GTP γ S stimulation of endosome fusion suggests a role for a GTP-binding protein in the priming of vesicles before fusion

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Guanosine 5'-(3-O-thio)triphosphate (GTP γ S), a non-hydrolyzable analogue of GTP, inhibits *in vitro* fusion among early endocytic vesicles in the presence of high concentrations of cytosol. In this report we show that fusion is remarkably stimulated by GTP γ S under conditions where cytosolic components are the limiting factors for the process. The amount of cytosolic factors required for maximal fusion activity is several-fold decreased by the presence of GTP γ S. Moreover, preincubation of vesicles in the presence of cytosol and GTP γ S allows fusion to proceed even in the absence of cytosol. Our results indicate that a GTP-binding protein facilitates the binding of cytosolic factor(s) required for endosome fusion to the endosomal membrane and stabilizes a dilution-resistant intermediate of the fusion process.

Introduction

Macromolecules are transported along a series of membrane-bound organelles during the processes of endocytosis and exocytosis. The transport is mediated, in part, by vesicle fusion events that allow the selective transfer of luminal and membrane-associated components between different compartments. In the exocytic pathway, the transport is carried out by vesicles that bud from one compartment and fuse with the compartment of destination. These events mediate a bulk outward transport of proteins synthesized in the endoplasmic reticulum (ER) (Wieland *et al.*, 1987). Specific recognition markers on the proteins being transported may modify the final destination of molecules. For example, signals have been identified that mediate the retention of some proteins in the ER (KDEL carboxy terminal sequence) (Munro and Pelham, 1987) or their transport to lysosomes (6-phosphomannosyl residues on N-linked sugar chains) (von Figura and Hasilik,

1986). In the endocytic pathway, fusion among vesicles derived from the cell surface brings about the formation of large endosomes, where ligands are exposed to acidic pH and to some acid hydrolases (Wileman *et al.*, 1985; Diment *et al.*, 1988). From this compartment, ligands are sorted to different intracellular organelles (e.g., lysosomes) or recycled back to the cell surface. Sorting and recycling processes are likely to require membrane-fusion steps.

Several transport steps along the exocytic and endocytic pathways that involve vesicle fusion events have been reconstituted in cell-free systems. It is widely accepted that fusion of biological membranes is not a spontaneous event but requires special mechanisms to take place. *In vitro* assays have shown that certain elements of the fusion machinery are similar in both endocytic and exocytic pathways. Fusion-dependent transport requires ATP and several membrane-associated and soluble proteins. One of these proteins, the N-ethylmaleimide-sensitive factor (NSF), has been purified (Block *et al.*, 1988; Wilson *et al.*, 1989) and shown to be active in numerous transport steps of the exocytic pathway (Malhotra *et al.*, 1988; Beckers *et al.*, 1989) and endocytic pathway (Diaz *et al.*, 1989b). A role for GTP-binding proteins in the transport process has also been proposed. At least two secretion-deficient yeast mutants have been identified that are defective in GTP-binding proteins (Salminen and Novick, 1987; Segev *et al.*, 1988). Nonhydrolyzable GTP analogues, such as GTP γ S [guanosine 5'-(3-O-thio)triphosphate], have an inhibitory effect on several *in vitro* assays that reconstitute different steps along the exocytic pathway of yeast (Baker *et al.*, 1988; Ruohola *et al.*, 1988) and mammalian cells (Melancon *et al.*, 1987; Beckers and Balch, 1989). G-proteins have been proposed to be involved in the exocytosis of neutrophils and other cell types (Barrowman *et al.*, 1986). We have shown in a previous report that GTP γ S also inhibits *in vitro* the fusion of endosomes (Mayorga *et al.*, 1989b). Inhibition was only observed at saturating concentrations of cytosol. A similar cytosol dependency has been reported for the

inhibitory effect of GTP γ S on transport between Golgi stacks (Melancon *et al.*, 1987). In this report we show that GTP γ S stimulates endosome-endosome fusion when assayed at concentrations of cytosol that are unable to support full fusion activity. The results suggest that a GTP-binding protein mediates the attachment of cytosolic factors required for vesicle fusion to the endosomal membrane. Furthermore, GTP γ S stabilizes aggregation of endosomes, a step that appears to precede fusion of endosomes *in vitro*.

