tation on a Long-Evans background and maintained pathogen-free in a barrier facility. Males were diagnosed as carriers at 12 months of age after we performed unilateral nephrectomy to detect tumors histologically in the excised kidney. Heterozygous male rats carrying the Eker mutation from a carrier breeding colony were bred to female rats of Eker stock, undiagnosed as to carrier status. Wild-type animals were bred from second generation tumor-free homozygous wild-type animals from Eker stock, maintained in a wild-type breeding colony.
16. The 16-week-old male and female animals were placed on a protein-free, high-carbohydrate diet for 96 hours before intraperitoneal injection of either saline diluent or 30 mg of DMN per kilogram of body weight dissolved in saline. All rats were observed for 8 months after injection, at which time all rats were euthanized and necropsied. A midsagittal section of each kidney was examined microscopically for quantitation of histological type, size, and number of renal neoplasms. At necropsy we examined all tissues and we noted no metastatic lesions by macroscopic observation or by microscopic examination of the lung and liver of any of the animals.
17. The carrier F1 group included both heterozygotes carrying the mutation and homozygous wild-type animals. Therefore, the impact of the Eker gene mutation on carcinogen susceptibility is in all likelihood an underestimate of the effect of this gene mutation.
18. D. R. Dietrich and J. A. Swenberg, *Mutat. Res.* **248**, 239 (1991).
19. Late Effects Study Group is an international consortium of pediatric oncology centers whose members study the long-term effects of treatment on children with cancer.
20. M. A. Tucker *et al.*, *J. Natl. Cancer Inst.* **78**, 459 (1987); A. T. Meadows, *Bull. Cancer* **75**, 125 (1988); *J. Clinical Oncol.* **3**, 532 (1985).
21. We thank A. Knudson, C. Barrett, and M. Andersen for discussions during the progress of these experiments. Founder rats carrying the Eker mutation were the gift of A. Knudson (Fox Chase Cancer Center, Philadelphia, PA). All animals were housed and treated in accordance with PHS policy following the guide for the Care and Use of Laboratory Animals (NIH no. 85-23), in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

12 November 1991; accepted 17 January 1992

Evidence of a Role for Heterotrimeric GTP-Binding Proteins in Endosome Fusion

MARIA I. COLOMBO, LUIS S. MAYORGA, PATRICK J. CASEY, PHILIP D. STAHL

Guanosine triphosphate (GTP)–binding proteins are required for intracellular vesicular transport. Mastoparan is a peptide component of wasp venom that increases nucleotide exchange in some classes of Gα subunits of regulatory heterotrimeric GTP-binding proteins (G proteins). Mastoparan and other compounds that increase nucleotide exchange by G proteins inhibited endosome fusion in vitro and reversed the effects of guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S), a nonhydrolyzable GTP analog. Addition of βγ subunits of G proteins to the fusion assay antagonized the stimulatory effect of GTP-γ-S, confirming the participation of G proteins. These results indicate that GTP-binding proteins are required for endosome fusion and in particular that a G protein is involved. Given the function of G proteins in signal transduction, these findings may provide insight into the mechanism by which endosomal vesicles become competent for fusion after their formation at the cell surface.

