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Cell, Vol. 36, 847–856, April 1984, Copyright © 1984 by MIT

0092-8674/84/04847-10 $02.00/0

Microinjection of mRNA Coding for an Anti-Golgi Antibody Inhibits Intracellular Transport of a Viral Membrane Protein

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Summary

Messenger RNA was prepared from a hybridoma cell line secreting a monoclonal antibody (53FC3) directed against a luminal epitope of a Golgi membrane protein (M_r = 135 kd) found in rodent cells. When this mRNA was microinjected into the cytoplasm of BHK cells, mouse IgG was seen to accumulate in the Golgi complex after 5–6 hr of incubation. No accumulation was seen in 3T3 cells which lack the epitope recognized by 53FC3. When microinjected BHK cells were infected with vesicular stomatitis virus, surface expression of the viral G protein was considerably reduced when compared with neighboring noninjected cells.

Introduction

The Golgi complex in eucaryotic cells plays a pivotal role in the intracellular transport and concomittant processing of newly synthesized secretory, lysosomal, and plasma membrane proteins (Palade, 1975; Rothman, 1981). Functions which have thus far been localized to this organelle include trimming and terminal glycosylation of asparagine-linked oligosaccharides (reviewed by Hubbard and Ivatt, 1981), fatty acylation of membrane proteins (Schmidt et al., 1979), and phosphorylation of mannose residues on lysosomal enzymes (von Figura and Klein, 1979; Hasilik and Neufeld, 1980; Reitman and Kornfeld, 1981). Much attention is now focused on the localization of these different functions to distinct compartments or cisternae within the Golgi stacks. One recent approach to this problem has been based on the use of drugs which may specifically block intracellular transport within the Golgi complex but not the synthesis of proteins to be transported. The rationale behind such an approach is that partially processed proteins should then accumulate immediately prior to the block. Thus by examination of these immature proteins, using biochemical and immunocytochemical techniques, one may be able to determine the positions of the various processing enzymes relative to the drug-induced transport block. The only drug that has been used so far with any degree of success is monensin (Tartakoff and Vassalli, 1977, 1978; Griffiths et al., 1983; Quinn et al., 1983). This drug, however, is far from ideal since it severely perturbs the normal morphology of the Golgi complex and may well have further undesirable effects. In addition to these problems it is still far from clear how monensin exerts its effects.

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As an alternative to the use of drugs, we considered the possibility of using antibodies to specifically interfere with Golgi functions. Currently, there are several well-characterized antibodies available that recognize specific Golgi proteins. These include polyclonal antisera against galactosyl transferase (Roth and Berger, 1982; Hiller and Weber, 1982) and mannosidase II (Novokoff et al., 1983) and both polyclonal and monoclonal antibodies against a 135K Golgi membrane protein (Louvard et al., 1982; Burke et al., 1982).

Introduction of specific antibodies into cells can be achieved relatively easily by microneedle injection and there are several well-documented experiments where specific cytoplasmic processes have been disrupted using this or similar methods (Mabuchi and Okuno, 1977; Yamaizumi et al., 1978; Antman and Livingstone, 1980; Lin and Feramisco, 1981). In the case of the Golgi complex, however, mere injection of antibodies into the cytoplasm may have no effect on function since all the available antisera appear to recognize protein domains on the luminal side of the Golgi membranes. Furthermore, all enzymic and receptor activities that can be assigned to the Golgi complex reside on the luminal side of the Golgi membranes.

The only obvious way, then, of introducing a specific antibody into the lumen of the Golgi complex is to microinject, not the antibody itself, but the messenger RNA coding for the heavy and light chains of the antibody. Since immunoglobulins are secretory proteins, one would expect the mRNAs to be translated on membrane-bound polysomes and the heavy and light chains to be translocated into the lumen of the rough endoplasmic reticulum (ER) where they are known to assemble to form functional antibodies (Colman et al., 1982; Valle et al., 1982). These antibody molecules would then be transported to the Golgi complex where they could bind to their specific antigen.

Since this approach requires a source of appropriate mRNA, one is clearly confined to the use of monoclonal antibodies. In this paper we describe experiments where we have microinjected BHK cells with mRNA coding for a monoclonal antibody (53FC3) against a 135K Golgi protein and we show that the antibody produced in these cells accumulates within the Golgi complex. We also examine the effects of microinjection of this mRNA on the cell surface expression of the G protein of vesicular stomatitis virus (VSV).

Results

53FC3 Recognizes a Luminal Domain of the 135K Protein

We showed previously (Burke et al., 1982) that most of the mass of the 135K protein was located on the luminal side of Golgi membranes since it was largely resistant to digestion by proteases added to sealed Golgi vesicles. We did not determine the location of the epitope recognized by 53FC3 although there was some circumstantial

evidence to suggest that it was on the luminal side. We have now repeated these digestion experiments and, as shown in Figure 1, the binding of 53FC3 to the 135K protein was not affected by protease treatment unless the vesicles were first made permeable using Triton X-100. The epitope recognized by 53FC3 is thus indeed located on the luminal side of Golgi membranes.

53FC3 Expressed in BHK Cells Accumulates in the Golgi Complex

To determine whether 53FC3 could bind to the 135K protein in vivo, mRNA coding for this antibody was injected into the cytoplasm of BHK cells. We have already shown by immunofluorescence microscopy that 53FC3 recognizes the 135K protein in these cells (Figure 2). Afer 5–6 hr incubation at 37°C, the cells were fixed and any synthesis of mouse IgG was detected using a specific antibody conjugated to rhodamine. As shown in Figure 3, the microinjected cells displayed a strong perinuclear fluorescence that was very similar in appearance to the pattern seen when BHK cells are stained in the conventional manner with the monoclonal antibody (Figure 2). If, prior to fixation, the microinjected cells were treated for 2.5 hr with cycloheximide to inhibit protein synthesis but not intracellular transport (Jamieson and Palade, 1968), essentially the same pattern was seen (Figure 4), indicating that mouse IgG was accumulating in what appeared to be the Golgi complex. Identical labeling patterns were found in every microinjected cell found expressing IgG (about 80 cells) in three separate experiments.