Results

GTP γ S stimulates endosome fusion at low cytosol concentrations

Fusion among early endocytic vesicles has been reconstituted in cell-free systems by several groups (Gruenberg and Howell, 1986; Braell, 1987; Diaz *et al.*, 1988; Woodman and Warren, 1988). The fusion assay employed in our studies utilizes two probes: 1) a mouse monoclonal antibody raised against dinitrophenol (anti-DNP IgG) that has been derivitized with mannose to make it a high-affinity ligand for the macrophage mannose receptor and 2) dinitrophenol-derivitized β -glucuronidase (DNP- β -glucuronidase), a glycoprotein recognized by the mannose receptor. These probes, in addition to being internalized by receptor-mediated endocytosis, form a stable complex when localized in the same compartment (Diaz *et al.*, 1988). In the standard assay, two separate populations of J774-E clone (mannose-receptor positive) macrophages are allowed to internalize the probes for 5 min at 37°C. Cells are homogenized and endosomal fractions are obtained by differential centrifugation. These membrane fractions are mixed in a reaction mixture containing salts, gel-filtered cytosol, and ATP. Fusion between endosomes loaded with different probes results in the formation of an immune complex that can be immunoprecipitated and quantitated by taking advantage of the enzymatic activity of β -glucuronidase.

In a previous report, we have shown that GTP γ S is able to inhibit *in vitro* vesicle fusion between early endosomes (Mayorga *et al.*, 1989b). The inhibitory effect was only observed at high concentrations of cytosol. Since fusion *per se* is cytosol dependent, the inhibitory effect of GTP γ S was only assessed at cytosolic concentrations that fully supported fusion. However, when GTP γ S was tested at concentrations of cytosol that supported submaximal fusion activity, a stimulatory effect was observed (Figure 1). GTP γ S concentration dependency was similar for both stimulation and inhibition with a half-maximal ef-

Figure 1. Effect of GTP and GTP γ S on *in vitro* endosome fusion at different cytosol concentrations. Endosomal fractions containing the fusion probes were mixed in fusion buffer supplemented with different concentrations of gel-filtered cytosol (20 μ l final volume). The samples were incubated in the presence of 20 μ M GTP γ S (●), 1 mM GTP (○), or without any addition (▲). After 60 min incubation at 37°C, the samples were lysed and immunoprecipitated, and the amount of complex formed was quantitated. Nonspecific background fluorescence was measured by incubation at 4°C and subtracted from all values. The data is representative of 1 of 5 independent experiments.

fect around 2 μ M GTP γ S (Figure 2A). The stimulatory effect was specific for guanosine triphosphonucleotides; ATP γ S [adenosine 5'-(3-O-thio)triphosphate] and GDP β S [guanosine 5'-(2-O-thio)diphosphate] were ineffective, and the stimulation was competed by GTP (Figure 2, A and B). While less effective than GTP γ S, GTP itself enhanced fusion at low cytosol concentrations, but it did not affect fusion at saturating concentration of cytosol (Figure 1). The presence of GTP may raise ATP levels in our preparations. Nucleoside diphosphokinases in cytosol can catalyze the transfer of γ -phosphate from GTP to ADP. Since fusion is ATP dependent, this may account for the stimulatory effect of GTP. However, the stimulatory effect of GTP was also observed when the control fusion reaction was carried out in the presence of 2 mM ATP (data not shown), suggesting that GTP itself facilitates fusion when cytosol is the limiting factor for the fusion reaction.

Characterization of the fusion process stimulated by GTP γ S

Fusion under GTP γ S stimulation may be mediated by an entirely different mechanism than the one normally responsible for endosome fusion at saturating levels of cytosol. Nonhydrolyzable ana-

Figure 2. Effect of nonhydrolyzable nucleotides on in vitro endosome fusion. (A) Fusion was carried out at a cytosol concentration of 0.09 mg protein/ml (●, ▲) or 1.8 mg protein/ml (○), in the presence of increasing concentrations of ATP γ S (▲) or GTP γ S (●, ○). Fusion was quantitated after 45 min incubation at 37°C, as described in Experimental Procedures. (B) Endosome fusion was assessed in the absence (control) or presence of different nucleotides at a cytosol concentration of 0.09 mg/ml. Values are the mean of 2 or 3 fusion reactions. Vertical lines represent standard deviation.

logues of GTP interact with a large number of GTP-binding proteins that can affect several intracellular processes, including fusion events (Bourne, 1988). To assess whether a different mechanism of vesicle fusion was activated by GTP γ S, the requirements for fusion in the presence of the GTP analogue were studied and compared with those already described for endosome-endosome fusion (Diaz *et al.*, 1988). GTP γ S-stimulated fusion was cytosol dependent (Figure 1 and Table 1). It required ATP and was affected by the ionic strength of the fusion buffer (Table 1). Treatment of vesicles and cytosol with NEM (N-ethylmaleimide) completely abrogated fusion (Table 1). More specifically, a monoclonal antibody raised against NSF, a protein required for vesicle transport along the secretory pathway (Block *et al.*, 1988), completely inactivated GTP γ S-induced fusion (Table 1). We have previously shown that this antibody inhibits endosome-endosome fusion in the standard fusion assay (Diaz *et al.*, 1989b). These results indicate that the mechanism mediating fusion in response to GTP γ S stimulation has the same requirements as that observed in the standard fusion assay.

