al. and Li *et al.* (*14*) found that the catalytic subunit (CS) of cAMP-dependent kinase activated outwardly rectifying Cl^- channels in excised patches of normal but not CF airway epithelial cells. In 18 inside-out patches from NL/T4 cells, 6 were found to have Cl^- channels (Fig. 4A). Five of these six were activated by 1 m*M* adenosine triphosphate (ATP) and 100 n*M* CS, but not by ATP alone. Chloride channels in the remaining patch were elicited by depolarizing voltage. Of 16 inside-out patches taken from CF/T43 cells, 5 were ultimately found to contain Cl^- channels. None of these were activated by the ATP or ATP plus CS; all were activated by depolarizing voltages. Chloride channels from CF/T43 and NL/T4 had conductances [28.7 ± 1.3 pS (n = 5) and 29.6 ± 2.0 pS (n= 5), respectively] (Fig. 4B) similar to those reported for CF and normal airway epithelial cells in primary culture (*14*).

The ultimate proliferation potential of the CF/T43 cell line remains to be determined. Nevertheless, the demonstrated proliferation capability is adequate to support many experiments. For example, a single cryopreserved vial of 10^6 cells at passage 9 may be expanded to 10^{12} cells at passage 19 (the highest passage currently known to manifest the CF regulatory phenotype). This exceeds by three orders of magnitude the number of CF cells in primary culture produced in 1 year by a large tissue culture core facility (*15*).

We have developed a human airway epithelial cell line that maintains specific differentiated properties and is therefore useful for studies of the biologic properties underlying CF. The extended proliferation capability of the CF/T43 cell line is certainly caused by the SV40T gene, since the T antigen is expressed and control cultures died within four passages. The retention of differentiated epithelial characteristics is probably due to the random site of gene insertion, which determines the expression of T antigen and its effects on cell proliferation and function. Our selection strategy, which focused on the ability of cells to form resistive barriers, may favor clones in which other differentiated cell functions are relatively preserved. The key features of the CF/T43 line—formation of functional tight junctions, reduced apical membrane G_{Cl}, and activation of apical Cl^- channels by Ca^{2+} ionophores but not by cAMP-dependent agonists—suggest that the molecular processes underlying CF are preserved. These cells will be instrumental in elucidating these mechanisms, in testing candidate complementary genes for correction of the observed CF abnormalities, and for developing and testing new therapeutic strategies.

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Regulatory Role for GTP-Binding Proteins in Endocytosis

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Guanosine 5′-triphosphate (GTP)–binding proteins have been implicated in the transport of newly synthesized proteins along the secretory pathway of yeast and mammalian cells. Early vesicle fusion events that follow receptor-mediated endocytosis as measured by three in vitro assays were blocked by guanosine 5′-*O*-(3-thiotriphosphate) and aluminum fluoride. The effect was specific for guanosine nucleotides and depended on the presence of cytosolic factors. Thus, GTP-binding proteins may also have a role in the transport of molecules along the endocytic pathway.

GUANOSINE 5′-TRIPHOSPHATE (GTP)–binding proteins may participate in various intracellular processes in addition to their well-characterized role in the transduction of hormonal and sensory signals (*1*). Analogs of GTP have been shown to affect exocytosis in permeabilized cells (*2*), and studies of yeast mutants defective in the secretory pathway have identified GTP-binding proteins that are required for normal secretion (*3*). These proteins have extensive homology with *ras* gene products (*4*). Ras proteins are GTP-binding proteins that appear to participate in the proliferation of eukaryotic cells (*5*). In mammalian cells, evidence for a regulatory mechanism mediated by GTP-binding proteins in the secretory pathway comes from the observation that transport from the endoplasmic reticulum to the Golgi apparatus (*6*) and among Golgi stacks (*7*) is inhibited by guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) (a nonhydrolyzable GTP analog) in cell-free systems. In addition, GTP-γ-S affects the in vitro recycling of phosphomannosyl receptors to the Golgi apparatus (*8*).