Since BHK cells do not normally secrete IgG it is possible that this accumulation may simply have been due to a nonspecific phenomenon and not to a specific interaction

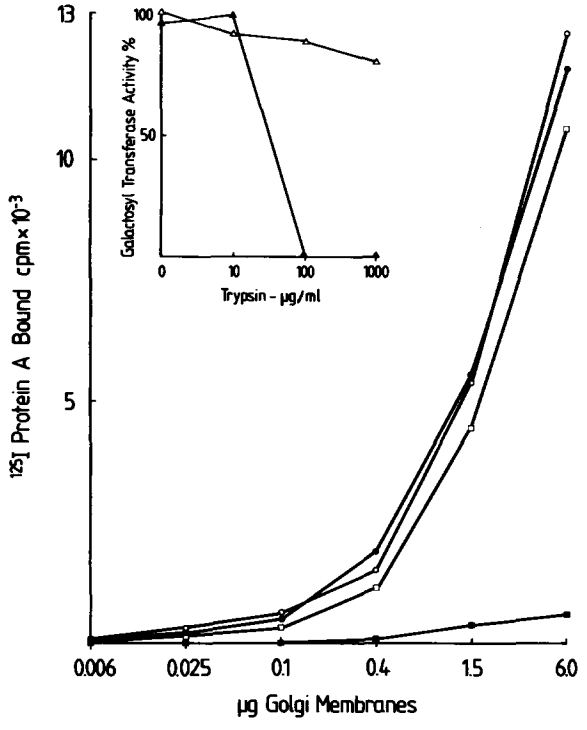


Figure 1. Effects of Trypsin on the Binding of 53FC3 to the 135K Golgi Membrane Protein

Rat liver Golgi vesicles were digested with 100 µg/ml trypsin in either the presence or absence of 0.4% Triton X-100 for 1 hr at 25°C. Membranes were then solubilized in Triton X-100 in the presence of protease inhibitors. Appropriately diluted samples were spotted onto nitrocellulose and labeled with 53FC3 and iodinated protein A as described in Experimental Procedures. Digestion conditions used were as follows: □ + Trypsin, − Triton; ■ + Tryspin, + Triton; ○ − Trypsin, − Triton; ● − Trypsin, + Triton. Inset: Effects of trypsin digestion in the presence (▲) or absence (△) of Triton X-100 (0.4%) on galactosyl transferase activity (a luminal marker) in rat liver Golgi vesicles. At 100 µg/ml trypsin in the presence of Triton, activity is completely abolished while in the absence of Triton it is largely unaffected.

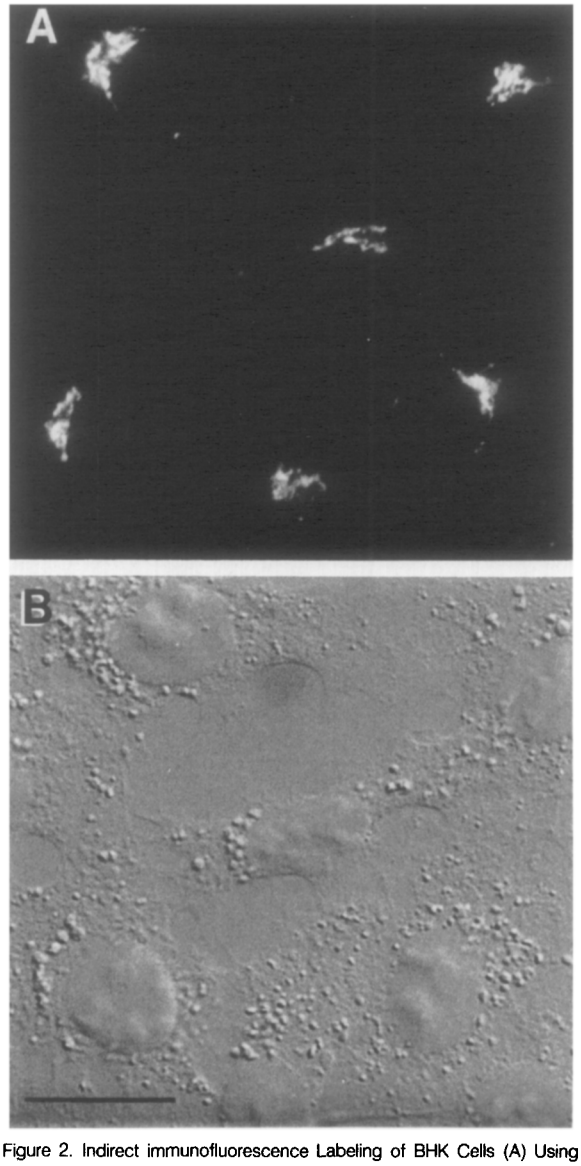


Figure 2. Indirect immunofluorescence Labeling of BHK Cells (A) Using 53FC3

The Golgi complex always occupies a distinctive reticular cap at one pole of the nucleus in interphase cells. (B) Corresponding field photographed using Nomarski optics. Bar = 20 µm.

