

A monoclonal antibody against a 135-K Golgi membrane protein

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A monoclonal antibody (53FC3) has been produced against a Golgi membrane protein with a mol. wt. of 135 000 which was originally identified using a polyclonal antiserum. Treatment of isolated, intact Golgi vesicles with protease caused a decrease in mol. wt. of 5000–10 000, whereas in the presence of Triton X-100, the protein was completely degraded. This shows that the protein spans the bilayer and that most of its mass is on the luminal side of Golgi membranes. Using two immunoelectron microscopic techniques, the protein was found in one or two cisternae on one side of the Golgi stack which, in normal rat kidney cells, had 4–6 cisternae. As an illustration of the use to which this monoclonal antibody can be put we present a light microscopic study of the disassembly and reassembly of the Golgi complex during mitosis.

Key words: Golgi complex/immunoelectron microscopy/immunofluorescence/mitosis/monoclonal antibody

Introduction

The Golgi complex in eukaryotic cells plays a pivotal role in the processing of secretory and lysosomal proteins as well as of proteins of the plasma membrane. As these proteins pass through the stacks of flattened Golgi cisternae they undergo a series of modifications which can include conversion of simple to complex oligosaccharides (reviewed by Hubbard and Ivatt, 1981), fatty acid acylation (Schmidt *et al.*, 1979), sulphation (Young, 1973), and the phosphorylation of mannose residues on those oligosaccharides bound to lysosomal proteins (von Figura and Klein, 1979; Hasilik and Neufeld, 1980; Reitman and Kornfeld, 1981). How these functions are distributed within the Golgi complex is still uncertain, but recent data suggests that the stack of Golgi cisternae can be subdivided into distinct functional domains. Immunocytochemical studies using anti-galactosyl transferase antibodies (Roth and Berger, 1982) and the galactose-specific lectin *Ricinus communis* agglutinin I (Griffiths *et al.*, 1982a, 1982b), have shown that complex oligosaccharides are constructed in the *trans* part of the Golgi stack. Biochemical studies have shown that 1,2- α -mannosidase can be separated from galactosyl transferase on sucrose gradients (Dunphy *et al.*, 1981) and these enzymes in turn can be separated from fatty acid acylation (Quinn *et al.*, 1982).

Further dissection of the Golgi complex, however, using purely biochemical methods, is confounded by two major difficulties. The first arises from the fact that the subcellular fractionation techniques currently available result in the destruction of any morphology by which specific sub-Golgi elements may be identified. Thus, any possible separation of Golgi functions may only be monitored as separations of enzymic activity with little or no possibility of referring back to the original Golgi structure. The second difficulty stems from

the fact that present fractionation procedures based on centrifugation techniques simply lack the required resolution. To circumvent these difficulties we therefore decided to take an alternative approach to the elucidation of the relationship between Golgi structure and function and to develop immunological markers for Golgi elements to complement the known enzymic markers. Antibodies against specific components of the Golgi complex would provide the unique ability to identify a particular Golgi-derived membrane in the intact cell using established immunocytochemical techniques and in the isolated state by means of an immunoassay.

In a previous report (Louvard *et al.*, 1982) we described the production of a polyclonal antiserum which, through the use of judicious adsorption steps, was directed largely against a single protein with a mol. wt. of 135 000 daltons found in rodent Golgi membranes. We realized, however, that in the long run, a collection of monoclonal antibodies against the Golgi complex might be of greater value. There are several reasons for this. Firstly, it would eliminate the necessity of having to adsorb out unwanted antibodies from crude antiserum. Secondly, even with the best designed adsorption protocols one may not be able to remove all of the minor unwanted antibodies. Thirdly, polyclonal antisera will be directed predominantly against the major antigenic component(s) of the Golgi preparation. When raising monoclonal antibodies on the other hand, one always has the possibility of screening for antibodies against minor components. A fourth advantage in raising monoclonal antibodies is that they can be produced in large quantities very easily. This would be of particular significance for those antibodies recognizing the cytoplasmic surface of Golgi membranes since they could be attached to a solid support and used as an immuno-adsorbant for the large scale purification of the membranes in the manner described by Ito and Palade (1978). This method has since been used to purify synaptic vesicles (Matthew *et al.*, 1981) and coated vesicles (Merisko *et al.*, 1982) and would circumvent the second difficulty referred to above since centrifugal separations would not be based on density alone.

In this paper we describe the production and characterization of a monoclonal antibody (53FC3) against Golgi membranes and show that it recognizes the same 135-K protein as our polyclonal antiserum. We have examined the location of this protein within the Golgi complex and its topology in the membrane. We have also used it as a marker to examine the fate of the Golgi complex during mitosis.

Results

Production of monoclonal antibody 53FC3

Hybrid cells secreting anti-Golgi antibodies were selected and screened for as described in Materials and methods. From a total of four fusions, 10 clones were identified which gave a typical Golgi-type staining in immunofluorescence studies on normal rat kidney (NRK) cells (Louvard *et al.*, 1982; Virtanen *et al.*, 1978). An example of this is shown in Figure 1 and is described more fully below. In addition, 34 cultures were identified containing hybridomas secreting antibodies against a variety of other cellular components, notably the cytoskeleton. Cloning of the anti-Golgi secreting hybrids, however, proved to be extremely difficult whether by