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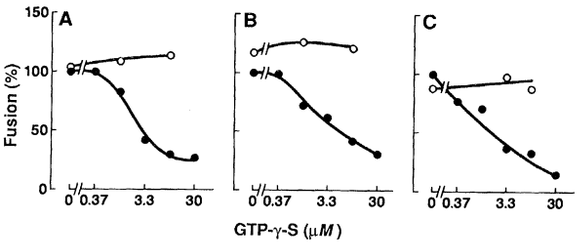

Fig. 1. Inhibitory effect of GTP-γ-S on three in vitro assays of fusion events after receptor-mediated endocytosis. Effect of increasing concentrations of GTP-γ-S on (**A**) endosome-endosome fusion, (**B**) fusion between plasma membrane–derived vesicles and endosomes, and (**C**) fusion-dependent endosomal proteolysis. Mixtures were incubated in the presence (O) or absence (●) of 1 m*M* GTP. For the experiments shown in (A) and (B), J774-E clone macrophages (a mannose-receptor positive cell line) were incubated (*11, 12*) either with aggregated anti-DNP IgG for 2 hours at 4°C (to load plasma membrane receptors) or with mannosylated anti-DNP IgG or DNP-β-glucuronidase for 5 min at 37°C (to load early endosomes). Cells were homogenized, and vesicle fractions that were enriched with either plasma membrane–derived vesicles or early endosomes were obtained by differential centrifugation. Anti-DNP IgG–containing vesicles were mixed with DNP-β-glucuronidase–loaded endosomes in fusion buffer [20 m*M* Hepes-KOH (*p*H 7.0), 250 m*M* sucrose, 0.5 m*M* EGTA, 1 m*M* dithiothreitol, 3 m*M* $MgCl_2$, dinitrophenol-derivatized bovine serum albumin (DNP-BSA, 250 μg/ml), 50 m*M* KCl, 1 m*M* ATP, 8 m*M* creatine phosphate, and creatine phosphokinase (31 U/ml)] in the presence of gel-filtered cytosol (1.7 mg of protein per milliliter). After incubation for 45 min at 37°C, fusion was assessed by the formation of complexes between anti-DNP IgG and DNP-β-glucuronidase as described (*11*). Fusion-dependent proteolysis (C) was assessed under the above fusion conditions with an endosomal fraction obtained from J774-E macrophages that had internalized immune complexes [anti-DNP IgG and radiolabeled DNP-BSA (*14*)] for 2 min at 37°C. Proteolysis was measured by the formation of trichloracetic acid–soluble radioactivity (*14*). Nonspecific background values were measured in the absence of cytosol (a condition that does not support fusion) and subtracted from each value. Fusion is expressed as a percentage of that observed in the absence of both GTP and GTP-γ-S. Each point represents the mean of two determinations. Similar results were obtained in three independent experiments.

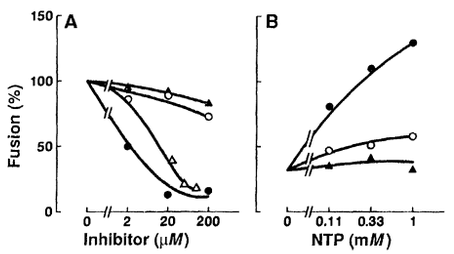

Fig. 2. Effect of nucleotides and aluminum fluoride on in vitro endosome-endosome fusion. (**A**) Different concentrations of nonhydrolyzable nucleotides [GTP-γ-S, ●; adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S), ▲; and guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S), O] and aluminum sulfate (△) were tested in an assay similar to that described in the legend to Fig. 1A. When the effect of aluminum was tested, EGTA was omitted from the fusion buffer and KF (5 m*M*) was added. (**B**) Reversal of the inhibitory effect of 20 μ*M* GTP-γ-S by increasing concentrations of NTP (GTP, ●; UTP, O; and CTP, ▲). Fusion is expressed as a percentage of that observed in the absence of inhibitory compounds and NTP other than ATP. Each point represents the mean of two determinations.

Macromolecules are transported between intracellular compartments during the processes of secretion and endocytosis. The fusion of vesicles originating from one compartment with the compartment of destination occurs in both processes. Because GTP-binding proteins have been implicated in the regulation of transport along the exocytotic pathway, they may play a similar role in the endocytic pathway (*9*). Therefore we investigated whether GTP analogs could affect transport of ligands after receptor-mediated endocytosis in cell-free systems.