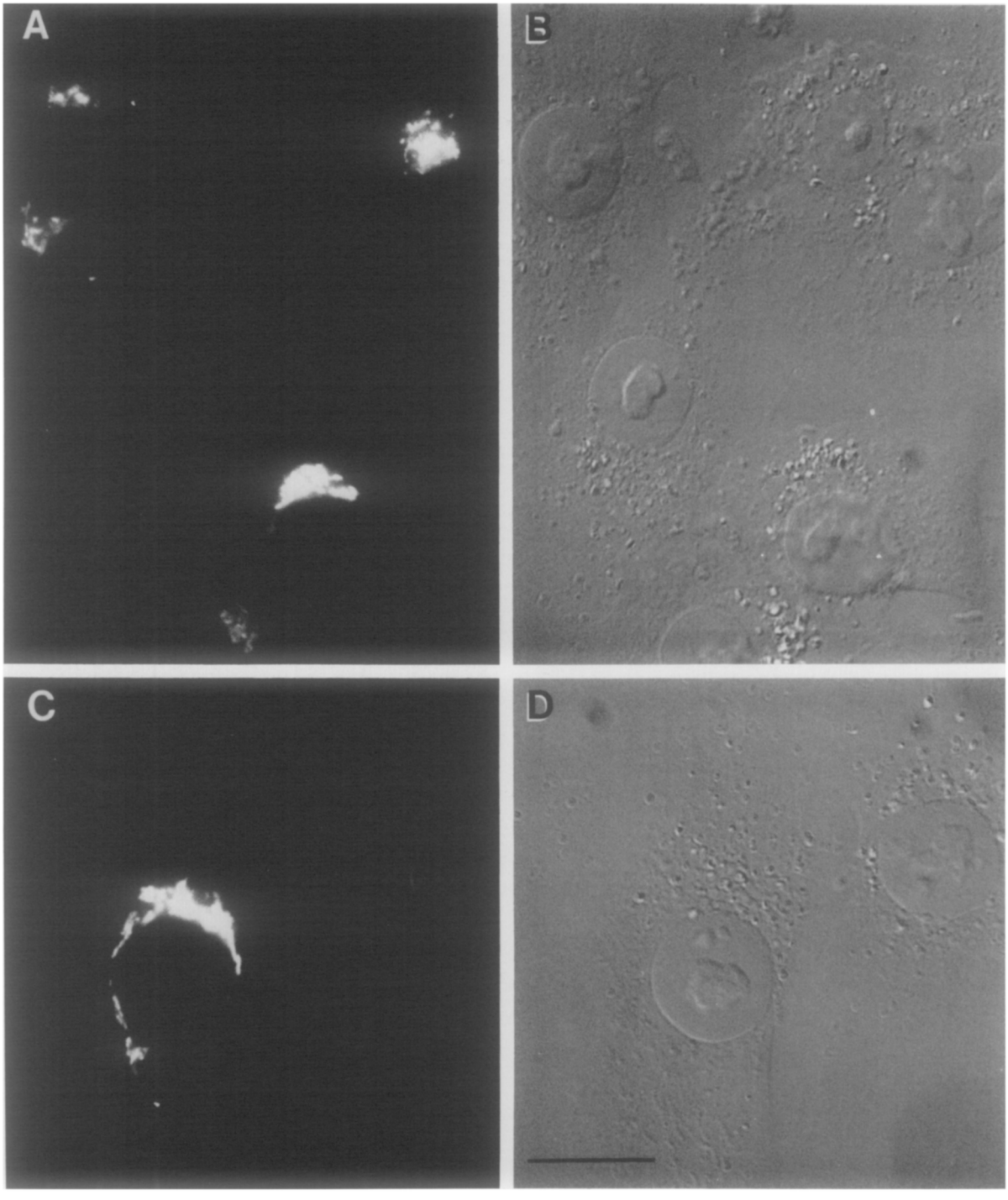


Figure 3. Direct immunofluorescence Labeling of BHK Cells Microinjected with 53FC3 mRNA (A, C)

Six hours after microinjection the cells were fixed in formaldehyde, permeabilized with Triton X-100 and then labeled with affinity-purified rabbit anti-mouse IgG that was conjugated to rhodamine. Note the similarity with Figure 2A. Corresponding fields photographed using Nomarski optics are shown in (B) and (D). Bar = 20 μM.

between 53FC3 and the 135K protein. To test this, mRNA from another hybridoma making an IgG directed against a viral antigen (Coronavirus E1, 471M1) was injected into BHK cells. After 5–6 hr incubation no intracellular accumulation could be seen (Figure 5A). However, to ensure that these cells were indeed synthesizing the IgG, 10 μM monensin was added to the medium for the last 3 hr of incubation in order to block secretion (Tartakoff and Vas-

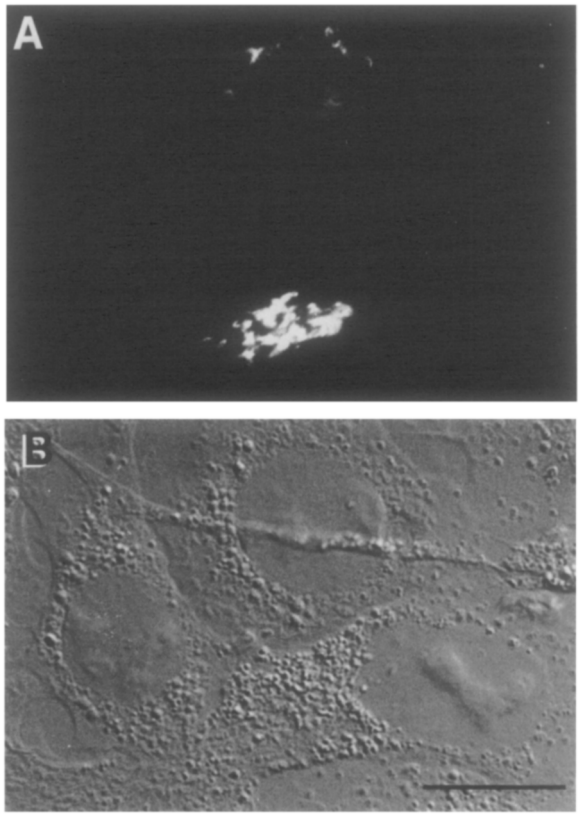


Figure 4. Direct Immunofluorescence Labeling of BHK Cells Microinjected with 53FC3 mRNA (A)

Five hours after microinjection cycloheximide was added to the medium to a final concentration of 10 μg/ml and the incubation continued for a further 2.5 hr. The cells were then fixed and labeled as described for Figure 3. The corresponding field photographed using Nomarski optics is shown in (B). Bar = 20 μm.

sali, 1977). When these cells were stained with rabbit anti-mouse IgG, a pattern resembling the ER was seen (Louvard et al., 1982), indicating that the mouse IgG was indeed being synthesized (Figure 5C). In the absence of monensin it is presumably secreted.

Similarly, when the 53FC3 mRNA was injected into 3T3 cells (Figure 5E) accumulation of mouse IgG could only be detected after treatment with monensin (Figure 5G). 3T3 cells contain the 135K protein as judged by immunofluorescence microscopy using polyclonal antisera, but lack the epitope recognized by 53FC3. This is true of all the mouse cells we have tested so far (results not shown).