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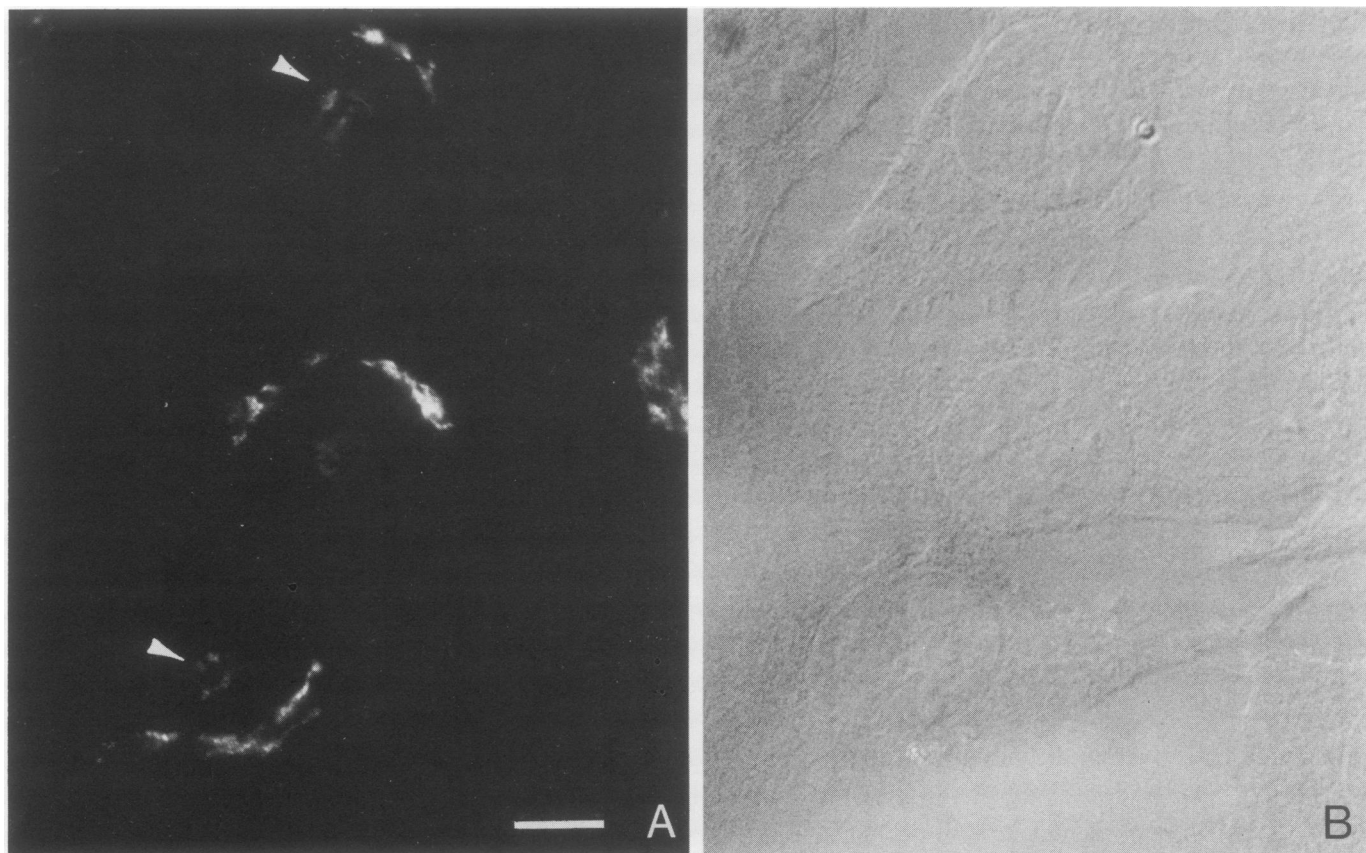


Fig. 1. The Golgi complex in interphase NRK cells revealed by indirect immunofluorescence labelling using 53FC3 (A). The reticular nature of the organelle is clearly seen with no obvious labelling of any vesicular structures. Note also the discrete patches of labelling above the nuclei (arrowheads). (B) Corresponding Nomarski photomicrograph. Bar equals 10 μm .

limiting dilution or by the soft agarose method. All but one of these cultures rapidly lost their anti-Golgi activity. This was especially surprising and disappointing since 2/3 of the other hybrids which were chosen for further study were successfully cloned and adapted to long term culture. A description of one of these, synthesizing an antibody against the nuclear lamins, is described elsewhere (Burke *et al.*, in preparation). In addition, most of these latter hybrids, after their first cloning in agarose, also showed 90–100% specific antibody-positive subclones suggesting that, in each well positive for cell growth after fusion, there were only, on average, one or two rapidly proliferating clones.

The anti-Golgi hybridoma, 53FC3, which was successfully cloned was shown to secrete an IgG by metabolic labelling with [^{14}C]leucine followed by analysis of secretory products by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (not shown). The subclass has not yet been determined.

Immunoprecipitation using 53FC3

[^{35}S]Methionine-labelled proteins, extracted from NRK cells with Triton X-114, were treated with 53FC3 followed by rabbit anti-mouse antibodies. The immune complexes were then bound to protein A-Sepharose, washed extensively, and fractionated by SDS-PAGE. The fluorograph presented in Figure 2 shows that the monoclonal anti-Golgi antibody was directed against a polypeptide with a mol. wt. of 135 000. The background level of other protein bands seen in both control and specific immunoprecipitations was low and invariably corresponded to major components of the original detergent extract. There is no doubt that this 135-K protein is the same as that recognized by our polyclonal antisera (Louvard *et al.*,

1982) since, as shown in Figure 3, specific immunofluorescence labelling of NRK cells by 53FC3 could be abolished by prior incubation of the fixed and permeabilized cells with the polyclonal rabbit anti-135 K antibody (Louvard *et al.*, 1982).

Immunoblotting

To examine the topology of the 135-K polypeptide in the Golgi membranes, vesicle fractions from rat liver Golgi were treated with increasing amounts of trypsin and the effect of this treatment on the 135-K polypeptide was monitored by immunoblotting. Since the monoclonal antibody did not detect the 135-K polypeptide blotted onto nitrocellulose paper, we used a high-titre, crude anti-Golgi antiserum prepared as described in Materials and methods. Since this polyclonal antibody reacted with Golgi proteins other than the 135-K polypeptide, we first affinity-purified the anti-135 K component of the antiserum using the immunoblotting protocol as described in Materials and methods.

