Characterization of the Mechanism of Endocytic Vesicle Fusion

in Vitro*

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A cell-free assay to monitor receptor-mediated endocytic processes has been developed that uses biotinylated transferrin and avidin linked β-galactosidase as receptor-associated and fluid-phase probes, respectively (Wessling-Resnick, M., and Braell, W. A. (1990) J. Biol. Chem. 265, 690-699). The fusion of vesicles from heterologous sources can be detected in this assay: endocytic vesicles from K562 cells (a human cell line) will fuse with vesicles from Chinese hamster ovary cells. Fusion between endocytic vesicles is inhibited upon treatment with N-ethylmaleimide but can be restored by the addition of untreated cytosol from either cell type. The in vitro fusion reaction is also inhibited by the nonhydrolyzable nucleotide analogs guanosine 5'-3-thiotriphosphate (GTPγS) and adenosine 5'-3-thiotriphosphate (ATPγS). Other nonhydrolyzable guanine nucleotides are found to inhibit the in vitro reaction in the following order of potency: GTPγS > ATPγS > a,β-methylene GTP (GTP-PCP). The inhibitory effects of the nonhydrolyzable analogs of GTP and ATP are not additive. Moreover, excess GTP relieves the inhibition by GTPγS more than it relieves the inhibition by ATPγS, while excess ATP preferentially alleviates ATPγS (not GTPγS) inhibition. These properties suggest that the two nucleotides exert their effects at distinct points in the fusion process. Although micromolar levels of excess Ca2+ also inhibit vesicle fusion, the inhibition exerted by GTPγS appears to proceed via a pathway independent of the divalent cation. The GTPγS-sensitive step in endocytic vesicle fusion is found to occur at a mechanistic stage prior to and distinct from the N-ethylmaleimide-sensitive step of the reaction. This situation permits the accumulation of a membrane vesicle intermediate in the presence of GTPγS; subsequent incubation of these vesicles with cytosol and GTP restores their fusion competence. Characteristics of in vitro endocytic vesicle fusion suggest that similarities exist with steps of the fusion mechanism involved with membrane traffic events of the secretory pathway.

A complex pattern of intracellular membrane vesicle traffic

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1 The abbreviations used are: CHO, Chinese hamster ovary; AvGal, avidin-β-galactosidase; B-Tf, biotin-transferrin; NEM, N-ethylmaleimide; NSF, NEM-sensitive factor; PNS, postnuclear supernatant; GTPγS, guanosine 5'-3-thiotriphosphate; ATPγS, adenosine 5'-3-thiotriphosphate; GDPβS, guanosine 5'-β-O-(2-thiodiphosphate); ADPβS, adenosine 5'-β-O-(2-thiodiphosphate); GTP-PCP, 5'-guanylylimidodiphosphate; GTP-PCP, a,β-methylene GTP; GTP, guanosine triphosphate; EGTA, ethyleneglycol(bis(oxyethylenenitrilo))tetraacetic acid; HEPES, 1 (2-hydroxyethyl) piperazineethanesulfonic acid; B IgG, biotinylated rabbit anti-goat IgG.
still remain to be characterized; for example, vesicles must somehow be targeted to and/or recognized by the appropriate receiving compartment, but it is as yet unclear which factors function in this capacity to direct vesicle traffic. It has been proposed that perhaps several small molecular weight GTP-binding proteins fill these roles in intracellular vesicle trafficking (22).

Although advances have been made in characterizing elements which mediate the cell-free stages of the secretory pathway, very little is known about in vitro endocytic events (1-5). It has been found by this lab (3) and others (4, 5) that in vitro fusion of endocytic vesicles requires at least one cytosolic NEM-sensitive factor. Recent experiments of Stahl and co-workers (23) have indicated the involvement of NSF, the Golgi factor, in the fusion of endocytic vesicles obtained from the J774 macrophage cell line. This group of investigators also has reported that endocytic vesicle fusion is sensitive to the nonhydrolyzable analog GTP? (24). Beyond these initial observations, however, little else is known about the relationship between these elements and the overall mechanism of the endocytic fusion reaction. The role of fusion-related proteins and of GTP-binding proteins is of great interest, as is the degree to which these functions are conserved across species lines. By utilizing the known characteristics of the cell-free fusion reaction, it is now possible to begin to investigate these relationships, as well as to study the mechanism of the interactions between endocytic vesicles in greater detail. We present here results which explore these events in consideration of the mechanistic elements known for other pathways of membrane traffic.