Surface Expression of VSV-G Protein Is Inhibited in Microinjected Cells Expressing 53FC3

Since the 53FC3 immunoglobulin appeared to accumulate in the Golgi complex, presumably by way of its specific interaction with the 135K protein, it seemed likely that this might disrupt in some way the normal functioning of the organelle. This might, for example, be reflected in the inhibition of surface expression of plasma membrane proteins. To investigate this, BHK cells were injected with 53FC3 mRNA and allowed to accumulate antibody intracellularly for 5 hr. At this point the cells were infected with a temperature-sensitive mutant of vesicular stomatitis virus, *ts045*, the G protein of which is synthesized but unable to leave the ER at the nonpermissive temperature of 39.5°C (Bergman et al., 1981). The cells were infected for 1 hr at 37°C and incubated for a further 1.5 hr at 39.5°C to allow accumulation of viral G protein in the ER. The cells were then transferred to the permissive temperature of 31°C for 30 min, which is sufficient time for the G protein to appear on the cell surface (Bergman et al., 1981). At the end of this incubation period the cells were fixed and labeled with rabbit anti-VSV-G protein without prior permeabilization so that only G protein on the cell surface was labeled. The cells were then treated with Triton X-100 and labeled with both rhodamine-conjugated sheep anti-rabbit IgG (to reveal surface G protein) and fluorescein-conjugated sheep anti-mouse IgG (to reveal 53FC3 in the Golgi complex). The results of this experiment are shown in Figure 6. Microinjected cells had considerably less surface G protein when compared with their neighbors. This lack of surface labeling was not due to inability of the VSV *ts045* to infect the microinjected cells since Triton X-100 treatment prior to G protein labeling resulted in strong internal staining in at least 95% of the cells expressing 53FC3 (Figure 7), approximately the same percentage as the noninjected cells. These data clearly indicate that 53FC3 in the Golgi complex can inhibit the surface expression of VSV-G protein.

It should be emphasized that we have never seen complete inhibition of surface expression of the viral G protein. We examined about 100 microinjected cells and found that 80%–90% of these showed considerable reduction in surface expression of G protein and about 40%–50% exhibited almost no surface G protein. It was, however, clear to us that the inhibition increased with the increasing amounts of injected mRNA. The fact that we could not obtain complete inhibition did have the advantage of showing that each microinjected cell examined was indeed infected.

In order to show that this inhibition of surface expression of VSV-G protein was not due to some trivial effect arising from the microinjection of hybridoma mRNA, we repeated the experiments described above using 417M1 mRNA. Since 417M1 IgG does not accumulate intracellularly (Figure 5) individual microinjected cells are difficult to identify. We therefore injected every cell on previously marked, and easily identifiable, areas of the glass coverslip. Out of 80 microinjected cells we could find no evidence for a reduction in the surface expression of the viral G protein (Figure 8). This suggests that 53FC3 does indeed exert a specific effect.

Discussion

In this paper we describe a novel method for blocking intracellular transport at the level of the Golgi complex using a monoclonal antibody directed against a luminal