Galactosyl transferase activity was used to monitor the effect of trypsin treatment. This enzyme is located within isolated Golgi vesicles on what *in vivo* would be the luminal side of the membrane. Fleischer (1981) has shown that this enzyme is resistant to trypsin treatment unless Triton X-100 is present during the digestion. We have carried out similar experiments using both trypsin and proteinase K. The results with trypsin are presented in Figure 4. In the presence of Triton X-100, galactosyl transferase activity was substantially reduced at trypsin concentrations in the range of 10–100 $\mu\text{g}/\text{ml}$ while in the absence of the detergent, and in five separate experiments, the activity was only reduced by 0–30%. In parallel experiments, protease-digested samples

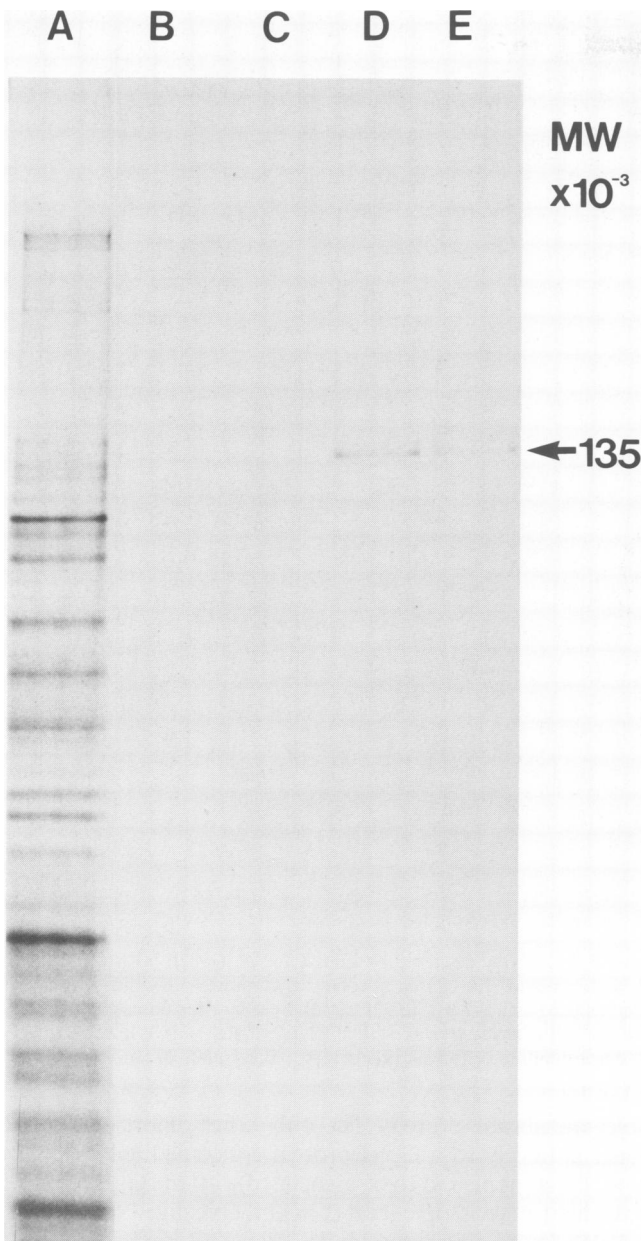


Fig. 2. Immunoprecipitation using 53FC3. **Lane A**, Triton X-114 extract of [35 S]methionine-labelled NRK cells. 100 times this amount was used in each immunoprecipitation. **Lanes B and C**, using control mouse IgG and washed in Triton X-100 (**B**) or Triton X-100 containing high salt (**C**). **Lanes D and E**, using 53FC3 and washed in either Triton X-100 (**D**) or Triton X-100 containing high salt (**E**).

were prepared for immunoblotting and labelled using the affinity-purified rabbit anti-135 K antibody. As shown in Figure 4, the 135-K polypeptide responded in the same way to trypsin treatment as did galactosyl transferase; the polypeptide was only extensively degraded by trypsin in the presence of Triton X-100 (Figure 4A–D). In the absence of Triton X-100, the mol. wt. of the polypeptide was reduced by 5000–10 000 daltons after treatment with trypsin. This 125 000-dalton ‘clipped’ form was resistant to further digestion even at trypsin concentrations as high as 1000 μ g/ml, indicating that most of the mass is on the luminal side of the membrane and that it spans the bilayer. This last suggestion is supported by the fact that the polypeptide appears to have a hydrophobic domain since it was selectively extracted by

Triton X-114 (Bordier, 1981) and it was such an extract which was then used to raise polyclonal antibodies which reacted with this polypeptide (Figure 4). We also used Na_2CO_3 -washed membrane fractions to prepare the monoclonal anti-Golgi antibody and it is known that Na_2CO_3 removes essentially all peripheral proteins leaving only those bound tightly to the membrane (Howell and Palade, 1982). These data clearly suggest that the 135-K polypeptide is anchored, with most of its mass on the luminal side of Golgi membranes, by a hydrophobic domain.

Immunofluorescence

Immunofluorescence labelling studies of interphase NRK cells using 53FC3 showed a brightly staining reticular network at one pole of the nucleus (Figures 1 and 3). The pattern was similar but not identical to that which we have previously seen using our polyclonal antisera. The major difference between the two lies in the fact that the polyclonal antiserum frequently appears to label vesicles in the proximity of the reticular network. 53FC3, on the other hand, labels only the reticulum and never labels vesicles (compare Figure 1 with Louvard *et al.*, 1982, Figure 1 and Green *et al.*, 1981, Figure 2c).

Immunoelectron microscopy

Two immunoelectron microscopic techniques were used to define the precise location of the 135-K polypeptide within the Golgi complex. Thin, frozen sections of baby hamster kidney (BHK) cells were incubated with 53FC3 followed by rabbit anti-mouse antibodies and protein A-gold. Despite the technical difficulties of locating minor antigens in thin sections using monoclonal antibodies, we were able to obtain specific labelling and show that it was entirely confined to Golgi cisternae (Figure 5A). A clearer picture was obtained using a more sensitive, albeit non-quantitative, immunoperoxidase technique which we used previously to characterise our polyclonal anti-Golgi antisera. The results of this are shown in Figure 5(B–D). Peroxidase label was found only in one or two cisternae on one side of the Golgi complex. The last cisterna on the labelled side of the stack was not stained, neither were any of the numerous small vesicles which may be found in the Golgi region. This again contrasts with results obtained with our polyclonal antiserum which appeared to show labelling of smooth and coated vesicles in the proximity of the Golgi complex and possibly more extensive labelling of the Golgi stack (compare Figure 5 with Louvard *et al.*, 1982, Figure 4).