MATERIALS AND METHODS

Materials and Cell Culture—Avidin-@-galactosidase (AvpGal) conjugate and biotinylated rabbit anti-lgG (B-IgG) were purchased from Sigma and dialyzed against uptake buffer (10% Triton X-100, 1% sodium dodecyl sulfate, 50 mg/ml bovine serum albumin) prior to use. Biotinylated transferrin (B-Tf) was obtained from Ciba-Geigy. Human K562 cells were obtained from American Type Culture Collection; these cells, as well as CHO cells, were maintained in minimum Eagle's medium containing 10 or 7.5% fetal calf serum (GIBCO), respectively. K562 cells were propagated in suspension between 10^6 and 10^7 cells/ml; CHO cells were passaged with trypsin (3). Preparation of cell-extracts—Cells (K562 or CHO) were harvested by centrifugation and washed three times in phosphate-buffered saline on ice. In experiments involving the uptake of B-Tf, K562 cells were incubated for 30 min at 37 °C in serum-free medium to ensure that all exogenous transferrin from the cell surface. Cells were disrupted at 2-7 °C by homogenization with a French press (25). Cells were sedimented against the side of the tube: care was taken not to dislodge the particulate matter. A 20-μl aliquot of the membrane fraction was then removed directly from the Nycodenz fraction. Some particulate material was found sedimented against the side of the tube: care was taken not to dislodge this mass. The vesicle fraction was gently resuspended by pipetting it up and down. Equivalent activity was recovered for both the B-Tf- and AvpGal-containing fractions; the total activity recovered was about 50% of that applied to the gradient. This may be due in part to the effect of Nycodenz, which was found to inhibit the fusion assay when present at >2.5% final concentration. For this reason, aliquots obtained from the gradients were used in minimal volumes (5-10 μl) in the assay in order to reduce the amount of contaminating Nycodenz.

RESULTS AND DISCUSSION

In Vitro Endocytic Vesicle Fusion Is Supported by Heterologous Components—A cell-free system which utilizes both an avidin-linked fluid-phase marker, AvpGal, and a biotinylated receptor-associated probe, B-Tf, has recently been described (6). The fusion of vesicles containing these endocytotic markers is detected by the avidin-biotin association reaction when they are placed in a common compartment. The resulting complex, AvpGal:B-Tf, may be assayed by a convenient enzyme-linked immunosorbent assay technique (3, 6). In vitro vesicle fusion monitored by these means has been characterized in some detail. This reaction is both time- and temperature-dependent, requires the presence of ATP, and proceeds independent of the progressive acidification known to occur in endocytic compartments.

Characterization of the reconstituted system has also revealed that the activity of at least one cytosolic factor is required for endocytic vesicle fusion: this function is lost upon treatment of cytosol with the alkylation agent NEM (3-6). Results presented in Table I demonstrate that cytosol from different cell types can provide the NEM-sensitive function that is required for in vitro endocytic vesicle fusion. Furthermore, our data indicate that fusion between vesicles originating from different cell types can occur. NEM prepared from K562 cells which have internalized AvpGal can be employed in cell-free fusion assays in conjunction with either K562
TABLE I

Heterologous fusion assay

| Experimental conditions | Control fluorescence units | NEM-treated PNS fluorescence units |
|------------------------|---------------------------|-------------------------------|
| K562 AviGal PNS + K562 B-TF PNS | 121.2 | 27.1 |
| + 2 mg/ml K562 cytosol | 88.7 | 82.7 |
| + 2 mg/ml CHO cytosol | 11.0 | 2.3 |
| K562 AviGal PNS + CHO B-IgG PNS | 12.5 | 13.5 |
| + 2 mg/ml K562 cytosol | 12.5 | 13.5 |
| + 2 mg/ml CHO cytosol | 12.5 | 13.5 |

PNS from cells having internalized B-Tf or CHO PNS from cells having pinocytosed B-IgG. In the latter assay, the avidin-biotin association signal is detected by using microtiter wells coated with anti-IgG antibodies (instead of anti-Tf antibodies) to precipitate the complex developed upon vesicle fusion. Under both of these experimental conditions, treatment of the PNS fractions with NEM resulted in nearly complete loss of activity, as expected from previous results (3–6). However, activity could be restored with the addition of either K562 or CHO cytosol (final concentration, 2 mg/ml), regardless of the origin of endocytic vesicles. Therefore, the results shown in Table I demonstrate that not only can the NEM-sensitive cytosolic factor function with the fusion apparatus of both cell types, but also that the membrane proteins involved in this process can recognize and mediate fusion with endocytic vesicles derived from a heterologous tissue source.

The observations summarized in Table I suggest that the fusion machinery involved in endocytosis may be highly conserved between different cell types and species; indeed, yeast cytosol has been found to support the fusion between endocytic vesicles derived from a heterologous tissue source.

The role of NSF in mediating endocytic vesicle fusion may be more substantial in J774 cells than in the lines we use. The idea that separate NEM-sensitive elements may mediate different cell-free fusion events is not surprising, in view of the fact that NSF functions in the secretory pathway involving exocytic membrane traffic, whereas the fusion of endocytic vesicles initiates the internalization pathway of membrane traffic. It is apparent that some cellular factor may function to direct this flow of vesicular traffic through appropriate compartments; therefore, distinct elements may be involved in order to recognize and target vesicles to the correct destination. Further work is necessary to explore the possibility that a number of related NEM-sensitive elements may mediate individual fusion events, conferring specificity in the fusion steps involved in membrane traffic.