Under standard conditions, early endosomes are able to fuse with plasma membrane-derived vesicles and with another population of early endocytic vesicles but not with late endosomes (Mayorga *et al.*, 1988). In another series of comparative experiments, we observed the same kind of specificity for GTP γ S-stimulated fusion. The GTP analogue promoted fusion among plasma membrane-derived vesicles and endosomes at low concentrations of cytosol. However, when DNP- β -glucuronidase was internalized for 5 min at 37°C and chased for 12 min to a late endocytic compartment, vesicles obtained from these cells were unable to fuse with 5-min endosomes

loaded with anti-DNP IgG in response to GTP γ S stimulation (data not shown).

Effect of GTP γ S on the kinetics of endosome fusion and fusion-dependent inactivation

Previous studies have shown that endosomes aggregate in vitro during the fusion reaction (Diaz *et al.*, 1989a). Within these aggregates, the limiting membranes of the endosomes fuse, leading to the formation of large vesicles. The products of these multiple fusion events are poorly fusogenic. We tested the effect of GTP γ S on the kinetics of fusion and on the fusion-dependent inactivation of the vesicles to assess whether the characteristics of the fusion reaction changed after stimulation by the GTP analogue. At low cytosolic concentrations, the addition of GTP γ S caused an increase in the fusion rate and extent of the fusion reaction (Figure 3A, left panel). A similar stimulation of the fusion rate was obtained by adding excess cytosol (Figure 3A, right panel). In contrast, GTP γ S strongly decreased the rate of fusion when added in the presence of excess cytosol (Figure 3A, right panel). We then monitored the fusogenic activity of vesicles after different periods of preincubation under conditions where fusion occurs to assess whether the kinetics of inactivation were affected by the presence of GTP γ S. In principle, a GTP-binding regulatory protein might keep vesicles in a fusogenic state permanently on binding of GTP γ S. This could lead to a slowing of the inactivation process. However, the results show that inactivation was even faster in the presence of GTP γ S (Figure 3B). Incubation in low concentrations of cytosol alone did not increase the inactivation rate (data not shown). Preincubation in the absence of KCl greatly decreased vesicle inactivation, suggesting

Table 1. Requirements for GTP γ S-stimulated fusion

Condition	Relative fusion
Reference condition: 0.1 mg protein/ml, 20 μ M GTP γ S	1.00
–GTP γ S	0.30 $\pm$ 0.05
–KCl	0.18 $\pm$ 0.02
–ATP	0.10 $\pm$ 0.02
–Cytosol	0.17 $\pm$ 0.03
+Concentrated cytosol, 2 mg protein/ml	0.09 $\pm$ 0.05
NEM preincubation	0.05 $\pm$ 0.03
Anti-NSF IgM	0.12 $\pm$ 0.02
Control IgM	0.90 $\pm$ 0.02

Values are the mean of at least 4 determinations $\pm$ SD.

In vitro fusion among endosomes was assessed by the use of buffers of different compositions. In some instances, cytosol and vesicles were pretreated with NEM. Fusion was expressed as a percentage of that obtained with the use of untreated endosomes resuspended in complete fusion buffer supplemented with 0.1 mg cytosolic protein/ml and 20 μ M GTP γ S and incubated for 60 min at 37°C. Nonspecific background fluorescence was measured by incubation at 4°C and subtracted from all values. The following conditions were tested: –GTP γ S, the nucleotide was not added to the reaction; –KCl, the salt was not included in the buffer; –ATP, an ATP-depleting system (5 mM mannose, 25 U/ml hexokinase) was substituted for the ATP-regenerating system (i.e., ATP, creatine phosphate, and creatine phosphokinase); –Cytosol, no cytosol was included in the fusion buffer; +Concentrated cytosol, final cytosol concentration in the reaction mixture was 2 mg protein/ml; NEM, vesicles and cytosol were incubated for 30 min at 4°C with 1 mM NEM before the fusion reaction, and excess NEM was quenched with 2 mM dithiothreitol; Anti-NSF IgM, 4A6-monoclonal antibody (0.1 μ g/assay) was added to the fusion reaction; Control IgM, nonspecific IgM fraction was added to the reaction (0.1 μ g/assay).

that, similar to what is observed under normal fusion conditions, inactivation is a consequence of the fusion process and not a result of incubation at 37°C (data not shown). These observations indicate that, in response to GTP γ S stimulation, vesicles are not permanently activated and that they lose their fusogenic activity on fusion. The increased rate of inactivation suggests that an irreversible step in the fusion reaction is accelerated by GTP γ S stimulation.