EVIDENCE THAT GTP-BINDING PROTEINS take part in vesicular transport comes from in vitro assays that reconstitute fusion between intracellular compartments and from the analysis of secretion-deficient mutants of yeast (*1, 2*). Non-hydrolyzable GTP analogs (such as GTP-γ-S) inhibit several steps of the secretory pathway reconstituted in vitro (*3*) and stimulate secretion in some preparations (*4*). In vitro endosome fusion can be stimulated or inhibited by GTP-γ-S, depending upon the assay conditions (*5, 6*). GTP-binding proteins behave as molecular switches that rapidly change from an active GTP-bound form to an inactive GDP-bound form. These proteins are regulated by interactions with other proteins that promote nucleotide exchange and GTP hydrolysis (7). Until recently, only monomeric GTP-binding proteins had been implicated in intracellular transport, such as the proteins YPT1, SEC4, ARF, and SAR1, which are required for secretion in yeast (*1, 2*). Moreover, several small GTP-binding proteins of the Rab family are localized on endosomal and secretory vesicles from mammalian cells (*8*). Rab 5 functions in fusion between early endosomes (*9*), and Rab 4 is apparently associated with a population of early endosomes that participate in transferrin receptor recycling (*10*). However, there are some indications that heterotrimeric GTP-binding proteins (G proteins) may also function in intracellular transport. Aluminum fluoride, which can activate G proteins but not monomeric GTP-binding proteins (*11*), can affect transport in the secretory (*12*) and endocytic pathways (*5, 13*). Also, the presence of Gα subunits of G proteins in specific intracellular compartments (*14*) and in rat liver fractions (*15*) suggests a function for G proteins in membrane trafficking. The experiments presented in this paper suggest a role for one or more G proteins in regulating endosome fusion.

G proteins are activated by ligand-stimulated receptors (*16*). Mastoparan is an amphiphilic tetradecapeptide toxin from wasp venom that accelerates nucleotide exchange in some α subunits (preferentially

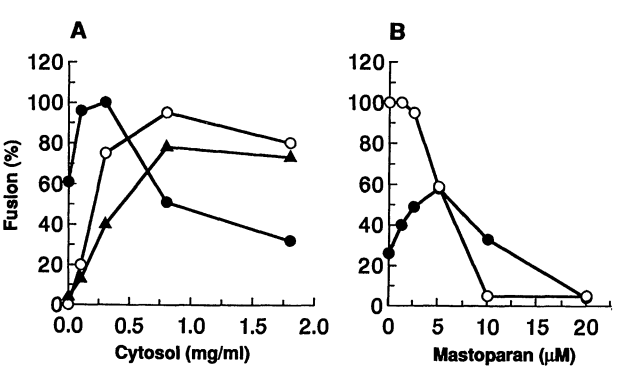

Fig. 1. Regulation of endosome fusion by mastoparan. Endosome fusion was assessed in a cell-free system with mannosylated antibody to dinitrophenol (DNP) and DNP-β-glucuronidase as probes (*27*). (**A**) Endocytic vesicles containing fusion probes were mixed with increasing concentrations of cytosol in the presence of 20 μM GTP-γ-S (●), 20 μM GTP-γ-S and 10 μM mastoparan (▲), or without additions (○). (**B**) Effect of increasing concentrations of mastoparan in the presence of GTP-γ-S (20 μM) at two different cytosolic protein concentrations, 0.1 mg/ml (○) and 2 mg/ml (●). Cytosol was prepared as described (*6*). Protein concentration was determined after gel filtration by the Bradford method (*28*). Incubations were carried out for 45 min at 37°C and the assay was stopped by cooling at 4°C. Values are expressed as percentages of the maximum fusion in the experiment.

M. I. Colombo, L. S. Mayorga, P. D. Stahl, Department of Cell Biology and Physiology, Washington University, School of Medicine, St. Louis, MO 63110.
M. I. Colombo and L. S. Mayorga, Instituto de Histologia y Embriologia, Facultad de Ciencias Medicas, Universidad Nacional de Cuyo (CONICET), Mendoza 5500, Argentina.
P. J. Casey, Section of Cell Growth, Regulation and Oncogenesis and Department of Biochemistry, Duke University Medical Center, Durham, NC 27707, USA.