Several assays have been developed to reconstitute fusion events of the endocytic pathway (*10*). Our approach was to generate two probes with high affinity for each other that are efficiently internalized by receptor-mediated endocytosis (*11*). The two macromolecules we used were dinitrophenol-derivatized β-glucuronidase (DNP-β-glucuronidase) and a mouse immunoglobulin G class monoclonal antibody to dinitrophenol (anti-DNP IgG); β-glucuronidase is a ligand for the mannose receptor of macrophages. Anti-DNP IgG was either derivatized with mannose to make it a ligand for the mannose receptor or aggregated to increase its affinity for the Fc receptor of macrophages. In the standard assay, two populations of cells were incubated with the probes for 5 min. The cells were then homogenized, and endosome-containing subcellular fractions were mixed in an in vitro system supplemented with adenosine 5′-triphosphate (ATP), ions, and cytosol. Fusion of two endosomes that contained different probes led to the formation of complexes between anti-DNP IgG and DNP-β-glucuronidase, and these complexes were immunoprecipitated and quantitated by measuring the enzymatic activity of β-glucuronidase.

When crude cell homogenates were incubated in the fusion assay, we observed a 30 to 40% inhibition of vesicle fusion in the presence of 20 μ*M* GTP-γ-S. When endosome-enriched fractions generated from the crude homogenate by differential centrifugation were used in the assay, GTP-γ-S inhibited fusion by more than 70%. Half-maximal inhibition occurred at 1 to 3 μ*M* GTP-γ-S (Fig. 1A). Other non-hydrolyzable nucleotides did not inhibit fusion (Fig. 2A). The inhibition by GTP-γ-S was reversed in the presence of GTP (Fig. 1A and Fig. 2B). Other nucleoside triphosphates (NTP) such as ATP, which was always present in the medium, cytidine 5′-triphosphate (CTP), and uridine 5′ triphosphate (UTP) were unable to reverse the inhibitory effect of GTP-γ-S (Fig. 2B). GTP-binding proteins are also activated by the combination of Al^{3+} and F^- (the effect is probably mediated by the AlF_4^- complex) (*1*). The combination of both ions inhibited vesicle fusion (Fig. 2A); the inhibition observed at the highest concentration (5 m*M* KF and 100 μ*M* aluminum sulfate) was reversed by the presence of 0.5 m*M* EGTA, suggesting that free Al^{3+} is required and that F^- alone is not sufficient for inhibition.

The effect of GTP-γ-S on Golgi transport is cytosol-dependent (*7*); inhibition is not observed when the ratio of cytosol to membranes is decreased. Similarly, the inhibitory effect of GTP-γ-S on endosome-endosome fusion was cytosol-dependent (Fig. 3A). Endosome fusion itself is cytosol-dependent (*11*), but the concentration of cytosol required to support fusion was much lower than that required to observe inhibition by GTP-γ-S. Thus, a factor present in cytosol is required for inhibition. The factor is probably a macromolecule because the cytosol was filtered through a G-25 Sephadex column before addition to the assay.

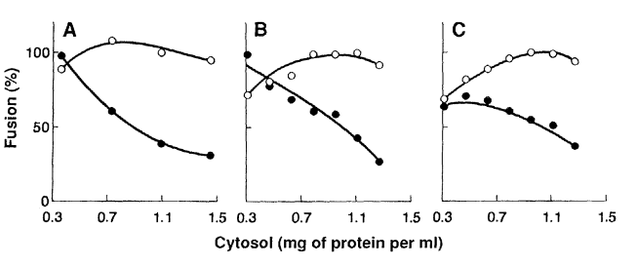

Fig. 3. Cytosol dependence of the inhibitory effect of GTP-γ-S on vesicle fusion. (**A**) Endosome-endosome fusion, (**B**) fusion between plasma membrane–derived vesicles and endosomes, and (**C**) fusion-dependent proteolysis were measured at different cytosol concentrations in the presence (●) or absence (O) of GTP-γ-S (20 μ*M*). Fusion is expressed as a percentage of that observed in the absence of GTP-γ-S and in the presence of 1.1 mg of cytosolic protein per milliliter. Each point represents the mean of two determinations. Similar results were obtained in four independent experiments.

