPγS (data not shown). Therefore, no factor requiring external ATP was lost during the endosome purification procedure. In the absence as well as in the presence of ATP, endosome fusion exhibited the same specificity to early compartments and was sensitive to inhibition both by anti-Rab7 antibodies and NEM. Considering these criteria, endosome fusions performed in the absence of ATP are similar to other well described homotypic fusion systems. Finally, the addition of ATPγS in the presence of an ATP-depleting system and GTPγS does not inhibit fusion. Altogether, these results show that either no ATP-binding protein is needed in endosome fusion in *D. discoideum* or that the fusion proceeds with tightly bound endogenous ATP.

The first hypothesis is not tenable because the addition of mammalian NSF can restore endosome fusion activity to NEM-inactivated *D. discoideum* PNS (16). Furthermore, evidence presented above suggests that Rab7 plays a role in endosome fusion. The fusion machinery seems therefore very close to the one described in mammalian cells and yeast that involves NSF, SNAPs, SNAREs, and Rab proteins (7). We therefore favor the
hypothesis that the nucleotide site of the ATP-binding proteins needed in this fusion assay, possibly D. discoideum NSF or an NSF-like protein, is inaccessible at this stage. Since the fusion activity originates from endosomes prepared from two different cell populations, this implies that ATP binding has occurred before the preparation of the PNS and therefore before the docking of the membranes. The order of the steps leading to membrane fusion would therefore differ from the initial model, accounting for synaptic vesicle fusion (8), but be consistent with more recent findings, that NSF is already present at the membrane fusion would therefore differ from the initial model, docking of the membranes. The order of the steps leading to mem-

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