α_o and α_i) of G proteins by a mechanism that is virtually identical to that of agonist-bound receptors (*17, 18*). GTP-γ-S activates GTP-binding proteins by holding such proteins in their active GTP-bound conformation. GTP-γ-S has a dual effect on endosome fusion in a cell-free system (*6*). It stimulates vesicle fusion under assay conditions containing low concentrations of cytosolic proteins and inhibits fusion in the presence of high concentrations of cytosol. Mastoparan reversed both activation and inhibition of fusion produced by GTP-γ-S (Fig. 1A). Both effects of GTP-γ-S were reversed by similar concentrations of mastoparan (Fig. 1B). Concentrations of mastoparan greater than 20 μM inhibited fusion at all concentrations of cytosol (Fig. 1B). This suggests that a mastoparan-sensitive GTP-binding protein functions in endosome fusion. This GTP-binding protein is probably a G protein, because mastoparan is much more active on this class of GTP-regulated proteins. Mastoparan stimulates the GTPase activity of G_o or G_i by 15- to 20-fold with an effective concentration (EC_{50}) of 5 to 10 μM (*18*). Mastoparan can also interact with the small GTP-binding proteins Rho and Rac (*19*). However, the half-maximal and maximal concentrations of mastoparan required for these effects are 20 μM and 100 μM, respectively, and the maximal stimulation is about fourfold. In our assay, the concentrations required are considerably lower (5 and 10 μM, respectively) (Fig. 1B). To evaluate the effect of mastoparan on endosome integrity, endosomes were loaded with three sizes of gold particles and incubated at low cytosol concentrations in the presence of GTP-γ-S and mastoparan (10 μM). Vesicle morphology, as revealed by electron microscopic analysis, was not affected, but endosome aggregation (*20*) and fusion were inhibited (*21*).

Some other amphiphilic peptides also activate nucleotide exchange in Gα subunits (*18*). Mellitin and HR1 (*18, 22*), two peptides with these properties, reversed the effect of GTP-γ-S on endosome fusion. The mean EC_{50}'s for mellitin and HR1 were 5 μM and 20 μM, respectively. HR2, a mast cell degranulating peptide not structurally related to mastoparan (*22*), had no effect. Mas17, a mastoparan analog that does not promote nucleotide exchange (*18*), did not reverse the effect of GTP-γ-S on endosome fusion. Hydrophobic amines such as benzalkonium chloride and methylbenzetonium, which enhance nucleotide exchange in G proteins (*18*), also inhibited fusion at concentrations similar to those that increase nucleotide exchange. Like mastoparan, benzalkonium chloride reversed the inhibitory effect of GTP-γ-S at high cytosol concentrations (*21*). The EC_{50}'s for benzalkonium chloride and methylbenzetonium were 20 μg/ml and 35 μg/ml, respectively. Triton X-100 was inactive at the same concentration, indicating that a detergent effect was not responsible for the inactivation (*21*). Moreover, as assessed by electron microscopy, vesicles remained intact in the presence of benzalkonium chloride (100 μg/ml). Both vesicle aggregation and fusion were inhibited by this amine (*21*).

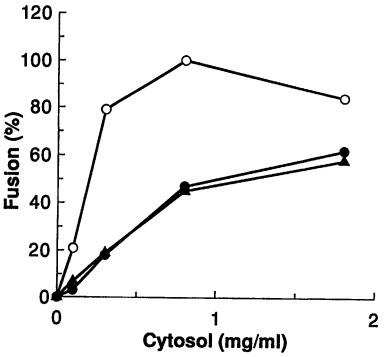

Fig. 2. Effect of mastoparan and GDP-β-S on endosome fusion in the absence of GTP-γ-S. Endosome fusion as described in Fig. 1 was assayed at different concentrations of cytosolic proteins in the presence of 10 μM mastoparan (●), 1 mM GDP-β-S (▲), or without any addition (○). Values are expressed as percentages of the maximum fusion obtained.

The effect of GTP-γ-S on endosome fusion might be due to the activation of a GTP-binding protein that is either part of the fusion machinery or involved in the regulation of the process. At high concentrations, mastoparan (Fig. 1B) not only reversed the effect of GTP-γ-S but also inhibited fusion completely. Moreover, mastoparan inhibited fusion in the absence of GTP-γ-S, suggesting that the activity of a GTP-binding protein, perhaps trimeric, is required for the fusion process (Fig. 2) At higher cytosol concentrations (1.5 mg/ml), mastoparan was less effective in inhibiting fusion, and higher concentrations of the peptide (>50 μM) were required (*21*).