In Vitro Endocytic Vesicle Fusion Is Inhibited by Nonhydrolyzable Nucleotide Analogues—A role for GTP hydrolysis during intracellular membrane vesicle transfer has also recently been characterized. Melançon et al. (19) found that the nonhydrolyzable GTP analog, GTPγS, inhibits vesicle transport between Golgi cisternae. The in vitro recycling of mannose 6-phosphate receptors to the trans-Golgi network also appears to require GTP hydrolysis (9). Vesicle transfer between the endoplasmic reticulum and the cisternal Golgi compartment is also blocked by the presence of GTPγS (25). Yeast SEC23 mutant cells, which are deficient in endoplasmic reticulum to Golgi transfer, have been utilized in a cell-free system which reconstitutes this transport step; these studies have also demonstrated inhibition by GTPγS (11). These latter results are of particular interest since the yeast gene products SEC4 and YPT1 are implicated to act in the secretory pathway and are known to be GTP-binding proteins (13, 14, 20). Stahl and co-workers (24) have also recently characterized the inhibition of J774 endocytic vesicle fusion by GTPγS.

Considering the functional differences between the different cell types in their apparent use of cytosolic NEM-sensitive factor(s), does there exist a role for GTPγS-sensitive factor(s) in our cell-free assay?

Fig. 1 demonstrates that the fusion between endocytic vesicles from K562 cells is also sensitive to the nonhydrolyzable analog, GTPγS. Preliminary experiments indicated that the presence of GTPγS affects the rate of the fusion reaction and that the inhibition by GTPγS required the addition of excess cytosol, relative to the level of cytosol needed to yield maximal fusion activity in the untreated assay. It was also found to be necessary to dialyze both cytosol and PNS fractions for these studies, presumably in order to remove endogenous nucleotides. Typically, cytosol was preincubated for 10 min at 37 °C in the presence of GTPγS in order to incorporate the guanine nucleotide analog and was added to the reaction at a final concentration of 4–6 mg/ml. Membrane additions to the assay mixture were reduced by 80% in order to increase the cytosolemembrane ratio. Similar conditions are also required to follow the inhibition of intra-Golgi vesicular transfer by GTPγS (19).
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The nonhydrolyzable GTP analog, GTP\(_\gamma\)S, inhibits the cell-free fusion reaction. The dose-response curve for inhibition by GTP\(_\gamma\)S is presented in terms of the ratio of fusion with GTP\(_\gamma\)S, relative to control (in the absence of GTP\(_\gamma\)S). Cytosol (final concentration, 6 mg/ml) added to the assay mixture was preincubated at 37 °C for 10 min in the presence of GTP\(_\gamma\)S; concentrations of the GTP analog represent those in the final assay volume. K562 vesicle fusion reactions were performed at 37 °C, and products were analyzed using the enzyme-linked immunosorbent assay technique previously described (5, 6), except that y-galactosidase activity was measured over a 90-min period. Data is from three separate experiments, in which the average maximal fusion signal (in the absence of GTP\(_\gamma\)S) was 132 fluorescence units.

**Table II**

Inhibition of fusion reaction

Fusion assays were performed as described for Fig. 1. Cytosol added to the reaction mixture was preincubated at 37 °C for 10 min with analog additions as indicated. In reactions including ATP and GTP, 50 \(\mu\)M MgCl\(_2\) was also included in the assay mixture. Inhibition was measured at 5 \(\mu\)M GTP\(_\gamma\)S and ATP\(_\gamma\)S in order to observe any possible additive effects, which would be most pronounced at levels below the EC\(_{50}\) value of 10 \(\mu\)M.

| Experimental conditions | Fluorescence units |
|------------------------|--------------------|
| Control                | 107.9              |
| 5 \(\mu\)M GTP\(_\gamma\)S | 82.8              |
| 5 \(\mu\)M ATP\(_\gamma\)S | 87.4              |
| 5 \(\mu\)M GTP\(_\gamma\)S + 5 \(\mu\)M ATP\(_\gamma\)S | 82.3          |
| 5 \(\mu\)M GTP\(_\gamma\)S + 100 \(\mu\)M GTP | 111.8          |
| 5 \(\mu\)M ATP\(_\gamma\)S + 100 \(\mu\)M ATP | 94.8            |
| 5 \(\mu\)M ATP\(_\gamma\)S + 100 \(\mu\)M GTP | 99.2            |
| 5 \(\mu\)M ATP\(_\gamma\)S + 100 \(\mu\)M ATP | 109.2           |
| Other nucleotides      |                    |
| Control                | 83.3              |
| 10 \(\mu\)M GTP\(_\gamma\)S | 49.9              |
| 10 \(\mu\)M GMP-PNP     | 65.1              |
| 10 \(\mu\)M GDP\(_\gamma\)S | 69.3              |
| 10 \(\mu\)M ADP\(_\gamma\)S | 73.4              |