Immunofluorescence labelling of mitotic cells

The fate of the Golgi complex in dividing cells is extremely difficult to follow using standard microscopic techniques. The reason for this lies in the fact that once the organelle has begun to disassemble at the onset of mitosis, all morphological criteria for the identification of its components are lost. An antibody against the Golgi complex or part of the Golgi complex thus provides a unique tool for studying the dissolution and reassembly of this organelle.

Figure 6 shows a gallery of cells at different stages of mitosis and labelled with both 53FC3 and Hoechst dye to indicate the mitotic stage of the cell. A very specific sequence of events was observed in NRK cells at the onset of cell division. During prophase (Figure 6A), the Golgi complex began to disassemble although, at this stage, the fragments so formed were large enough so as to be easily visible. Most striking, however, was that these large fragments became evenly dis-

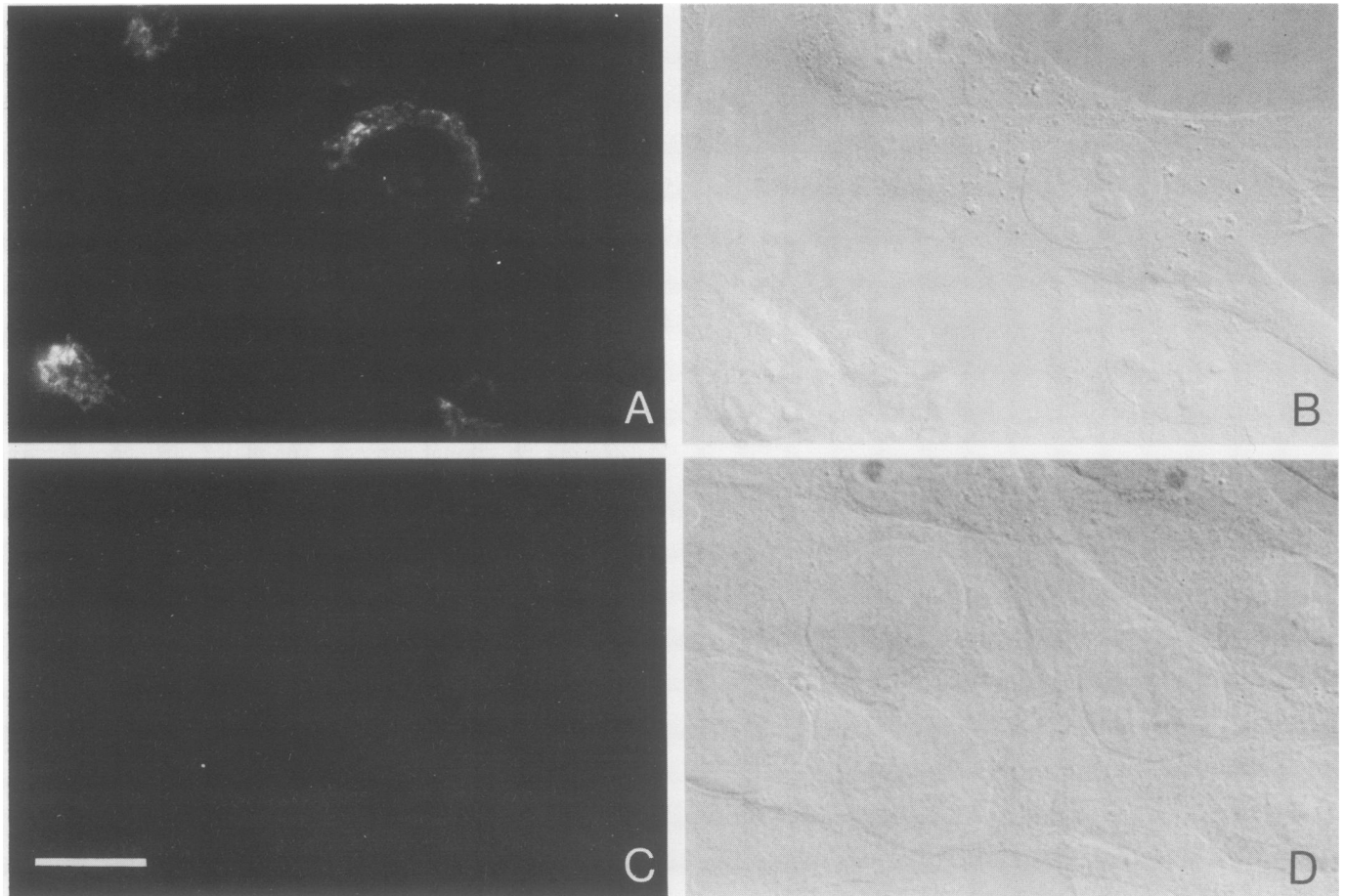


Fig. 3. Immunofluorescence competition experiment between 53FC3 and the rabbit anti-135 K antibody described by Louvard *et al.* (1982). NRK cells, grown on glass coverslips were fixed and permeabilised as described in Materials and methods and incubated for 30 min at room temperature with either non-immune rabbit serum (A) or the rabbit anti-135 K antibody (C). Each coverslip was then labelled as normal with 53FC3 followed by rhodamine-conjugated rabbit anti-mouse IgG. In (A) a distinctive reticular network may be seen at one pole of the cell nucleus, typical of the Golgi complex, in (C), however, the specific staining by 53FC3 is completely abolished by the rabbit antibody. (B) and (D), corresponding Nomarski photographs. Bar equals 20 μm .

tributed about the prophase nucleus. By metaphase (Figure 6B), most but not all of the large fragments had completely broken up, presumably into small vesicles below the limit of resolution of the microscope (0.2 μm). At this stage the cytoplasm appeared to be filled with diffuse fluorescence. However, some brightly staining large aggregates remained close to the poles of the mitotic spindle. This material disappeared in anaphase (Figure 6C). During telophase (Figure 6D), the Golgi complex appeared to reassemble, presumably by specific vesicle fusion. As we have previously reported (Louvard *et al.*, 1982), this occurred at two locations within each daughter cell, as they began to spread out but before their cytoplasmic junction became broken. The major site of reassembly in each cell occurred at the pole of the nucleus most distant from the mid body. The second, relatively minor site appeared close to the mid body at either pole of the remains of the mitotic spindle. Whether the two Golgi regions in each daughter cell actually join to form one large unit is uncertain. However, it is interesting to note that in many interphase cells, apart from the large reticulum at one pole of the nucleus, a smaller, possibly unconnected reticulum lying above the nucleus is frequently visible (see Figure 1).