To further explore the possibility that the activity of a GTP-binding protein is an absolute requirement in the fusion process we assessed the effect of guanosine 5′-*O*-(2-thiodiphosphate (GDP-β-S). GDP-β-S is a GDP analog that cannot be phosphorylated (*23*); it inactivates GTP-binding proteins by holding these proteins in their GDP-bound, inactive form. The inhibition of fusion observed when GDP-β-S was included in the reaction mixture (Fig. 2) suggests that a GTP-binding protein is part of the fusion machinery or is an obligatory element for its assembly.

Activation of Gα subunits of G proteins can be specifically blocked by the addition of the βγ subunit complex (Gβγ). To confirm the participation of G proteins in endosome fusion, we tested the effect of Gβγ on fusion stimulated by GTP-γ-S. Vesicles were incubated with a low concentration of cytosol for 10 min at 37°C in the presence of increasing concentrations of Gβγ before GTP-γ-S was added. Purified Gβγ from bovine brain inhibited fusion stimulated by GTP-γ-S. This inhibition was abrogated by heat denaturation of Gβγ (Fig. 3A). On the other hand, incu-

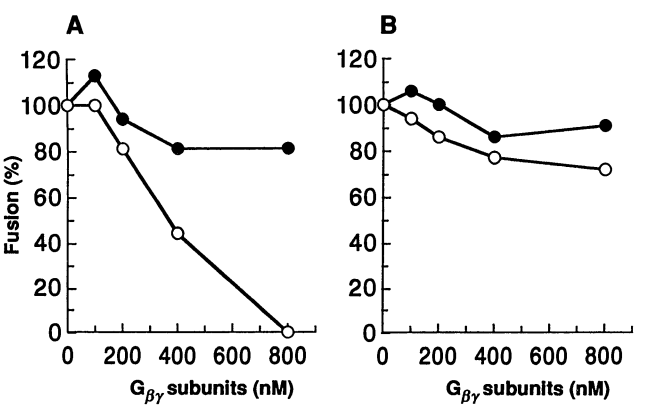

Fig. 3. Inhibition of GTP-γ-S–stimulated fusion by purified Gβγ subunits of G proteins. Endocytic vesicles were resuspended in fusion buffer containing a low concentration of cytosol (0.2 mg/ml). (**A**) The mixture was incubated with increasing concentrations of untreated (○) or heat-inactivated (●) Gβγ subunits. After 10 min at 37°C, 20 μM GTP-γ-S was added, and the mixture was incubated for 45 min. (**B**) The mixture was incubated for 10 min at 37°C with 20 μM GTP-γ-S. Increasing concentrations of untreated (○) or heat inactivated (●) Gβγ subunits were then added, and incubation was continued for 45 min. Values are expressed as percentages of the fusion obtained with the addition buffer alone. Gβγ subunits were purified from bovine brain as described (*29*). The purified proteins (2.5 mg/ml) were present in 50 mM tris-Cl (pH 8.0) containing 1 mM EDTA, 1 mM DTT, sodium cholate (1%), 50 mM NaCl and AlF_4 (6 mg $MgCl_2$, 10 mM NaF, 20 μM $AlCl_3$). To eliminate the AlF_4, EDTA was added to give a final concentration of 10 mM. Before adding Gβγ subunits to the fusion reaction (1/10 volume), the stock solution was diluted with homogenization buffer containing 1 mM EDTA and cholate (0.1%) to keep the proteins in solution. The same buffer was used as a control; the detergent had a slightly inhibitory effect at the concentrations used (0.01%). The purified Gβγ subunits were inactivated by boiling the samples 10 min. The fusion assay was done in silconized tubes to prevent absorption of Gβγ to the tubes.

bation of the vesicles and cytosol with GTP-γ-S before the addition of Gβγ produced a less marked effect (Fig. 3B), consistent with the reduced interaction of Gβγ with Gα subunits bound to GTP-γ-S. Like mastoparan, Gβγ partially inhibited fusion in the absence of GTP-γ-S (*21*).