Combined data from a series of dose-response studies yield an EC\(_{50}\) of 10 \(\mu\)M GTP\(_\gamma\)S for inhibition of the fusion reaction (Fig. 1). This value is an order of magnitude higher than results reported for J774 endocytic vesicle fusion (24), in vitro transfer between endoplasmic reticulum and Golgi (25), and Golgi-to-Golgi transport (19), but it is comparable to determinations made for vesicle transport between prelysosomes and trans Golgi (9) and for in vitro SEC gene product dependent transfer in yeast (11). Unlike observations reported for several of these cell-free systems, we find that ATP\(_\gamma\)S, a nonhydrolyzable ATP analog, blocks endocytic vesicle fusion as well as GTP\(_\gamma\)S. However, as shown by results summarized in Table II, the effects of GTP\(_\gamma\)S and ATP\(_\gamma\)S are not additive. The concentrations employed in this experiment (5 \(\mu\)M) are lower than the determined value for EC\(_{50}\) (10 \(\mu\)M), in order that any possible synergism between ATP\(_\gamma\)S and GTP\(_\gamma\)S would be observed. Furthermore, the presence of excess ATP completely prevents inhibition by GTP\(_\gamma\)S, whereas the addition of excess ATP only partially alleviates the block by this nonhydrolyzable analog. Conversely, excess GTP does not completely relieve inhibition by ATP\(_\gamma\)S, while excess ATP does prevent the effects of ATP\(_\gamma\)S. The differential effects on the pattern of inhibition exerted by these nucleotide triphosphates are reliably reproducible, although the magnitude of their action is somewhat variable. The minor overlap in the ability of ATP and GTP to mitigate inhibition is most likely due to the presence of nucleotide kinases that influence the composition of the nucleotide pool available for relief of inhibition (25). It should be noted that it is necessary to add the nucleoside triphosphates during the preincubation of cytosol at 37 °C to prevent inhibition by the nucleotide analogs: the inhibitory effects of GTP\(_\gamma\)S and ATP\(_\gamma\)S are not reversed under these conditions by subsequent addition of either GTP or ATP. The fact that subsequent addition of ATP will not restore fusion after ATP\(_\gamma\)S pretreatment apparently explains why the effects with ATP\(_\gamma\)S can be observed at all, since the fusion assay typically contains ATP. The results presented in Table II imply that the hydrolysis of both ATP and GTP is required for in vitro endocytic vesicle fusion, albeit at distinct stages of the process. Finally, other nonhydrolyzable guanine nucleotide analogs were also found to block the in vitro fusion reaction, with the following order of potency: GTP\(_\gamma\)S > GMP-PNP > GDP-PCP. GDP\(_\gamma\)S and ADP\(_\gamma\)S also exhibited inhibitory effects, similar to results obtained for in vitro vesicle transport between Golgi cisternae (19).

The sensitivity of K562 vesicle fusion to ATP\(_\gamma\)S is yet another feature which distinguishes this mechanism from that utilized in vitro by J774 vesicles. Although our results demonstrating inhibition by GTP\(_\gamma\)S correlate fairly well with the data of Mayorga et al. (24), those researchers do not find that vesicle fusion is affected by the presence of ATP\(_\gamma\)S in their cell-free assay. Our result is, however, in accord with the requirement for ATP exhibited by both in vitro assay systems (3, 4, 6). For K562 cells, at least, the hydrolysis of ATP is probably involved in the fusion process, although the possibility that this apparent energy requirement might not exist in other cell types cannot be ruled out.

In Vitro Fusion Is Suppressed in the Presence of AlF\(_3\), GDP, and Mg\(^{2+}\)—Members of the class of regulatory GTP-binding proteins, known as G proteins, become activated by the presence of both A\(^{1+}\) and F\(^-\) ions, presumably by the formation of the anionic complex AlF\(_3\) (26). It has been postulated that when GDP is bound in the nucleotide-binding site of these proteins, AlF\(_3\) stimulates the otherwise inactive G protein by mimicking the terminal y-phosphate of GTP. In an analogous manner, GTP\(_\gamma\)S might chronically activate the GTP-binding factors involved in vesicle trafficking by preventing the deactivation process triggered by the hydrolysis of the bound nucleotide.

Effects of AlF\(_3\) have also been observed in the intracellular vesicle transport systems which are sensitive to GTP\(_\gamma\)S. In the presence of 50 \(\mu\)M A\(^{1+}\) and 5 \(\mu\)M F\(^-\), transfer between Golgi stacks is markedly reduced (19). Similar inhibitory effects are reported for vesicle transport between the endoplasmic reticulum and the Golgi apparatus (25). The presence of A\(^{1+}\) and F\(^-\) reduced transfer from yeast endoplasmic reticulum by 35% in a cell-free system (11). These additions are also found to inhibit J774 endocytic vesicle fusion (24). The addition of either ion alone had minimal effects in all of these in vitro systems.

The observation that endocytic vesicle fusion was inhibited by GTP\(_\gamma\)S, coupled with the observations made for steps of the secretory pathway, prompted us to study possible AlF\(_3\) effects in the K562 cell in vitro fusion assay. However, unlike the studies discussed above, it was observed that the additional presence of GDP and Mg\(^{2+}\) was required in order to
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witness inhibitory effects of AlF\(_{3}\) and F\(^-\). These results are summarized in Table III. In the presence of 100 μM Mg\(^{2+}\), the fusion assay is reduced to 80–90% of control values. A further reduction in fusion signal is observed when 100 μM GDP is also incorporated in the reaction mixture; however, no effect is observed in the absence of added Mg\(^{2+}\) (data not shown). When 50 μM AlF\(_{3}\) and 5 mM F\(^-\) are present along with GDP and Mg\(^{2+}\), the fusion reaction is inhibited 30–50%. Control experiments performed with Cl\(^-\) in place of F\(^-\) indicate that this effect is specific for the presence of the latter anion. The fusion reaction was not inhibited by 5 mM F\(^-\) alone, in the absence of AlF\(_{3}\), or in the absence of GDP and Mg\(^{2+}\) (results not shown). The effects of AlF\(_{3}\) and F\(^-\) could be reversed by the addition of 50 μM desferal and 100 μM GTP to the reaction mixture.