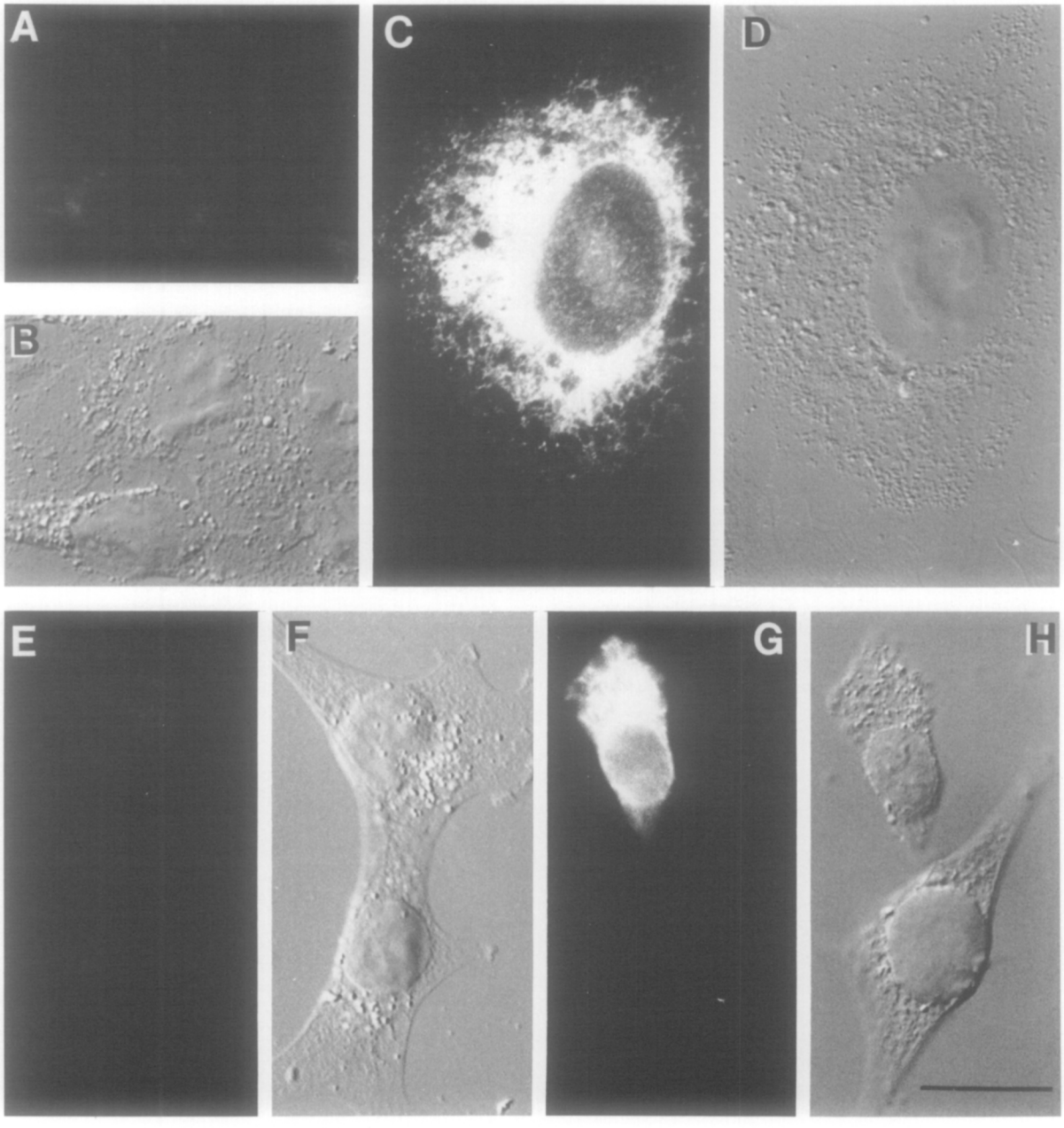
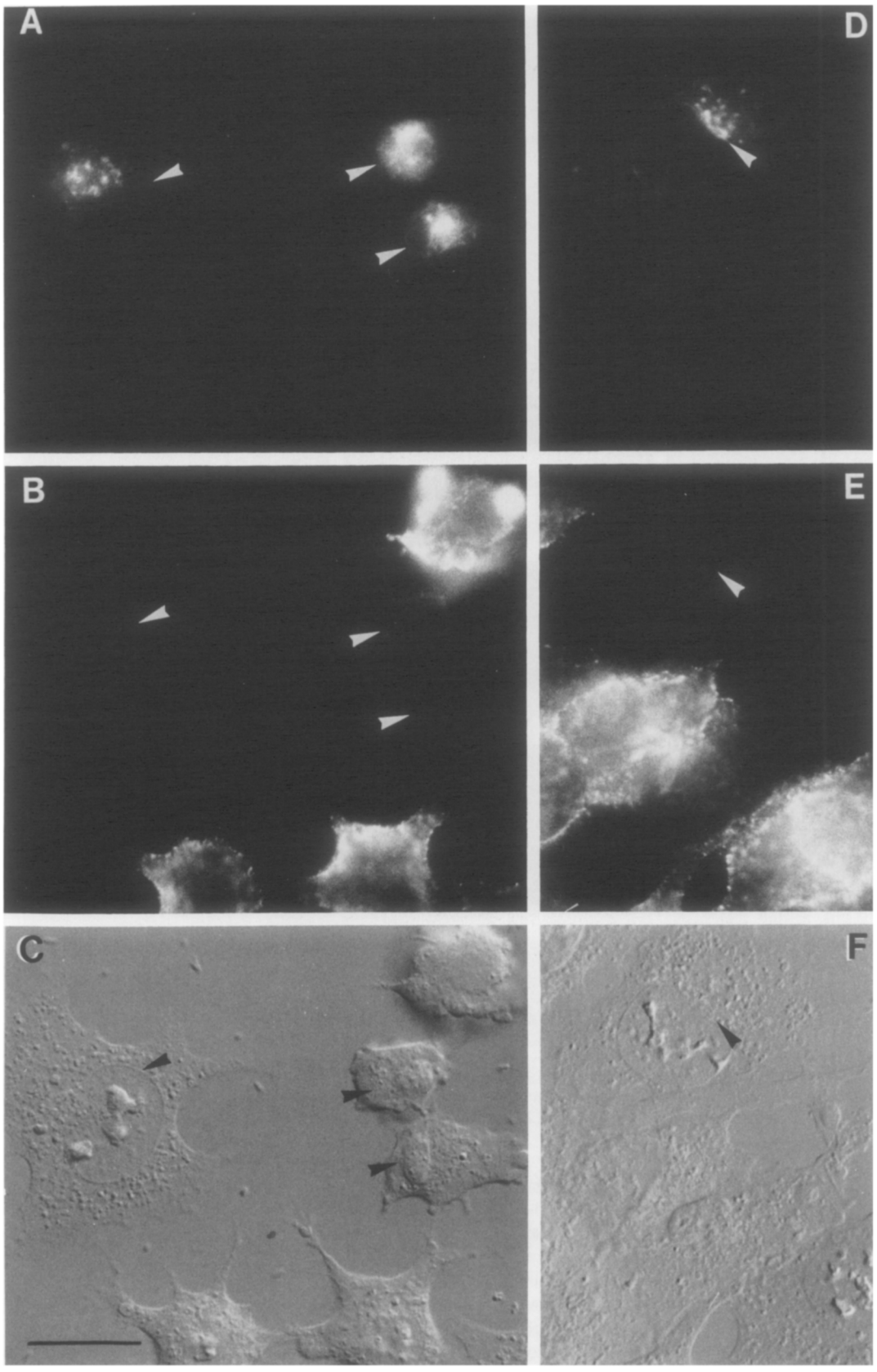


Figure 5. Direct Immunofluorescence Labeling of Microinjected Cells

BHK cells microinjected with 417M1 mRNA (A and C) and 3T3 cells with 53FC3 mRNA (E and G) were incubated for 6 hr and then processed for fluorescence microscopy as described in Figure 2. In (C) and (G) 10 μM monensin was added to the medium for the final 3 hr of incubation. In both cases there is accumulation of intracellular IgG only in the presence of monensin. (B), (D), (F), and (H) are the corresponding fields photographed using Nomarski optics. Bar = 20 μm.