Effect of GTP γ S on the morphology of endosome aggregates

Vesicle pellets observed by electron microscopy after incubation in standard fusogenic conditions or in the presence of GTP γ S displayed, in general, similar morphological characteristics (Figure 4, A and B). Aggregates of endosomes containing colloidal gold of different sizes were observed in both instances. After GTP γ S stimulation, however, vesicles containing two sizes of gold were often observed to be connected by a tubular

structure, presumably formed during the fusion reaction. It was not rare to observe several vesicles connected by intervesicular tubules (Figure 4, B–F). These connections were only seldom seen in aggregates obtained after incubation in standard fusogenic conditions (Figure 4A). A quantitation of these structures showed about a fivefold increase in the frequency of tubular attachments per aggregate (100 aggregates from 3 independent experiments were quantitated for both standard and GTP γ S-stimulated fusion). The presence of these bridges allowed us to assess the morphological characteristics of the vesicles fusing in vitro. Small vesicles containing colloidal gold particles were often observed in the process of fusing to each other as well as with tubular, annular, and multivesicular endosomes (Figure 4, B–F). Interestingly, multilamellar bodies were sometimes found connected with endosomes (Figure 4F).

Aggregates are stabilized by GTP γ S

The rate of fusion between endocytic vesicles is strongly affected by vesicle dilution (Braell, 1987; Mayorga *et al.*, 1989a). It would be expected that endosome aggregation might yield a dilution-resistant intermediate in the fusion reaction. However, this intermediate has not been observed by the use of our biochemical assay. The aggregated state was only observed in the electron microscope by the use of a fast method of fixation (Diaz *et al.*, 1989a). This suggests that endosomal aggregates are very unstable structures that can form and dissociate in a reversible way. A common effect of nonhydrolyzable GTP analogues is to block the reversibility of processes mediated by GTP-binding proteins. For this reason, and because of the morphological observation above, it was of interest to assess whether the nonhydrolyzable GTP analogue could affect the reversibility of some steps of the fusion process. Specifically, we tested the effect of GTP γ S on the formation of a dilution-resistant prefusion state.

Fusion was inhibited when vesicles were diluted before the fusion reaction (Figure 5A). The dilution-dependent inhibition was similar for both normal and GTP γ S-stimulated fusion. A 10-fold dilution was able to abrogate fusion completely in both situations. This indicates that the binding of endosomes was not irreversible in the presence of GTP γ S. An irreversible binding step would have strongly increased the affinity of endosomes for each other, and fusion should have become more resistant to dilution. However, a significant amount of fusion was observed after a 10-fold dilution when endosomes were first incubated for

Figure 3. Kinetics of *in vitro* fusion and fusion-dependent inactivation in the presence of GTP γ S. (A) Fusion-dependent immune-complex formation was assessed after vesicles were incubated for different times at 37°C in the presence (●) or absence (○) of 20 μ M GTP γ S. Fusion buffer was supplemented with 0.1 mg (left panel) or 2 mg (right panel) of cytosolic proteins/ml. (B) Vesicles containing anti-DNP IgG or DNP- β -glucuronidase were incubated separately for different periods of time at 37°C in fusion buffer. Vesicles were then mixed and allowed to fuse for 60 min at 37°C. Preincubation and fusion were carried out under the same conditions. (○, fusion buffer contained cytosol at 1.5 mg protein/ml; ●, fusion buffer contained cytosol at 0.1 mg protein/ml and 20 μ M GTP γ S). Fusion was expressed as a percentage of that observed without preincubation. The experiments were repeated 3 times with similar results.

a short time at 30°C in the presence of GTP γ S (Figure 5B, left panel). This suggests that, in the presence of GTP γ S, aggregates are stabilized in a prefusion step. This intermediate state seems to be reversible under normal conditions, because a dilution-resistant state was not detected when vesicles were incubated in the absence of GTP γ S before dilution (Figure 5B, right panel). GTP γ S, therefore, does not appear to increase the affinity of these vesicles for each other, but it may induce an irreversible aggregated state between endosomes. The formation of a dilution-resistant complex between endosomes may be related to the observation that endosomes inactivate more rapidly after incubation in the presence of GTP γ S (Figure 3B) and to the appearance of a network of interconnected endosomes observed by electron microscopy (Figure 4).

Cytosol is not activated by preincubation in the presence of GTP γ S

An important characteristic of the GTP γ S effect on endosome-endosome fusion is that it is a function of the cytosol concentration (Figure 1). A possible explanation for this observation is that some cytosolic factors are activated by GTP γ S. To assess this possibility, cytosol was pretreated with GTP γ S, gel filtered to remove free nucleotide,

and used at different concentrations in a fusion assay in the presence or absence of GTP γ S. Preincubation of cytosol with GTP γ S did not activate or inactivate any soluble factor because cytosol that had been exposed to GTP γ S gave results comparable with control cytosol in all the conditions tested (Figure 6). This indicates that, if a GTP-binding protein were present in cytosol, it has an absolute requirement for membranes to become active.