The data presented in Table III indicate that AlF\(_{3}\), in the presence of GDP and Mg\(^{2+}\), can suppress in vitro endocytic vesicle fusion. These results are compatible with the notion that, with the diphasate nucleotide, the anion complex substitutes for GTP in some as yet unknown binding site. It is interesting to note that GDP and Mg\(^{2+}\) both can potentiate AlF\(_{3}\) effects on members of the regulatory class of G proteins (26). It is not known whether AlF\(_{3}\) prevents the GTP hydrolysis required for endocytic vesicle fusion or whether it affects another element which may regulate this reaction. However, AlF\(_{3}\), in combination with GDP and Mg\(^{2+}\), only partially blocks the fusion reaction; the reason for this incomplete inhibition is not clear.

**Calcium Inhibits in Vitro Endocytic Vesicle Fusion**—Along with GTP, calcium has been implicated to be essential in vesicle transport between the endoplasmic reticulum and the Golgi apparatus. Beckers and Balch (25) have demonstrated that transport of protein in semi-intact cells is blocked by the total absence of calcium. In contrast, endocytic vesicle fusion does not appear to require calcium. As demonstrated by results shown in Fig. 2, the addition of 100 μM EGTA has little effect on the fusion reaction (open circle). The in vitro assay typically includes 20 μM EGTA, and the addition of excess calcium to about this level has little effect. However, above the chelating capacity of the EGTA present in the system, additional calcium inhibits endocytic vesicle fusion: at 100 μM Ca\(^{2+}\), the reaction is inhibited to background levels (Fig. 2, closed triangle). Beckers and Balch (25) also observe an inhibition of secretory vesicle transport with these levels of Ca\(^{2+}\) ion: although a minimal level of calcium is apparently required for vesicular transport from the endoplasmic reticulum to cis-Golgi cisternae, higher levels (>0.3 μM) inhibit this transport.

It should be noted that there is a calcium-dependent, ATP-independent component in the fusion of vesicles in the cell-free system derived from J774 cells (4). In contrast, we do not observe any fusion in the absence of ATP, regardless of the presence or absence of calcium, with vesicles derived from CHO or K562 cells. The requirement for calcium in steps involved in the secretion of proteins may be unique to the exocytic pathway, whereas the suppression of intracellular vesicle fusion by higher levels of the divalent cation may be a common feature shared between different pathways of membrane traffic. Future work will hopefully highlight other differences and similarities between exocytic and endocytic vesicular traffic in order to clarify mechanistic and regulatory factors which serve to distinguish the two pathways.

A possible reason for our observation of Ca\(^{2+}\) inhibition is that the effects of nonhydrolyzable guanine nucleotides are exerted in some Ca\(^{2+}\)-dependent manner. GTP binding proteins have been observed to regulate intracellular Ca\(^{2+}\) levels (27). Could GTP\(_{\gamma}\)S be inhibiting endocytic vesicle fusion by stimulating the release of Ca\(^{2+}\) from membranous stores, for example? As the inset of Fig. 2 demonstrates, the addition of EGTA to the reaction mixture does not suppress the inhibitory effects of the nonhydrolyzable GTP analog. This result indicates that the mechanism of inhibition by GTP\(_{\gamma}\)S probably does not involve alterations in the concentration of Ca\(^{2+}\) in the assay mixture. It is most likely that these agents inhibit endocytic vesicle fusion via different and distinct mechanisms. Along these lines, Beckers and Balch (25) have distinguished that the Ca\(^{2+}\)-requiring step in vesicle transfer between CHO endoplasmic reticulum and Golgi occurs after the GTP\(_{\gamma}\)S-sensitive step. In this regard, it should be noted that Baker et al. (14) have also recently demonstrated that the YPT1 gene product and Ca\(^{2+}\) act at distinct steps during in vitro transport between the endoplasmic reticulum and Golgi in yeast.