component of some Golgi cisternae. We show that after microinjection of the appropriate mRNA, BHK cells are able to synthesize the IgG which is transported to the Golgi complex where it can recognize its specific antigen and accumulate. If these microinjected cells are subsequently infected with VSV, surface expression of the viral G protein is greatly inhibited despite the fact that viral infection appears to have occurred normally. How this inhibition of surface expression takes place is not clear. There are, however, two rather different although not mutually exclusive possibilities. The first is that binding of the antibody may inhibit some specific function of the 135K protein which is essential for intracellular transport. At present, we have no idea what the function of this protein might be. The second possibility is that by cross-linking proteins within the Golgi membranes the organelle may simply be unable to function efficiently. This explanation would, of course, require that the 135K protein exists as an oligomer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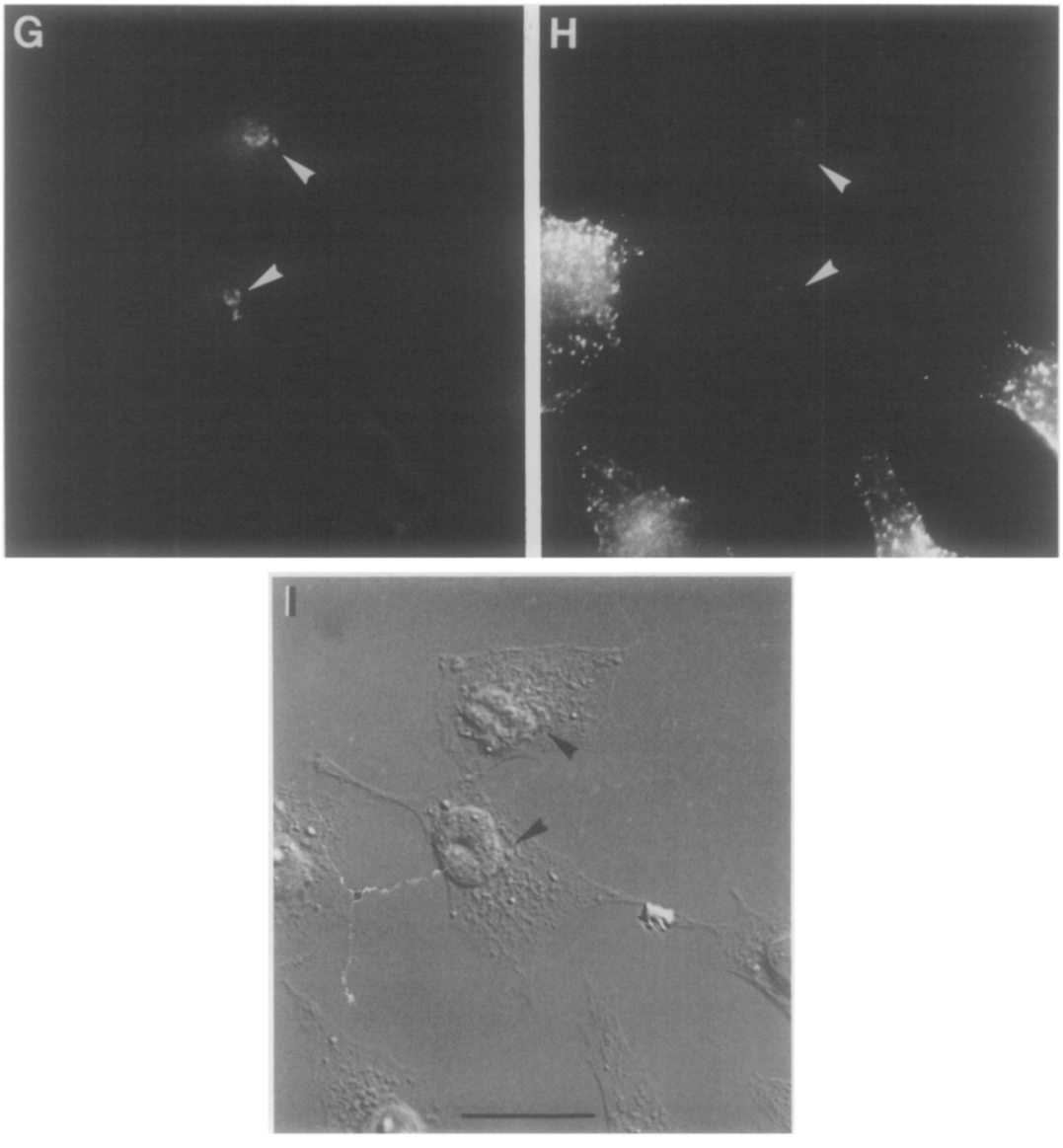

Figure 6. Indirect Double-Label Immunofluorescence Microscopy of BHK Cells

BHK cells were microinjected with 53FC3 mRNA, incubated for 5 hr and then infected with VSV-*ts045* as described in the text. The cells were fixed in formaldehyde and then VSV-G protein on the cell surface was labeled with rabbit anti-G antibody. The cells were permeabilized with Triton X-100 and incubated with affinity-purified second antibodies. These were sheep anti-rabbit IgG conjugated to rhodamine and sheep anti-mouse IgG conjugated to fluorescein. Neither antibody cross-reacted with the other. Each field is shown as a group of three photographs (A) to (C), (D) to (F), and (G) to (I). Microinjected cells, identified by their labeling with the fluorescein-conjugated anti-mouse IgG are shown in (A), (D), and (G). The same fields showing cell surface G protein visualized with the rhodamine-conjugated antibody are shown in (B), (E), and (H). The corresponding fields photographed using Nomarski optics are (C), (F), and (I). It is clear that the microinjected cells (arrowheads) have very little G protein on the cell surface. Bar = 20 μm.

in vivo. Furthermore, one would have to argue that cross-linked proteins within Golgi membranes are not dealt with in the same manner as those on the cell surface despite the fact that the luminal face of Golgi membranes and the external surface of the cell are topologically equivalent. Plasma membrane proteins cross-linked with antibodies are rapidly internalized and directed to lysosomes (Silverstein et al.,1977). Our results indicated, however, that the bulk of 53FC3 remains in the Golgi complex for at least 2.5 hr.