GTP γ S mediates association of cytosolic factors with vesicle membranes

Since the cytosol requirement for fusion is greatly decreased in the presence of GTP γ S, the effect of the putative GTP-binding protein may be related to the interaction of cytosolic factors with the membranes. To assess this possibility, vesicles were preincubated separately with or without cytosol in the presence or absence of GTP γ S. Potassium chloride was deleted from the incubation to prevent fusion from proceeding. After the preincubation, samples were centrifuged to remove the nucleotide and cytosol. The vesicles were then resuspended in complete fusion buffer, and fusion was assessed in the absence or presence of increasing concentrations of cytosol. When vesicles were preincubated in the presence

Figure 4. Clustering and fusion of endosomes under normal conditions or after GTP γ S stimulation. Subcellular fractions containing colloidal gold of 5, 10, or 20 nm loaded in 5-min endosomes were incubated in vitro for 60 min at 37°C in fusion buffer containing 1.5 mg of cytosolic proteins/ml (A) or 0.1 mg of cytosolic proteins/ml and 20 μ M GTP γ S (B–F). After fusion, the samples were fixed in suspension, pelleted, and analyzed for transmission electron microscopy. Bars = 100 nm.

Figure 5. Effect of GTP γ S on a dilution-resistant prefusion state. (A) Vesicles were diluted at 4°C to the corresponding volume with fusion buffer supplemented with 1 mg of cytosolic proteins/ml (O) or with fusion buffer containing 0.1 mg of cytosolic proteins/ml and 20 μ M GTP γ S ($\bullet$). Samples were then incubated 30 min at 37°C. Fusion was expressed as a percentage of that observed under the most concentrated condition. Values are the mean of duplicate determinations. (B) Vesicles were resuspended in 10 μ l of fusion buffer supplemented with 0.15 mg of cytosolic proteins/ml and 20 μ M GTP γ S (left) or with 1.5 mg of cytosolic proteins/ml (right) and incubated at 30°C for different periods of time. After the appropriate time of preincubation, 1 aliquot was stopped by cooling on ice (O), and another aliquot was diluted 10-fold with the corresponding fusion buffer and incubated at 37°C for an additional period of 30 min ($\bullet$). All samples were brought to the same volume, and the fusion-dependent immune complex formed was quantitated. The 30°C incubation was used to slow the fusion reaction. Data are from 1 of 3 separate experiments.

of cytosol and GTP γ S, subsequent fusion (in a second incubation) was observed in the presence of potassium chloride and ATP even without the addition of cytosol (Figure 7A, left panel). This suggests that cytosolic factors required for fusion associate with the membranes in the presence of GTP γ S during the preincubation step. Consistent with this hypothesis are the observations that 1) presence of cytosol was required during the preincubation to obtain cytosol-independent fusion (Figure 7A, left panel), and 2) no further activation of fusion was observed by adding cytosol to the reaction after vesicles were exposed to cytosol and GTP γ S during the preincubation phase of the experiment (Figure 7A, right panel). Preincubation with cytosol in the absence of GTP γ S did not affect the subsequent cytosol dependency of the fusion reaction (Figure 7A, right panel), suggesting that the interaction of soluble factors with membranes is reversible under these conditions.

A test for the hypothesis that GTP γ S mediates an irreversible binding of cytosolic factors to the membranes is whether the cytosol can be depleted of fusion factors by preincubation in the presence of membranes and GTP γ S. Depleted cytosol should be unable to support further fusion.

Figure 7B shows that, after a 10-min incubation in the presence of GTP γ S and membranes, the capability of cytosol to support fusion was significantly reduced. Incubation without GTP γ S (Figure 7B) or without membranes (Figure 6) did not affect the fusion-promoting properties of cytosol.

Discussion

Protein transport in eucaryotic cells is a multidirectional process that is carried out by a complex series of membrane fusion events. Macromolecules endocytosed by the cell are transported to a heterogeneous network of vesicular structures that constitute the endosomal-lysosomal compartment where they are stored, degraded, or recycled back to the cell surface (Wileman *et al.*, 1985). Newly synthesized proteins that are translocated into the endoplasmic reticulum are transported via the Golgi apparatus to different intracellular organelles where they exert their function or, alternatively, they are secreted to the extracellular medium (Pfeffer and Rothman, 1987). Since, in most instances, macromolecules do not traverse lipid bilayers, their intracellular transport is mediated by vesicles budding from one compartment with subsequent recognition and fusion

Figure 6. Effect of GTP γ S-treated cytosol on in vitro endosome fusion. Gel-filtered cytosol was incubated for 15 min at 37°C in the presence (Δ , $\blacktriangle$) or absence ($\circ$, $\bullet$) of 20 μ M GTP γ S in homogenization buffer containing 1 mM dithiothreitol, 1 mM ATP, and 1.5 mM MgCl₂. After the incubation, the cytosols were filtered through G-25 Sephadex and assayed for their ability to support fusion in the presence ($\bullet$, $\blacktriangle$) or absence ($\circ$, Δ) of 20 μ M GTP γ S. Fusion reactions were incubated for 60 min at 37°C, and the immune complex formed was quantitated. The data represent 1 of 2 experiments.

with a second compartment. An efficient mechanism of directed movement, recognition, and fusion must exist to allow vesicle-dependent transport along these complex intracellular pathways.