**GTP Hydrolysis Is Required Prior to NEM-sensitive Stage in Vesicle Fusion**—The results discussed thus far suggest that the GTP\(_{\gamma}\)S-sensitive factor affecting endocytic vesicle fusion is most likely a cytosolic factor. The relevant evidence for this conclusion is that (a) the concentration of cytosol needed to exhibit the GTP\(_{\gamma}\)S effect is in excess of the 2 mg/ml concentration that maximally stimulates vesicle fusion, and (b) it is necessary to preincubate this cytosolic fraction with GTP\(_{\gamma}\)S at 37 °C to provide the inhibitory effect. One puzzling feature of the results presented in Fig. 1 is that the apparent EC\(_{50}\) for inhibition was quite high, about 10 μM: this is an

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**TABLE III**

Effects of AlF\(_{3}\), GDP, and Mg\(^{2+}\)

Fusion assays were performed using cytosol which had been preincubated for 10 min at 37 °C in the presence of the agents listed. For further description of experimental details, see text and conditions described for Fig. 1.

| Experimental conditions | Fluorescence units |
|------------------------|--------------------|
| Control                | 61.3               |
| 100 μM MgCl\(_{2}\)     | 52.4               |
| 100 μM GDP + 100 μM MgCl\(_{2}\) | 43.6 |
| 50 μM AlNH\(_{4}\)(SO\(_{4}\)) + 5 mM NaF + 100 μM GDP + 100 μM MgCl\(_{2}\) | 29.8 |
| 50 μM AlNH\(_{4}\)(SO\(_{4}\)) + 5 mM NaCl + 100 μM GDP + 100 μM MgCl\(_{2}\) | 42.7 |

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**FIG. 2.** Calcium inhibits the in vitro fusion of endocytic vesicles. The levels of endocytic vesicle fusion occurring at 37 °C with increasing doses of exogenously added CaCl\(_{2}\) are presented (○), with comparison to background fluorescence from a control incubation performed at 4 °C (●). Vesicle fusion was assayed in the presence of 10 μM ruthenium red; this reagent inhibits mitochondrial calcium flux (32), preventing any sequestration of the added calcium in mitochondria during the incubation. At this level, the ruthenium red did not interfere with the in vitro assay. In some reactions, 100 μM EGTA (O) was added, beyond the 20 μM level normally present, to ensure effective chelation of any endogenous free calcium. Inset, results (in fluorescence units) of in vitro fusion experiments performed in the presence or absence of 100 μM GTP\(_{\gamma}\)S and/or EGTA, as indicated.
order of magnitude higher than that reported for other vesicular transport systems, for example (19, 24, 25). Is it possible that excess cytosol and GTPyS are necessary in order to compete with an unaffected counterpart to the guanine nucleotide-binding factor, which is already bound to the vesicle surface? The fact that limiting amounts of membrane are also required to observe this inhibitory effect supports this idea. However, in order to explore this possibility, it would first be necessary to maintain the endocytic vesicles at 37 °C in order to incorporate GTPyS into the membrane-associated guanine nucleotide-binding sites. This is problematic, since under these conditions, we have observed that vesicles will fuse into an endosome-like compartment (6), thereby depleting the supply of fusion-competent vesicles loaded with our probes. As a result, the overall signal will be diminished, making it difficult to distinguish the direct effects of the guanine nucleotide.

One way to avoid this difficulty is to temporarily block vesicle fusion with NEM. As demonstrated by the results of Table I, alkylation with NEM halts vesicle fusion by modifying cytosolic proteins critical for fusion, subsequent addition of unmodified cytosol (after quenching the NEM) restores fusion activity. In order to study the effects of GTPyS in this situation, it is necessary that the NEM-sensitive step occur after the nonhydrolyzable nucleotide acts to block the reaction, which is demonstrated by the results shown in Table IV. In these experiments, PNS fractions containing the endocytic vesicles are first treated with NEM at 4 °C, mixed with NEM-treated cytosol (which had been preincubated in the presence or absence of GTPyS), and preincubated at 37 °C. Vesicles cannot progress to the point of fusion under these conditions, but would be expected to accumulate at an intermediate stage, determined by which critical factor (NEM sensitive or GTPyS sensitive) was inhibited in the reaction. Additional cytosol, also preincubated in the presence or absence of GTPyS, is subsequently added to restore the NEM-modified factor. If the point of action of the GTPyS-sensitive factor precedes that for the NEM-sensitive factor, the accumulated intermediate should have bypassed the GTPyS-sensitive point, unless nonhydrolyzable analog was present during the preincubation of the vesicles. The subsequent addition of unmodified cytosol would then restore vesicle fusion, even if it was pretreated with GTPyS. This scenario is indeed observed: GTPyS-pretreated cytosol restores fusion nearly as well as untreated cytosol (Table IV), unless GTPyS was also present during the preincubation step. Similar effects are observed when the effects of ATPyS-treated cytosol are compared when it is added during or after the preincubation (Table IV). These results are compatible with a sequence of events during the vesicle fusion process which places the point of action of both the ATP and GTP nucleotides prior to the NEM-sensitive stage of the mechanism. Other experiments have indicated that GTPyS- and ATPyS-sensitive steps are distinct (see discussion regarding Table II).

If a membrane-bound GTP-binding factor is the primary target for the inhibition induced by GTPyS, then the preincubation of NEM-treated vesicles with GTPyS should directly affect that factor. This should produce a shift in the dose-response curve for GTPyS, relative to that seen when GTPyS-treated cytosolic protein is added to vesicles not previously exposed to the analog. As Fig. 3 demonstrates, this is indeed the case: vesicles blocked with NEM, and first exposed to GTPyS at 37 °C, display a greater sensitivity to the nonhydrolyzable analog, with an apparent EC50 of 1 μM. For comparison, the dose-response curve is also shown for vesicles which are NEM-treated, but not exposed to GTPyS during this preincubation (open circles). This shift in the EC50 value correlates quite well with results from other in vitro fusion systems (19, 24, 25). Furthermore, this result also supports the notion that in the absence of the preincubation at 37 °C, a competition between the GTPyS-treated component(s) and its native counterparts occurs in association with the membrane component of the reaction.