Despite the fact that the mechanism of this inhibition is still not clear, there are nevertheless certain conclusions which may be drawn. First, and most important, our data strongly support the view (Rothman, 1981) that plasma membrane and secretory proteins must share a common pathway through at least that part of the Golgi complex prior to the one or two cisternae in each Golgi stack in which the 135K protein is located (Burke et al., 1982). Second, since after microinjection we could never find any evidence for the 135K protein appearing on the plasma

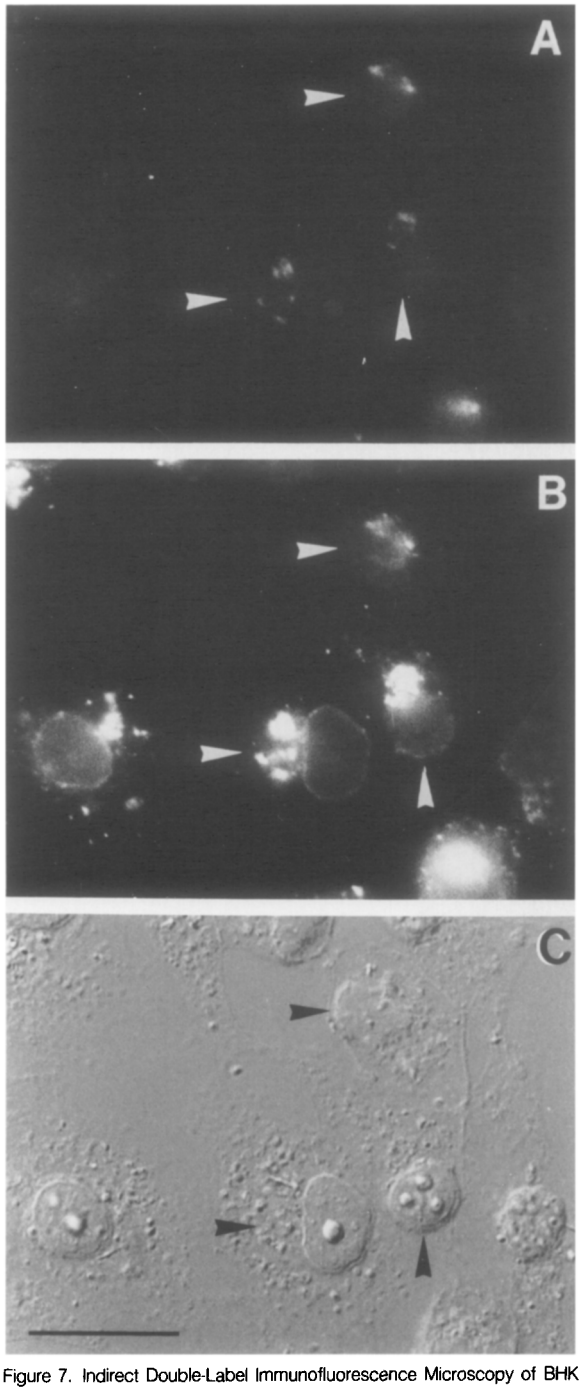

Figure 7. Indirect Double-Label Immunofluorescence Microscopy of BHK Cells

Cells were treated exactly as described in Figure 6 except that the cells were permeabilized with Triton X-100 prior to labeling with rabbit anti-VSV-G so that internal G protein was also labeled. (A) Microinjected cells (arrowheads) identified by their labeling with the fluorescein-conjugated antibody. (B) The same field showing internal VSV-G protein labeling with the rhodamine-conjugated antibody. (C) Nomarski photograph of the same field. The microinjected cells are clearly infected since they contain large amounts of intracellular G protein. Bar = 20 μm.

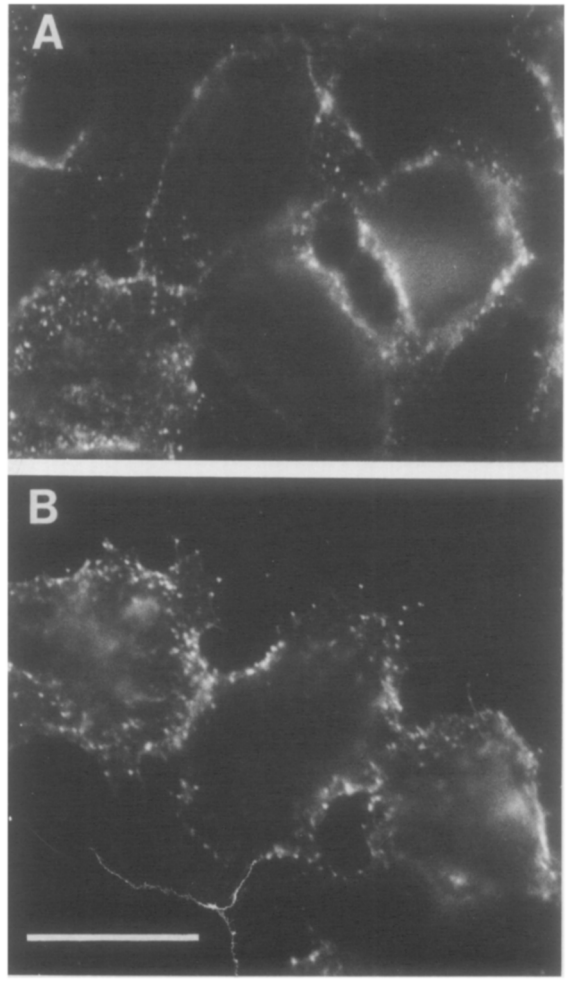

Figure 8. Indirect Immunofluorescence Microscopy of BHK Cells

BHK cells growing within a marked area on a glass coverslip were all microinjected with 417M1 mRNA, incubated for 5 hr and infected with VSV-*ts045* as described in the text. The cells were fixed and labeled with antibodies exactly as described in Figure 6. Only labeling of G protein on the cell surface is shown since there is no appreciable intracellular accumulation of 417M1 IgG. (A) All the cells within this field (part of a group of 80) were microinjected with 417M1 mRNA. (B) Noninjected cells. Clearly microinjection with 417M1 mRNA has little or no effect on the surface expression of VSV-G protein. Bar = 20 μm.

membrane, it would appear that simply attaching a secretory protein, an immunoglobulin, to an integral, Golgi membrane protein is insufficient to make it move from its normal location to the exterior of the cell.

Our observations also explain the rather restricted species specificity of the monoclonal antibody. As described previously (Burke et al., 1982) the hybridoma was obtained from a fusion involving spleen cells from a mouse immunized with rat liver Golgi membranes. Clearly, it would create problems for a hybridoma (or any cell) to secrete antibody directed against a component found on the luminal side of its own Golgi membranes. It is not surprising then that although the antibody recognizes the 135K pro-

tein in most rodent cells tested, it does not recognize the protein in mouse cells.