In the last few years, it has become apparent that GTP-binding proteins play an important role in transport along the endocytic and exocytic pathway. Two GTP-binding proteins have been identified that are required for normal secretion in yeast. The *YPT1* gene product is a 23-kDa protein that seems to play a role in vesicle transport from ER to Golgi (Segev *et al.*, 1988). The *SEC4* gene encodes a 23.5-kDa GTP-binding protein that associates with secretory vesicles and plasma membrane (Salminen and Novick, 1987). The activity associated with *SEC4* appears to be required for a later step in the yeast secretory pathway. These two proteins show homology with the ras p21 protein. The latter is also a GTP-binding protein whose activity is regulated by GTP hydrolysis (McCormick, 1989). *Ras* gene products are reportedly involved in both secretion and endocytosis (Barbacid, 1987). In vitro, nonhydrolyzable analogues of GTP inhibit several transport steps along the exocytic and endocytic pathways. Transport between ER and Golgi, in both mammalian (Beckers and Balch, 1989) and yeast sys-

tems (Baker *et al.*, 1988; Ruohola *et al.*, 1988), is inhibited by GTP γ S. Transport between Golgi stacks (Melancon *et al.*, 1987) and recycling from endosomes to trans Golgi (Goda and Pfeffer, 1988) is also inhibited by GTP γ S. On the other hand, a number of studies have shown that GTP γ S stimulates the fusion-dependent release of secretory granule contents in permeabilized cells (Gomperts and Fernandez, 1985).

Similarly, we have reported that GTP γ S inhibits fusion among two populations of early endosomes as well as fusion between plasma membrane-derived vesicles and endosomes in cell-free assays (Mayorga *et al.*, 1989b). Moreover, transport from an early protease-free endosomal compartment to protease-positive endosomes is also inhibited by the GTP analogue (Mayorga *et al.*, 1989b). Similar to what is observed in Golgi transport, the effect is cytosol dependent, inhibition being observed only at high concentrations of cytosol. In the present report, we show that when the effect of GTP γ S is assessed at concentrations of cytosol that do not support full fusion activity, a prominent activation is observed. In the presence of the GTP analogue, the cytosol concentration required to obtain maximal activity decreases 5- to 10-fold. Addition of more cytosol to the assay reverses the stimulation, and inhibition of fusion is observed. It is unlikely that GTP γ S mediates nonspecific fusion events among membrane-bound compartments. Our data indicate that fusion is specific for early endocytic compartments under normal conditions and after GTP γ S stimulation.

GTP induces dramatic changes in the biological properties of GTP-binding proteins; nevertheless, GTP hydrolysis per se is seldom required for the activation. On binding, the nucleotide causes a conformational change in the protein, and hydrolysis is required to allow the protein to cycle between an active and an inactive state. In signal transduction, G proteins (GTP-binding regulatory proteins) are activated by interaction with an appropriate receptor-ligand complex (Stryer and Bourne, 1986; Gilman, 1987). On stimulation, they interchange GDP for GTP and remain active until GTP is hydrolyzed. Hence, their activity is highly stimulated by nonhydrolyzable analogues of GTP. During protein synthesis, GTP hydrolysis is required for the cyclic interaction of the Tu elongation factor-aminoacyl-tRNA complex with the ribosome in the process of elongation (Moldave, 1985). GTP γ S inhibits the process by preventing recycling of the elongation factor after the binding of complex to ribosome has occurred (Moazed and Noller, 1989). Fusion between endosomes proceeds in the presence of GTP γ S, which indi-