The function of G proteins in signal transduction has been well characterized. However, recent studies implicate this class of GTP-binding proteins in vesicular transport. $G\alpha_{i\text{-}3}$ regulates transport of proteoglycans through the Golgi (*24*). Aluminum fluoride, which activates G proteins (*11*), blocks in vitro transport along the secretory (*12*) and endocytic pathways (*5, 13*). However, aluminum fluoride effects alone are difficult to interpret because fluoride is also known to inhibit many cellular phosphatases. Our observations that mastoparan reverses both the activation of fusion by GTP-γ-S at low cytosol concentrations and the inhibition of endosome fusion by GTP-γ-S at high cytosol concentrations suggests that a G protein is indeed involved in endosome fusion. Furthermore, our in vitro assay was specifically inhibited by the addition of Gβγ subunits. Our data do not allow us to decipher whether one or more G proteins may be regulating fusion. The effects of mastoparan and Gβγ may seem paradoxical, because mastoparan is expected to increase nucleotide exchange of G proteins whereas Gβγ has the opposite effect. However, a speculative model that includes two G proteins can resolve this paradox. In this model the activation of one G protein by mastoparan induces the dissociation of its Gβγ subunit. The resultant increase in the concentration of free Gβγ would favor binding of Gβγ to the second (stimulatory) G protein and block the function of that G protein. A similar subunit exchange model has been proposed to explain the roles of G_i and G_s in the regulation of adenylyl cyclase (*16*). Factors that participate in the vesicular transport among Golgi stacks interact with membranes by means of GTP-binding proteins. The release of the coat protein β-COP by brefeldin A is inhibited by GTP-γ-S and aluminum fluoride (*25, 26*). GTP-γ-S induces the binding of β-COP and ARF, a small GTP-binding protein, to membranes, and Gβγ also blocks these binding events (*26*). These data indicate that factors related to vesicular transport associate with membranes by means of GTP-binding proteins, some of which seem to be G proteins. We have suggested that GTP-γ-S mediates the irreversible binding of factors to endosomal membranes necessary for priming before fusion (*6*). Because the effect of mastoparan is competed by excess cytosol, it appears that one or more G proteins may influence the binding of cytosolic factors to endosomal membranes.

REFERENCES AND NOTES

1. W. E. Balch, *Trends Biochem. Sci.* **15**, 473 (1990).
2. M. F. Rexach and R. W. Schekman, *J. Cell Biol.* **114**, 219 (1991).
3. W. E. Balch, *J. Biol. Chem.* **264**, 16965 (1989); Y. Goda and S. R. Pfeffer, *FASEB J.* **3**, 2488 (1989).
4. R. D. Burgoyne, *Nature* **328**, 112 (1987).
5. L. S. Mayorga, R. Diaz, P. Stahl, *Science* **244**, 1475 (1989).
6. L. S. Mayorga, R. Diaz, M. I. Colombo, P. Stahl, *Cell Regulation* **1**, 113 (1989).
7. H. R. Bourne, D. A. Sanders, F. McCormick, *Nature* **349**, 117 (1991).
8. P. Chavrier, R. G. Parton, H. P. Hauri, K. Simons, M. Zerial, *Cell* **62**, 317 (1990).
9. J. P. Gorvel, P. Chavrier, M. Zerial, J. Gruenberg, *ibid.* **64**, 915 (1991).
10. P. van der Sluijs *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6313 (1991).
11. R. Kahn, *J. Biol. Chem.* **266**, 15595 (1991).
12. P. Melancon *et al.*, *Cell* **51**, 1053 (1987).
13. M. Wessling-Resnick and W. A. Braell, *J. Biol. Chem.* **265**, 16751 (1990).
14. L. Ercolani *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4635 (1990).
15. N. Ali, G. Milligan, H. Evans, *Biochem. J.* **261**, 905 (1989).
16. L. Stryer and H. R. Bourne, *Annu. Rev. Cell Biol.* **2**, 391 (1986); A. G. Gilman, *Annu. Rev. Biochem.* **56**, 615 (1987).
17. T. Higashijima, S. Uzu, T. Nakajima, E. Ross, *J. Biol. Chem.* **263**, 6491 (1988).
18. T. Higashijima, J. Burnier, E. Ross, *ibid.* **265**, 14176 (1990).
19. G. Koch, B. H. Haberman, C. Mohr, I. Just, K. Aktories, *FEBS Lett.* **291**, 336 (1991).
20. R. Diaz, L. Mayorga, P. Stahl, *J. Biol. Chem.* **264**, 13171 (1989).
21. M. Colombo *et al.*, unpublished results.
22. M. Tuichibaeu, N. Akhmedova, I. Kazarov, A. Korneev, A. Gagei'gans, *Biochem. USSR* **53**, 219 (1988).
23. F. Eckstein, D. Cassel, H. Leukovitz, M. Lowe, Z. Selinger, *J. Biol. Chem.* **254**, 9829 (1979).
24. J. L. Stow *et al.*, *J. Cell Biol.* **114**, 1113 (1991).
25. J. G. Donaldson, J. Lippincott-Schwartz, R. D. Klausner, *ibid.* **112**, 579 (1991).
26. J. G. Donaldson, R. A. Kahn, J. Lippincott-Schwartz, R. D. Klausner, *Science* **254**, 1197 (1991).
27. R. Diaz, L. S. Mayorga, P. D. Stahl, *J. Biol. Chem.* **263**, 6093 (1988).
28. M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).
29. P. J. Casey, M. P. Graziani, A. G. Gilman, *Biochemistry* **28**, 611 (1989).