Discussion

We have described the production and characterization of a monoclonal antibody (53FC3) which recognizes a 135-K

Golgi membrane protein. The only other report of a monoclonal antibody to a Golgi protein concerned a 110-K polypeptide that appeared to be associated with the Golgi complex at the light microscopic level (Lin and Queally, 1982). Since it was also found on occasion in the nucleus it is presumably not a membrane protein and would not therefore be such a reliable marker of the Golgi complex. We previously identified this 135-K protein using a Golgi antiserum made in rabbits (Louvard *et al.*, 1982). We showed that polyclonal antibodies were preferentially made to this protein despite the fact that rabbits were injected with Golgi membrane fractions containing several hundred different proteins. The fact that the monoclonal anti-Golgi antibody is also directed against this 135-K protein serves to emphasize its immunogenicity. We neither understand the reason for this immunogenicity nor the function of the polypeptide itself though the two may conceivably be related. The protein is, however, an excellent marker for the Golgi complex.

Perhaps not surprisingly, the gross specificity of 53FC3 differed in some respects from that of our polyclonal antiserum. It became clear to us immediately that 53FC3 produced a more restricted labelling pattern both at the light and at the electron microscopic level. This difference could arise if the polyclonal anti-Golgi antibody contained minor antibodies to other Golgi-associated components. Such minor components might be missed in immunoprecipitation experiments but

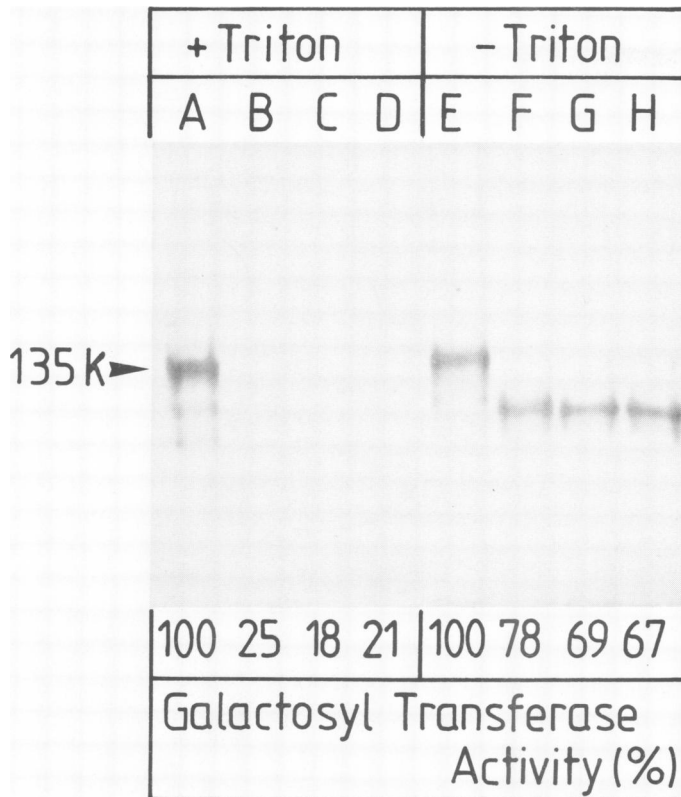


Fig. 4. The effects of increasing concentrations of trypsin on the 135-K protein in isolated rat liver Golgi vesicles. Duplicate 10 μ l samples of Golgi membranes (2 mg/ml) were treated with 0 (A and E), 10 (B and F), 100 (C and G), or 1000 μ g/ml (D and H) of trypsin either in the presence (A–D) or absence (E–H) of Triton X-100. Following incubation with the enzyme, the samples were either assayed for galactosyl transferase activity or were fractionated on a 7.5% SDS-PAGE, blotted onto a nitrocellulose filter and labelled with affinity purified rabbit anti-135 K antibodies. In the Triton-treated samples (A–D) the 135-K band was completely degraded by 10 μ g/ml trypsin, and at the same time galactosyl transferase activity was reduced by 75% over the non-enzyme treated control (A). In intact Golgi vesicles (E–H), when digested with 10 μ g/ml trypsin (F), the 135-K protein showed a 5000–10 000 dalton drop in mol. wt. leaving a protected fragment of ~125 000 daltons which remained resistant to trypsin even at a concentration of 1000 μ g/ml. At the same time, galactosyl transferase activity fell by only 22–33% over the control non-digested sample (E).

might not go unnoticed in immunocytochemical studies, especially those which rely on enzymic staining reactions such as the immunoperoxidase technique and which by their very nature introduce a large signal amplification. Clearly, this may give false emphasis to minor species of antibodies against other Golgi proteins.

An alternative but equally likely possibility is that the monoclonal antibody does not detect the 135-K protein present in membrane compartments recognised by the polyclonal antibody either for reasons of sensitivity (compare intensity of staining in Figure 5 with Figure 4 in Louvard *et al.*, 1982) or because the protein is in a form which obscures the single epitope recognised by the monoclonal antibody. This would explain, for example, why lysosomes degrading Golgi membranes are always stained by the polyclonal antibody (Louvard *et al.*, 1982) but never by the monoclonal antibody. The conditions prevailing within the lysosomal compartment could easily destroy the single epitope but considerable degradation would be needed to destroy all the epitopes recognised by the polyclonal antibody. In any event both the polyclonal and monoclonal antibodies are extremely useful

markers of the Golgi complex and any real differences in labelling would make them of greater analytical value.

We also noticed a difference in the specificity between 53FC3 and the rabbit anti-Golgi antibodies. Both antibodies would only recognize rodent Golgi membranes but, in addition, 53FC3 failed to recognize the 135-K polypeptide in at least some mouse cell lines. This is not in fact surprising since the antibody itself is made by mouse cells and must, of course, be secreted *via* the cell's own Golgi complex. One would expect that the cell might be placed under a considerable disadvantage if the antibody which it was secreting was able to bind to a component within its own Golgi complex. Naturally this could only occur if the antigenic determinant recognized by the antibody was found on the luminal face of the Golgi membranes, a postulate supported by our protease digestion studies on isolated Golgi vesicles.