Figure 7. Effect of vesicle preincubation with GTP γ S on the cytosol dependency of endosome fusion in vitro. (A) Vesicles containing the fusion probes were preincubated separately for 10 min at 37°C in KCl-free fusion buffer containing different concentrations of cytosol. Preincubation was carried out in the presence (●) or absence (○) of GTP γ S. KCl was not included during the preincubation to inhibit fusion and avoid fusion-dependent inactivation (see Figure 3B and text). After preincubation, the vesicles were diluted 300 times in homogenization buffer, and GTP γ S was added to match the same final concentration of nucleotide in all samples. Samples were centrifuged 50 000 $\times$ g for 7 min to remove cytosol and GTP γ S and resuspended in complete fusion buffer without cytosol (left) or supplemented with increasing concentrations of cytosol (right). Samples were then incubated for 60 min at 37°C, and the immune complex formed was quantitated. Vertical lines represent standard deviation ($n = 3$). Some data shown in the left panel are the result of a single determination (i.e., without standard deviation). Similar results were obtained in 3 independent experiments. (B) Gel-filtered cytosol (0.12 mg protein) was incubated with blank vesicles, i.e., an endosomal fraction obtained from cells that had not internalized any ligand (0.2 mg vesicle protein) in 0.3 ml of KCl-free fusion buffer for 10 min at 4°C (Δ), at 37°C (○), or at 37°C in the presence of 20 μ M GTP γ S (●). Samples were centrifuged at 50 000 $\times$ g for 7 min. GTP γ S was added to the supernatants (preincubated cytosols) to match GTP γ S concentrations in all samples. Preincubated cytosols were used at different concentrations in a normal fusion assay in the presence of 1 mM GTP to compete GTP γ S.

cates that GTP hydrolysis is not required for the process. It is possible that the effect of GTP γ S is mediated by a regulatory GTP-binding protein. This kind of mechanism has been proposed to explain the cytosol-dependent inhibitory effect of GTP γ S on Golgi transport (Melancon *et al.*, 1987). However, our data suggest that GTP γ S is affecting the reversibility of one or more steps of the fusion process. A GTP-binding protein may function as classical G-proteins do during signal transduction in plasma membrane. It is then likely that one or more GTP-binding proteins have a direct role in the fusion mechanism. More specifically, our data indicate that GTP-binding proteins facilitate the binding of the cytosolic factors required for fusion to the endosomal membrane. Recycling of factors after the fusion reaction may require GTP hydrolysis. GTP γ S would induce an "irreversible" attachment of factors to the membrane, decreasing considerably the cytosol concentration required for fusion. The N-ethylma-

leimide sensitive factor recently characterized to be required for endosome fusion (Diaz *et al.*, 1989b) may be one of them. GTP γ S also seems to affect the reversibility of a prefusion aggregated state. It is not clear from our experiments whether there is an absolute GTP requirement for the binding of factors. In the normal assay, GTP is not added to the system; nevertheless, fusion occurs. Membranes may contain enough GTP associated with them to support binding of cytosolic factors. Alternatively, the binding of necessary factors may not have an absolute GTP requirement, but the nucleotide may facilitate the interaction of factors with membranes. This may be a limiting condition for efficient fusion within the cell. Another possibility is that the GTP-binding protein is not required for the constitutive fusion process, but can trigger fusion on stimulation by facilitating binding of factors to the endosomal membrane.

Morphological observation of fusion events after GTP γ S stimulation showed the formation of

networks of endosomes connected by tubular structures. The exact mechanism for the formation of these tubules is not well understood. These morphological phenomena may be related to the stabilization of an aggregated state in the presence of GTP γ S. More stable aggregates may increase the frequency of multiple bridge formation between endosomes. The presence of these bridges facilitates the morphological identification of subcellular structures active in fusion. This may prove to be a convenient experimental model for future morphological analysis.

It is possible that the stimulatory effect of GTP γ S at low cytosol concentrations and the inhibitory effect at high concentrations are related to the same mechanism. An excess of factors bound irreversibly to the membranes may inhibit fusion by steric hindrance or may promote futile reactions that consume some additional factor present in limiting concentration (Bourne, 1988). Alternatively, two GTP-binding proteins may be active in endosome fusion: one that mediates the binding of cytosolic factors and another that provides a regulatory role in fusion by exerting an inhibitory effect in the presence of a factor present in cytosol.

This is the first report of activation by GTP γ S of an intracellular transport process reconstituted *in vitro*. If this were a common mechanism, why has it not been observed in other reconstituted transport systems? Several possibilities may explain this discrepancy. First, fusion among early endocytic vesicles may represent a single-step process; the sequence of events aggregation-fusion-inactivation that has been proposed (Diaz *et al.*, 1989a) is consistent with this model. In contrast, reconstitution of transport in the secretory pathway utilizes an active "donor" fraction, which is forming transport vesicles as the reaction proceeds. These transport vesicles then find acceptor membrane domains with which they fuse. If GTP γ S induces the irreversible binding of the recognition and fusion factors, it may impair the recycling of these components after a first round of fusion. This would inhibit further transport and, as a result, strongly affect both ER to Golgi and Golgi-Golgi transport. Endosome fusion, as measured in our assay, however, could be unaffected. Second, because reconstitution of transport in the secretory pathway is a multiple-step process, it may be modulated by some other GTP-binding proteins that are not active in endosome fusion. GTP γ S inhibition of Golgi transport causes the accumulation of non-clathrin-coated vesicles (Orci *et al.*, 1989). Based on this observation, a role in the uncoating step has been proposed for the GTP-binding protein active in this system. Similar

coats have not been described in the endosomal system. Third, GTP γ S may have differential effects on the endocytic and exocytic pathways. The existence of two distinctive GTP-binding proteins in the secretory pathway of yeast, that appear to affect different transport steps along the pathway, indicates that a family of these proteins may be active in vesicular transport processes.