Other fusion events beside those that occur between two populations of early endosomes have been reconstituted. Plasma membrane–derived vesicles, generated by homogenization of cells whose external Fc receptors were saturated with aggregated anti-DNP IgG, can also fuse with early endosomes in a cell-free system (*12*). This process mediates the transport of cell-surface molecules to an endocytic compartment. Endosomes contain acid proteases that can digest internalized proteins (*13*), and therefore, the fusion of vesicles generated shortly after internalization (which do not contain proteases) with protease-containing compartments (that is, endosomes and probably also Golgi-derived vesicles) should trigger the proteolytic processing of internalized ligands. This fusion-dependent proteolysis can be reconstituted and monitored by the onset of degradation of an internalized radiolabeled protein (*14*). Fusion between plasma membrane–derived vesicles and endosomes and fusion-dependent proteolysis were both inhibited by GTP-γ-S (half-maximal inhibition at 1 to 3 μ*M* GTP-γ-S), and the effect was completely reversed by 1 m*M* GTP (Fig. 1, B and C). Moreover, the inhibitory effect was only observed at high concentrations of cytosol (Fig. 3, B and C).

Cell-free reconstitutions of fusion processes of both the exocytotic and endocytic pathways have several common requirements. Fusion requires cytosolic proteins and energy in the form of ATP (*10, 11, 15*). In vitro transport requires an *N*-ethylmaleimide–sensitive factor (*11, 15*). This factor has been purified to homogeneity (*16*) and shown to be required in both the secretory and the endocytic pathways (*17*). We show here that a putative GTP-binding protein may also participate in the regulation of fusion events after receptor-mediated endocytosis. Therefore, a common mechanism may support fusion in the endocytic and exocytotic pathways. However, some specific components must exist to avoid transport to inappropriate compartments and to allow individual modulation of both pathways. Distinctive markers on the limiting membrane of the compartments and specific regulatory components of the fusion process may account for the independence of the two pathways.

The exact role of GTP-binding proteins in intracellular transport is unknown. Most GTP-binding proteins are activated on binding GTP and become deactivated on hydrolysis of the nucleotide. This property may allow GTP-binding proteins to serve as regulatory components in vesicular transport. It may also provide a mechanism for coupling the energy of GTP hydrolysis to the transport process (*9*).

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Antigen-Specific Helper Function of Cell-Free T Cell Products Bearing TCR $V_\beta 8$ Determinants

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Although the T cell receptor (TCR) αβ heterodimer and its encoding genes have been characterized, a cell-free form of this receptor, which is needed for the study of functional or ligand-binding properties of the receptor, has not previously been isolated. When the cell-free supernatant products of activated cloned T helper (T_H) cells were found to mediate helper activity with antigen specificity identical to that of intact T cells, experiments were carried out to determine whether this functional activity was mediated by a cell-free form of TCR-related material. A disulfide-linked dimer indistinguishable from the T cell surface αβ heterodimer was precipitated from cell-free supernatants of cloned T_H cells with F23.1, a monoclonal antibody specific for a TCR $V_\beta 8$ determinant. Moreover, when cell-free T_H products were bound to and eluted from immobilized F23.1, these affinity-purified materials had antigen-specific and major histocompatibility complex–restricted helper activity that synergized with recombinant lymphokines in the generation of B cell antibody responses. These findings suggest that the factor isolated from T cell supernatants is a cell-free form of the TCRαβ dimer.

THE TCRαβ HETERODIMER HAS been identified, and both its glycoprotein structure (*1*) and its encoding genes (*2*) have been characterized. Recent DNA-mediated gene transfer experiments have directly demonstrated that this αβ dimer mediates the antigen-specific and major histocompatibility complex (MHC)–restricted recognition function of many helper and cytotoxic T cells (*3, 4*). To date, however, it has not been possible to assess the specificity or functional properties of the TCR in soluble or cell-free form. A system in which cell-free TCR material can be isolated and studied would permit analysis of the TCR-ligand interactions and could be used to test whether TCR structures can act as signaling molecules in the interaction of T cells with other cells. The cell-free products of T_H cell clones provide antigen-specific and MHC-restricted helper activity in the generation of hapten-specific immunoglobulin G (IgG) antibody responses (*5*). Because this specificity closely reproduces the recognition specificity of the intact T_H cells, presumably mediated by the αβ TCR, we studied the cell-free supernatants of activated T_H cells for the presence of TCR-like products.

Cloned antigen-specific and MHC-restricted type 2 T_H cells (*6*), which secrete interleukin-4 (IL-4) but not IL-2 (*7*), were activated in the presence of specific antigen and appropriate antigen-presenting cells

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