Such a phenomenon might also go some way to explain why we had such difficulties in obtaining viable anti-Golgi secreting hybridomas, while hybridomas obtained in the same fusions and secreting antibodies against other cellular components were relatively easy to handle. If these cells were secreting antibodies which could interact with their own Golgi membranes there would be considerably greater than normal selective pressure against such hybrids. Non-secretors would thus accumulate very rapidly.

Finally, as an illustration of the use to which such an antibody can be put, we studied the fate of the Golgi complex during mitosis. At the turn of the century the Golgi was seen to fragment during mitosis (for a review, see Wilson, 1925) and these observations were confirmed by electron microscopy (e.g., Robbins and Gonatos, 1964; Zeligs and Wollman, 1979), though here the difficulty was that the fragmented Golgi vesicles could not be unambiguously identified. Specific anti-Golgi antibodies should remove this difficulty and even at the light microscopic level we have been able to show in overview the striking changes that occur. Within a matter of minutes the entire Golgi complex is dispersed into small vesicles which rapidly re-associate in telophase. A detailed study of this process may well lend insight into the normal functioning of the Golgi complex.

Materials and methods

Cells

NRK cells were maintained in minimum essential medium (MEM) containing 10% (v/v) foetal calf serum (FCS). BHK cells were grown in MEM (Glasgow) containing 5% (v/v) FCS. All cells were grown at 37°C in a humidified CO₂ incubator (7.5% CO₂, 92.5% air).

Immunization and cell fusion

5-week-old BALB/c mice were injected s.c. with 30 μ g of rat liver Golgi membranes washed with Na₂CO₃ (Howell and Palade, 1982) (a gift from K. Howell, EMBL) and emulsified in complete Freund's adjuvant. After 3–4 weeks the animals were treated with a further 30 μ g in incomplete adjuvant given i.p. Ten days after this injection a small blood sample was taken from each animal and the serum tested for specific anti-Golgi antibodies by indirect immunofluorescence on NRK cells (below). Each positive animal was given a second boost after 10 days. This consisted of 70 μ g of antigen in phosphate buffered saline (PBS) pH 7.6 given i.p. Three days after this final boost, the spleen was removed from the animal, the cells dissociated and fused with the SP2/0-Ag14 myeloma line (Schulman *et al.*, 1978) according to procedures described by Galfre *et al.* (1977). After fusion, the cells were gently suspended in MEM (Dulbecco) containing 20% (v/v) FCS, penicillin/streptomycin, hypoxanthine, aminopterin, and thymidine and distributed into 10 x 24 well tissue culture trays. After 2–3 weeks, supernatants from cultures positive for hybrid cell growth were screened for specific antibodies by indirect immunofluorescence microscopy (below). Positive cultures were cloned in agarose (Indubiose, Industrie Biologique Francaise; Coffino and Scharff (1971) and injected into pristane-primed mice to form ascites tumours (Koprowski *et al.*, 1977).