30. We thank S. Gonzalo for excellent technical assistance, K. Blumer for helpful discussions, D. James and P. Weidman for critical reading of the manuscript, and C. Adles for assistance with tissue culture. Supported in part by Department of Health, Education, and Welfare Grants GM 42259 and AI 20015. L.S.M. is supported by a Rockefeller Foundation Biotechnology Career Development Award.

13 November 1991; accepted 28 January 1992

Chloroplast DNA Evidence on the Ancient Evolutionary Split in Vascular Land Plants

LINDA A. RAUBESON* AND ROBERT K. JANSEN†

Two groups of extant plants, lycopsids and psilopsids, alternatively have been suggested to be the living representatives of the earliest diverging lineage in vascular plant evolution. The chloroplast DNA (cpDNA) gene order is known to contain an inversion in bryophytes and tracheophytes relative to one another. Characterization of tracheophyte cpDNAs shows that lycopsids share the gene order with bryophytes, whereas all other vascular plants share the inverted gene order. The distribution of this character provides strong support for the fundamental nature of the phylogenetic separation of lycopsids and marks the ancient evolutionary split in early vascular land plants.

PHYLOGENETIC RELATIONSHIPS among the major extant lineages of vascular land plants are poorly resolved. Most recent systematic treatments consider each group a division and recognize no hierarchical relationships between these taxa (*1*). Explicit phylogenies have been produced by only a few workers (*2–5*). When a basal lineage has been hypothesized, it has varied between the psilopsids and lycopsids. Psilopsids (*6*) have been suggested as the earliest diverging clade primarily by neontologists (*2, 5*). These plants consist of dichotomizing aerial axes arising from prostrate rhizomes and appear similar, at least superficially, to some of the earliest appearing fossil vascular plants. Psilopsids lack roots even in the embryo, and the shoots bear emergences that may not be homologous to leaves. Alternatively, paleobotanists have suggested the basal placement of the lycopsids (*7*) on the basis of the stratigraphic occurrence of lycopsids in the fossil record and the

L. A. Raubeson, Department of Biology, Yale University, New Haven, CT 06511–7444.
R. K. Jansen, Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269–3042.

*To whom correspondence should be addressed, at the Department of Biological Sciences, Mount Holyoke College, South Hadley, MA 01075.
†Present address: Department of Botany, University of Texas, Austin, TX 78713.