Several GTP-binding proteins have been purified from mammalian membranes whose functions are unknown; some of them may be active in vesicle transport (Ohmori *et al.*, 1989). Identification of the molecular components of vesicle recognition and fusion mechanisms will provide insight into the role of GTP and GTP-binding proteins in vesicle transport processes.

Experimental Procedures

Cells and materials

J774-E clone, a mannose-receptor positive macrophage cell line, was grown in minimum essential medium containing Earle's salts and supplemented with 10% fetal calf serum. HDP-1, a mouse IgG1 monoclonal antibody specific for dinitrophenol (anti-DNP IgG) was isolated according to Otsuka *et al.* (1984) and derivatized with mannose as previously described (Diaz *et al.*, 1988). β -glucuronidase was isolated from rat preputial glands as described (Keller and Touster, 1975). Bovine serum albumin and β -glucuronidase were dinitrophenol derivatized with the use of dinitrofluorobenzene (Diaz *et al.*, 1988). Anti-NSF (N-ethylmaleimide-sensitive factor) monoclonal antibody (4A6 IgM) was a generous gift from James E. Rothman (Princeton University, Princeton NJ). ATP γ S was obtained from Boehringer-Mannheim Biochemicals. All other chemicals were obtained from Sigma. Cytosol was the high spin supernatant of a cell homogenate obtained as described (Diaz *et al.*, 1988) and stored at -80°C . Cytosol aliquots (200 μl) were gel filtered through 1 ml G-25 Sephadex spin columns just before use in the fusion assay. Protein concentration after filtration was ~ 3 mg/ml. Proteins were measured according to Bradford (1976) with the use of bovine serum albumin as standard.

Preparation of endosomal fractions

Early endosomes were loaded with mannosylated anti-DNP IgG or with DNP- β -glucuronidase by a 5-min uptake at 37°C , as previously described (Diaz *et al.*, 1988). Cells (1×10^8 cells) were washed sequentially with 150 mM NaCl, 5 mM EDTA, 10 mM phosphate buffer pH 7.0 and with 250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH pH 7.0 (homogenization buffer) and homogenized in the latter buffer (2 ml), as previously described (Diaz *et al.*, 1988). Homogenates were then centrifuged at $800 \times g$ for 5 min to eliminate nuclei and intact cells. The supernatants were quickly frozen in liquid nitrogen and stored at -80°C . For the experiments, two 0.2-ml aliquots of postnuclear supernatants, each containing a different probe, were quickly thawed, diluted up to 3 ml with homogenization buffer, and pelleted for 1 min at $37\,000 \times g$. The supernatants were centrifuged for additional 5 min at $50\,000 \times g$. The pellets of the second centrifugation were enriched with 5-min endosomes (Diaz *et al.*, 1989a).

Fusion reaction

Endosome-enriched pellets containing the probes were resuspended (0.5 mg proteins/ml) in fusion buffer (250 mM su-

crose, 0.5 mM EGTA, 20 mM HEPES-KOH pH 7.0, 1 mM dithiothreitol, 1.5 mM MgCl₂, 50 mM KCl, 1 mM ATP, 8 mM creatine phosphate, 31 U/ml creatine phosphokinase, and 0.25 mg/ml DNP-BSA), supplemented with different concentrations of gel-filtered cytosol, mixed in a final volume of 10–20 μ l, and incubated at 37°C for different periods of time. The reaction was stopped on ice, and the immune complex formed was immunoprecipitated and quantitated with the use of 4-methylumbelliferyl β -D-glucuronide, as previously described (Diaz *et al.*, 1988).

Electron microscopy

Colloidal gold particles of 5, 10, and 20 nm were coated with mannosylated bovine serum albumin by the use of standard techniques (Roth, 1983). Gold particles were internalized into three separate populations of J774-E clone macrophages by a 5-min incubation at 37°C. Endosome-enriched fractions were obtained as described above and used for fusion reactions under the same conditions used for the biochemical assay. Fusion was stopped by adding 1% glutaraldehyde (prepared in homogenization buffer) at room temperature to preserve endosome aggregates. After 10 min, samples were centrifuged for 15 min at 12 000 $\times$ g, and the pellets were processed for transmission electron microscopy (Harding *et al.*, 1983).

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