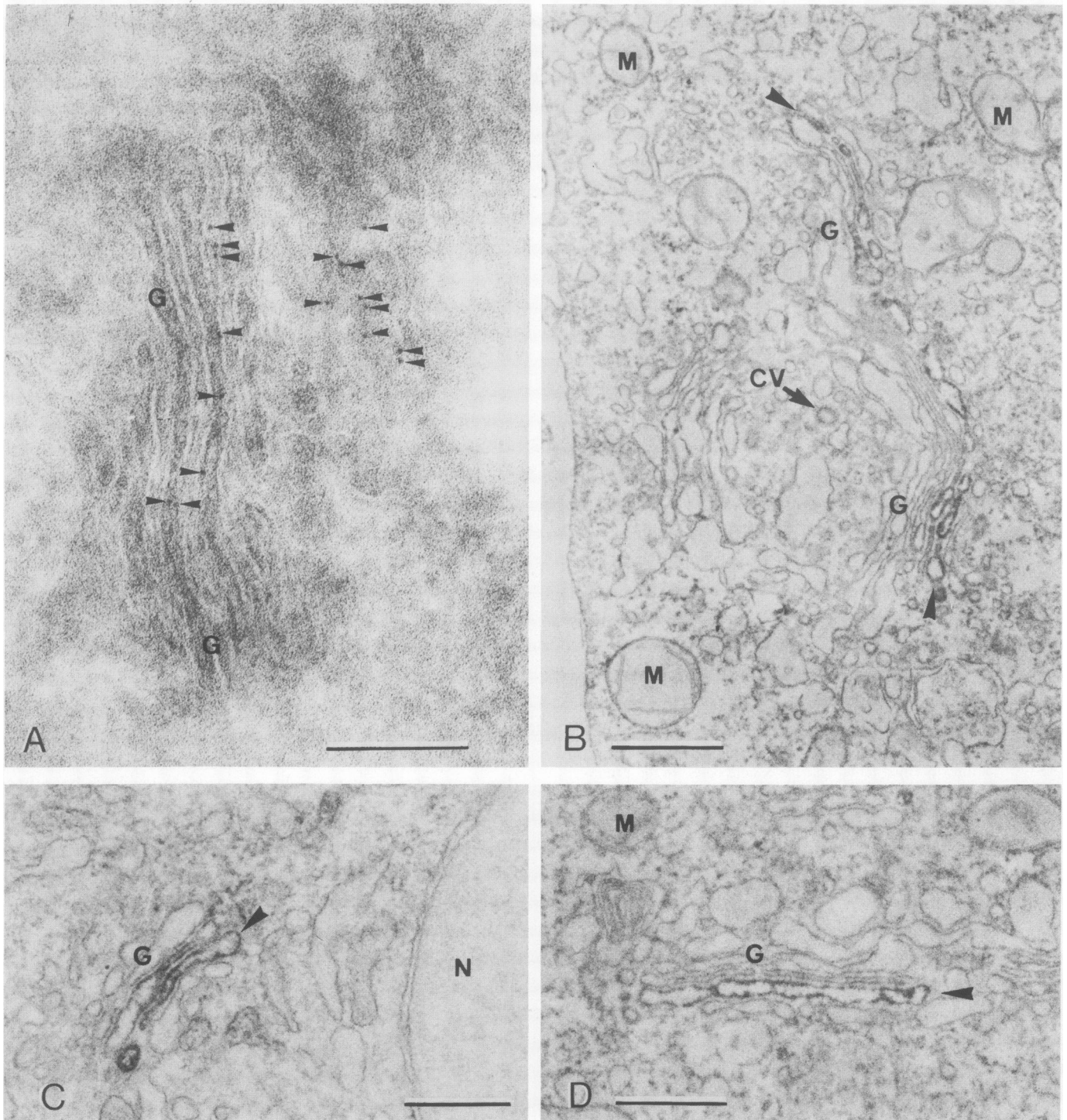


Fig. 5. Immunoelectron microscopic localisation of the 135-K protein in BHK cells (A) and NRK cells (B–D). In (A) a thin frozen section of BHK cells was labelled first with 53FC3 followed by rabbit anti-mouse IgG and protein A conjugated to colloidal gold. Since the contrast needed to visualize the Golgi cisternae tended to obscure the gold particles we have indicated their positions with small arrowheads. In (B–D) the location of the 135-K protein recognized by 53FC3 was visualized by means of an immunoperoxidase technique. It is clear that only one, or at most two, cisternae of the Golgi stacks are labelled (large arrowheads) with few, if any vesicles being labelled. CV, coated vesicle; G, Golgi stack; M, mitochondrion; N, nucleus. Bar equals (A) 0.1 μm , (B) 0.5 μm (C and D) 0.2 μm .

Screening of hybrids

A novel high resolution immunofluorescence procedure was used for screening culture supernatants. NRK cells were grown in Falcon 60-well Terasaki plates. The cells were then fixed and permeabilized according to the method of Ash *et al.* (1977). For labelling with antibody the liquid in each well was carefully aspirated and replaced with 25 μl of culture supernatant. After 20 min incubation at room temperature, the entire plate was washed 3 times in PBS containing 0.2% gelatin (Ash *et al.*, 1977) and then 25 μl of rhodamine-conjugated rabbit anti-mouse IgG was similarly pipetted into each well. After

a further 20 min incubation at room temperature the plate was then washed four times with PBS/gelatin and then twice with PBS. Each well was then examined under epi-illumination using a Zeiss photomicroscope III fitted with a plate holder of our own design and made by Zeiss, FRG, and using either a X25 oil/glycerol/water immersion objective or a X63 water immersion objective.