### Identification of a Reaction Intermediate Blocked by the Presence of GTPyS

The results described in Table IV and Fig. 3 indicate that upon incubation at 37 °C, a reaction intermediate is produced which has advanced past the point in the fusion mechanism which is sensitive to GTPyS. One interpretation of our results is that the presence of GTPyS induces the binding of some cytosolic factor(s) to the membrane surface, which then somehow inhibits subsequent fusion of the vesicle. If this is so, then a vesicle fusion intermediate might be isolable in the presence of GTPyS. Our cell-free assay involves the use of crude PNS fractions, which contain both cytosol and membrane components required for the fusion process. As a result, such an intermediate could be present in these fractions, and therefore, it would be possible to isolate and purify it. This intermediate could then be studied in greater detail to understand its role in the fusion process.

### Table IV

| Preincubation conditions | Fusion assay conditions | Fluorescence units |
|-------------------------|------------------------|--------------------|
| Untreated               | Control                | 156                |
| +GTPyS                  | +GTPyS                 | 124                |
| +ATPyS                  | +ATPyS                 | 127                |
| +GTPyS                  | Control                | 82                 |
| +ATPyS                  | +ATPyS                 | 69                 |
| +GTPyS                  | Control                | 80                 |
| +ATPyS                  | +GTPyS                 | 75                 |
| +ATPyS                  | Control                | 64                 |

For further details, see text.
endocytic vesicle fusion. The major problem in the identification of such a reaction intermediate stems from the difficulties involved in isolating the vesicle fraction such that the integrity of vesicles is not affected.

Isolation of J774-derived vesicles for such purposes (28) has involved simply sedimenting the vesicle fraction by centrifugation. However, the cell-free fusion system based on the J774 vesicles involves the use of two receptor-associated probes, which will remain membrane-bound even if this isolation method incurs substantial levels of vesicle breakage and rescaling. One of our probes, B-Tf, remains receptor-associated in an analogous manner. However, AvpGal, a fluid-phase marker, will be lost if the vesicles break and rescale. We find that this loss does indeed occur when our vesicles are isolated by sedimentation and resuspension: the signal of the B-Tf-containing vesicle population is retained, while a substantial portion of the signal from AvpGal-containing fractions is lost during or after sedimentation (results not shown). We also find that under these conditions, large membrane aggregates are formed during resuspension, such that a second, low-speed centrifugation step (analogous to the postnuclear supernatant centrifugation) is necessary.

For these reasons, we sought to develop a technique in which the membrane fraction may be isolated in a gentle, nondisruptive manner. This was accomplished by using a Beckman Airfuge and gently underlaying the postnuclear supernatant fraction with 2.5 volumes of a 0.25 M sucrose solution, which in turn was underlayered with isotonic Nycodenz as described under "Materials and Methods." The formation of the Nycodenz step gradient cushions the vesicle fraction as it sediments, while soluble markers are separated in the upper part of gradient. The membrane "pellet," isolated on top of the Nycodenz cushion, is found to resuspend quite readily and does not appear to contain large sedimentable aggregates.

Using this step gradient sedimentation technique, we are able to isolate an active membrane fraction: the functional activities of both B-Tf- and AvpGal-containing fractions are equally recovered. This observation implies that the integrity of the endocytic vesicles is maintained under These conditions of centrifugation. Fig. 4 demonstrates that this also allows the isolation of the GTP-γS-sensitive reaction intermediate. These PNS fractions are first blocked by alkylation with NEM and then preincubated in the presence or absence of GTP-γS, as indicated. The presence of GTP-γS is found to exert its effect on the membrane component of the PNS, since the activity of vesicles isolated under these conditions is inhibited, even when untreated cytosol is added to the assay for fusion activity. However, the activity of the GTP-γS-blocked intermediate can be partially recovered after isolation on the gradient. This second incubation step is also performed in the presence of NEM-treated cytosol, again to block fusion activity. In the absence of GTP, no restoration of activity occurs, but in its presence, about 68% of control activity can be recovered. These results are consistent with the idea that GTP is now able to replace the GTP-γS in the factor bound to the vesicle surface, and thus can relieve the inhibition of vesicle fusion.

Our results with GTP-γS treatment of endocytic vesicles fit nicely with the models proposed for the role of GTP-binding factors in vesicle transport in the secretory pathway. It appears likely that the sensitivity of GTP-γS witnessed in all these vesicle transfer reactions may be ascribed to a common stage in the membrane fusion process. Rothman and colleagues (15-19) have characterized the intra-Golgi vesicular transfer in some detail. They have found that GTP-γS exerts analogous effects on the secretory pathway; that the hydrolysis of GTP is required prior to action of NSF (17). In fact, they have been able to isolate an intermediate vesicle fraction, since in the presence of GTP-γS, a class of coated vesicles accumulates in the cell-free system reconstituting intra-Golgi transfer (29). Rothman's laboratory has been able to identify other stages, including the uncoating of vesicles prior to the membrane fusion step completing the transport process (17).