While microinjection of both polyclonal and monoclonal antibodies has previously been used to modify the behavior of cytoplasmic proteins, we believe this is the first report of a monoclonal antibody being used to block the intracellular transport of a plasma membrane protein. Furthermore, the antibody was introduced into the intracellular transport pathway by microinjection of specific mRNA coding for the antibody heavy and light chains. Thus we utilized the cells' own secretory machinery to deliver the antibody to the correct location. Although we have not thus far attempted to extend our results to the biochemical level, there is no reason to believe that this cannot be done. Cleveland et al. (1983), for instance, have recently been able to demonstrate biochemically that tubulin biosynthesis is depressed in cells microinjected with purified tubulin. Clearly the procedures that we have employed here are not only applicable to the study of the Golgi complex, but may be used to investigate the function of any protein specific to any of the compartments that comprise the intracellular transport pathway. The only requirement is the availability of the appropriate monoclonal antibody.

Experimental Procedures

Cell Culture

3T3 cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and containing penicillin/streptomycin (Gibco). BHK cells were grown in Glasgow MEM containing 5% FCS, 10% tryptose phosphate broth (Gibco), and penicillin/streptomycin. Hybridomas were grown in Dulbecco's MEM containing 4.5 g/L glucose, 15% FCS, and penicillin/streptomycin. All cells were grown at 37°C in a humidified incubator with a 7.5% CO_2, 92.5% air atmosphere. For bulk growth of hybridomas, 5 L glass spinner culture vessels gassed with 5% CO_2, 95% air were used.

Dot Immunobinding Assay

Rat liver Golgi membrane vesicles (Bergeron, 1979) were treated with trypsin (10–1000 μg/ml) as described by Fleischer (1981) and modified by Burke et al. (1982) either in the presence or absence of 0.4% Triton X-100. Digestion was stopped by the addition of 25 mg/ml ovomucoid to give a final concentration of 3 mg/ml. After a 5 min incubation on ice the Triton X-100 concentration of all samples was brought to 0.4%. The samples were then diluted as appropriate (see Results) in PBS containing 0.2% Triton X-100 and 40 μg/ml PMSF. Two microliter aliquots (4-fold serially diluted) were spotted 1 cm apart onto a nitrocellulose filter (Glenney et al., 1982; Howe and Hershey, 1981). The filter was then air-dried and soaked in PBS containing 2% bovine hemoglobulin and 0.2% Triton X-100 (PBS/Hb/Tx). After 30 min the filter was transferred to 20 ml of 53FC3 culture supernatant and incubated for a further 45 min at room temperature. Following three 15 min washes in PBS/Hb/Tx the filter was incubated for 45 min at room temperature in the same solution containing rabbit anti-mouse IgG appropriately diluted. The filter was again washed three times and finally labeled with ^{125}I-protein A (New England Nuclear; 600,000 cpm/ml in PBS/Hb/Tx) for 45 min. After extensive washing the individual dots were cut out and counted. Galactosyl transferase was assayed as described by Bretz and Stäubli (1977) as modified by Bretz et al. (1980).

Immunofluorescence Microscopy

BHK or 3T3 cells were grown on glass coverslips and, following microinjection of mRNA or infection with VSV-*ts045* (Warren et al., 1983) were fixed and labeled with antibodies using the procedures described by Ash et al. (1977). Antibodies used were affinity-purified (Ternynck and Avrameas, 1976) and coupled to rhodamine (Brandtzaeg, 1973).

Microinjection

Messenger RNA at a concentration of 1 mg/ml was microinjected into the cytoplasm of BHK or 3T3 cells grown on glass coverslips. The procedures employed for microinjection were either those described by Ansorge (1982) or by Timm et al. (1983). Both techniques gave identical results.

Preparation of mRNA

Cells, grown to a density of 6 × 10^5/ml, were treated with 10 μg/ml cycloheximide (CHX) and the cells harvested in a Beckman J6-B centrifuge at 2,500 rpm for 10 min. The cells were then washed successively in PBS containing CHX; 100 mM MKCl, 2 mM Mg acetate, 5 mM DTT, 10 μg/ml CHX, 20 mM HEPES (pH 7.4), and finally in 15 mM KCl, 1.5 mM Mg acetate, 5 mM DTT, 10 mM HEPES (pH 7.4). After resuspension in 2 volumes of this last wash buffer, the cells were left on ice for 15 min and then broken by 20 strokes in a loose fitting Dounce homogenizer. One tenth by volume of 0.9 M KCl, 10 mM HEPES (pH 7.4) was added to the homogenate and the nuclei removed by centrifugation in a Sorvall SS34 rotor at 1,000 rpm for 5 min at 4°C. Microsomes were also collected by centrifugation in the SS34 at 20,000 rpm for 30 min at 4°C. They were resuspended in 120 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl (pH 7.5) and then SDS was added to a final concentration of 2%. RNA was then extracted in chloroform-phenol. Following the addition of Na acetate to 0.2 M the RNA was precipitated in ethanol at −20°C. The RNA was redissolved in 0.5 M NaCl, 1 mM EDTA, 0.5% SDS, 10 mM Tris-HCl (pH 7.5) and mRNA was prepared by affinity chromatography on oligo dT cellulose, as described by Blobel and Dobberstein (1977). After a final ethanol precipitation, the mRNA was redissolved in water to a concentration of 1 mg/ml. A small aliquot from the mRNA preparation was translated in vitro in the presence of dog pancreas microsomes to confirm that mRNA for the immunoglobulin heavy and light chains was indeed present (Meyer and Dobberstein, 1980; Warren and Dobberstein, 1978).

Acknowledgments

We thank Alix Cockroft for excellent technical assistance Wilhelm Ansorge, Henrik Garoff, and Beate Timm for help with the microinjections, David Meyer and Susanne Stämpfli for the in vitro translations, Bernhard Dobberstein for helpful discussion, Thomas Kreis and Dan Cutler for critical reading of the manuscript, and Annie Steiner for the typing.

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Received November 18, 1983; revised February 3, 1984

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