Is GTP hydrolysis required for the "uncoating" of vesicles, and is this a limiting step for the fusion process? The Golgi-associated vesicles isolated by Rothman and co-workers do not contain clathrin, although other vesicle-associated proteins may have a function analogous to that of clathrin (29). During the initial stages of the endocytic pathway, vesicles are formed with clathrin coats, which are lost soon after internalization (31). If GTP hydrolysis is associated with the removal of the clathrin coat, the thesis may be put forward that this is a prerequisite step for the fusion of endocytic vesicles. Thus, the presence of GTP-γS in the cell-free system would prevent the removal of the clathrin lattice, thereby blocking membrane fusion. Due to the limitations of the in vitro system, the characteristics of the vesicle population which participates in the fusion reaction are not known; future work exploring the composition of the components of the fusion reaction should greatly aid in our understanding of the reaction mechanism and the role of GTP hydrolysis. The fact that we are now able to isolate an active vesicle fraction by using the step gradient approach opens the possibility of completing morphological studies in conjunction with our investigation of the cell-free system.

The requirement for GTP hydrolysis during endocytic vesicle fusion also prompts questions about the nature of the GTP-γS-sensitive factor. Other classes of proteins which contain GTP-binding sites, and which may act as GTPases,
include G proteins involved with signal transduction, initiation and elongation factors of protein synthesis, tubulins, and proteins related to the ras oncogene (27). Interestingly, the YPT1 gene product has been identified as a member of the latter class of GTP-binding proteins (30) and has been found associated with putative Golgi structures in yeast (21). A mammalian homolog of this protein has also been localized to the Golgi apparatus. A second GTP-binding protein, the SEC4 gene product, has a functional role in the yeast exocytic pathway and is found on the surface of secretory vesicles (20). The YPT1 gene product has up to 70% homology with YPT1 (30). These small (20-25 kDa) proteins perhaps constitute a new class of GTP-binding factors functioning in intracellular vesicular transport. It is not yet clear whether the GTPyS-sensitive factor involved in endocytic vesicle fusion is also an element of this class.

Bourne (22) has proposed that perhaps the GTPyS-sensitive elements of vesicle traffic are involved in targeting and recognition function. Such a factor would bind to or interact with factors which associate with the vesicle surface. Once the vesicle is targeted to the appropriate cellular destination, GTP hydrolysis might signal this recognition event. In this manner, an endocytic vesicle could be directed to fuse with endosomal domains, while an endoplasmic reticulum-derived vesicle could simultaneously be targeted to the Golgi. It is possible, then, that a number of small-molecular-weight GTP-binding elements could function as the "traffic signals" for vesicle localization within the cell.

Summary—Fig. 5 depicts the sequence of events during the in vitro endocytic vesicle fusion described above. An early stage of the endocytic pathway involves the hydrolysis of GTP, perhaps due to the involvement of a ras-like protein in vesicle targeting. In this way, GTPyS, by preventing hydrolysis, might perhaps block the recognition step which permits fusion to proceed. Other activities one could envision for such factors would be in the uncoating of vesicles prior to the fusion reaction itself. This possibility is prompted by analogy with the results reported by Rothman and co-workers (17), which demonstrate a "coated" intermediate in vesicular transfer between Golgi stacks that is produced by exposure to GTPyS.

A subsequent stage of vesicle traffic involves cytosolic NEM-sensitive factor(s). Thus, alkylation by NEM provides a convenient means to study individual steps of the fusion mechanism, particularly those involving GTP hydrolysis. Our results demonstrate the ability to capture the GTPyS-blocked intermediate, which is produced during incubation of the NEM-treated fractions at 37°C, and which corresponds to Stage II of the fusion mechanism (Fig. 5). The isolation of this membrane complex is also enabled by the use of the step gradient technique described here, which may prove highly applicable in future in vitro studies involving the small, limited volumes that are currently used. One feature revealed by these studies is that the function of this reaction intermediate may be recovered upon incubation with cytosol and GTP. Currently, we are exploring the parameters which produce this intermediate vesicular species during the fusion mechanism, as well as investigating its precise role in the cell-free reaction.

Finally, further stages of the endocytic vesicle fusion mechanism require other factors, particularly membrane-associated proteins, since it has been found that even mild trypsinization of the vesicle surface will disrupt the in vitro reaction (3, 4). Our results indicate that the membrane-associated fusion machinery may be fairly well conserved, since vesicles derived from K562 cells are able to fuse with vesicles obtained from CHO cells. Events subsequent to vesicle fusion also are witnessed in the in vitro system, including endosomal functions involved in the sorting and sequestration of internalized proteins.
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material. Given the prevalence of GTPyS-sensitive steps in vesicle traffic within the cell, it is interesting to speculate that perhaps these steps also involve GTP-binding factors, or other regulatory factors, which serve to target sorted fractions to the appropriate destination within the cell.

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