Polyclonal anti-Golgi antisera

To improve upon the titre of our previous anti-Golgi antisera (Louvard *et*

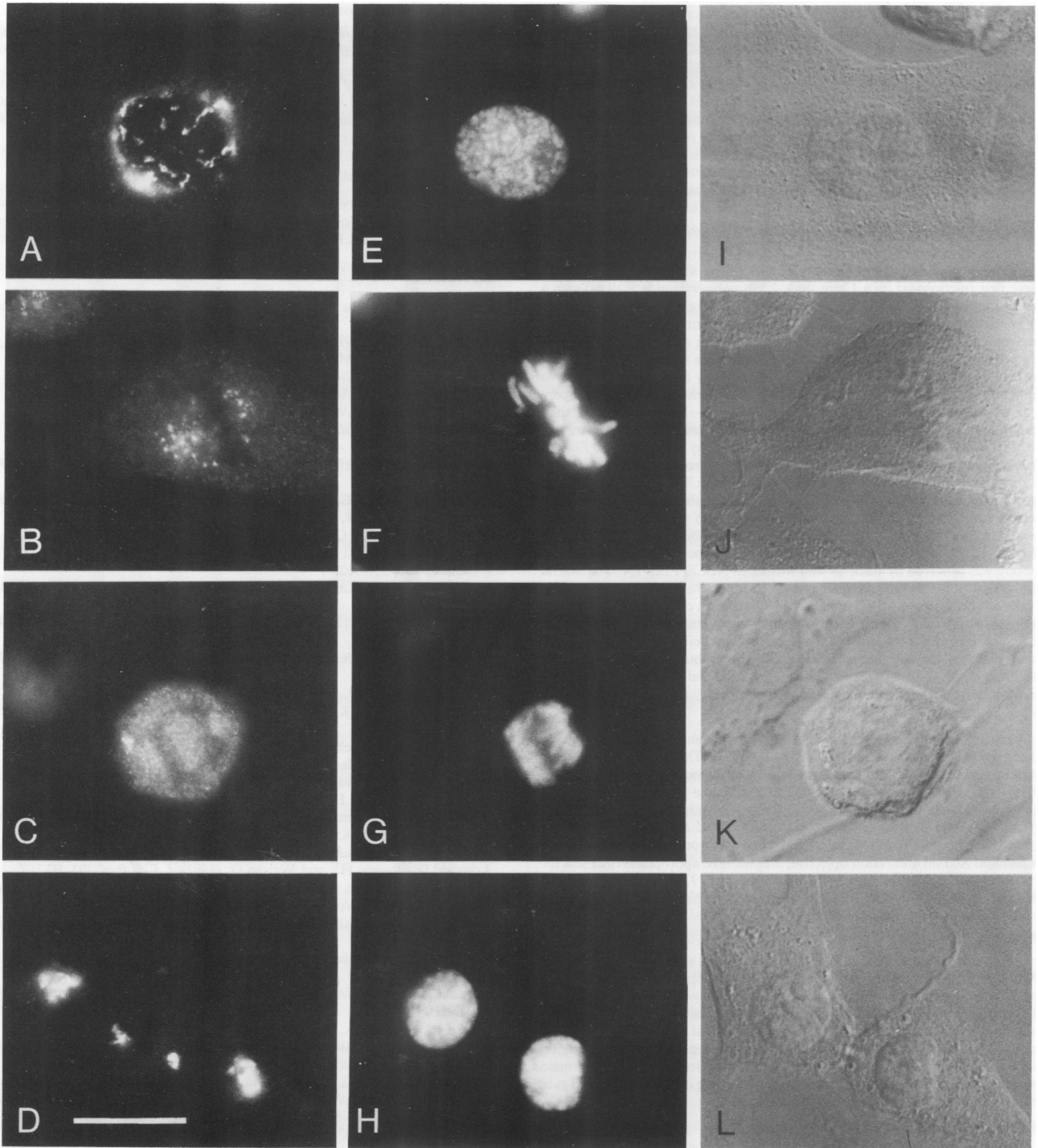


Fig. 6. The Golgi complex in mitotic NRK cells revealed by immunofluorescence (A–D) using 53FC3. (A) Prophase cell. The Golgi complex is partially fragmented and distributed about the prophase nucleus. (B) Metaphase. Almost all discernible structure has disappeared although some large fragments remain near the poles of the mitotic spindle. (C) Anaphase. (D) Late telophase. Two reassembly points for the Golgi complex are visible in each of the daughter cells. I–L are the corresponding Nomarski photographs, while E–H are of the same cells labelled with Hoechst dye (33258) which indicated the stage of mitosis reached. Bar equals 20 μ m.

al., 1982), rat liver Golgi membrane fractions (Bergeron, 1979) were extracted with Triton X-114 (Bordier, 1981) and the detergent subsequently removed by sucrose density gradient centrifugation (Simons *et al.*, 1978). The resulting hard pellet of protein was dispersed in PBS by sonication and injected into rabbits as described previously (Louvard *et al.*, 1982). Immunofluorescence ex-

periments showed that the titre of anti-Golgi antibody was ~200 times higher than that of the previous antiserum (Louvard *et al.*, 1982).

Immunoblotting

Immunoblotting was performed essentially as described by Burnette (1981)

but with the following modifications. Following electroelution, the nitrocellulose filter was soaked at room temperature in PBS pH 7.4 containing 10% (v/v) new born calf serum (NBCS) and 0.2% Triton X-100 (NBCS/PBS/TX) for 30 min followed either by undiluted affinity purified rabbit anti-135 K (below) for 1.5 h or by crude rabbit anti-Golgi diluted 1:500 in NBCS/PBS/TX for 1 h. The filter was then washed three times for 15 min in NBCS/PBS/TX followed by the same medium containing 1/1000 dilution of sheep anti-rabbit IgG conjugated to horse radish peroxidase (Pasteur Institute) for 1 h. After washing three times in NBCS/PBS/TX as described above, the labelled bands were then visualized by means of the peroxidase reaction described by Graham and Karnovsky (1966) using diaminobenzidine as substrate. The reaction was stopped by rinsing the filter in PBS.

Affinity purification of the polyclonal anti-135 K antibody

An immunoblotting procedure was used to affinity-purify the anti-135 K component of the rabbit antiserum. Total rat liver Golgi proteins (0.5 mg) were first fractionated on a 1 mm thick 7.5% polyacrylamide SDS gel with a single sample slot 12 cm wide and then blotted overnight onto a nitrocellulose filter (as above). Following transfer, the filter was immersed in PBS at 4°C and two narrow (0.5 cm) strips were cut from each side. These strips were then labelled with crude rabbit anti-Golgi antibodies and the labelled bands visualized using a peroxidase-linked second antibody (see above). These two antibody-labelled strips were then used to locate precisely the 135-K polypeptide on the remainder of the filter. This band (1–2 mm wide) was then carefully excised, a process which was greatly simplified by first visualizing the protein bands on the filter itself. This was achieved by immersing the filter in 0.2% Ponceau S (Serva Heidelberg) in 3% trichloroacetic acid (TCA) for 5 min and then destaining in distilled water for 1 min. The filter strip was then transferred immediately to NBCS/PBS/TX and incubated for 1.5 h at room temperature. During this period the Ponceau S stain was released. The strip was then labelled overnight at 4°C using crude rabbit anti-Golgi antiserum diluted 1:100 in NBCS/PBS/TX. Following this incubation, unbound antibody was removed by three 15 min washes in NBCS/PBS/TX. The bound anti-135 K antibody was then eluted by immersing the nitrocellulose strip, at room temperature, in 1.5 ml of a 0.2 M HCl solution containing 0.2% gelatin adjusted to pH 2.8 with 2 M glycine also containing 0.2% gelatin. After 2 min the strip was removed and the solution, containing the eluted antibody, neutralized by the addition of 0.5 ml 1 M Tris (unbuffered). Following the addition of 20 μ l of 20% Triton X-100, the undiluted solution was used immediately for further immunoblotting experiments.

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed and stained with first antibody using the procedures described by Ash et al. (1977). The second antibody was rabbit anti-mouse IgG which had been affinity-purified (Ternynck and Avrameas, 1976) and then conjugated to rhodamine (Brandtzaeg, 1973). In some cases, cells were further labelled for 2 min with 2 μ g/ml Hoechst dye (number 33258) in PBS (Berlin et al., 1978). Fixed and labelled cells were observed using epi-illumination on a Zeiss photomicroscope III equipped with a planapo X63 oil immersion objective, and with the appropriate filter sets for rhodamine and Hoechst dye 33258 (Berlin et al., 1978). Labelling with the Hoechst dye, a DNA-specific stain, allowed unambiguous identification of mitotic cells and the stage they had reached in mitosis.

Immunoprecipitation

NRK cells were labelled with [³⁵S]methionine and were extracted in Triton X-114 as described by Bordier (1981). Immunoprecipitation was carried out essentially as described before (Louvard et al., 1982) with the following minor modifications. Since the immunoprecipitation was carried out using a mouse antibody, a non-immune mouse IgG (an anti-Semliki Forest virus E2 monoclonal antibody) was used for the preadsorption step. Following incubation with the specific (mouse) antibody and prior to addition of protein A-Sepharose, each sample was incubated with 20 μ g of affinity-purified rabbit anti-mouse IgG for 30 min at room temperature.

Protease treatment

Golgi membrane vesicles (Bergeron, 1979) were treated with trypsin essentially as described by Fleischer (1981). Duplicate samples (10 μ l) of Golgi membrane vesicles [5 mg protein/ml in 10% (w/v) sucrose, 5 mM HEPES pH 7.0, 1 mM MgCl₂] were treated with 5 μ l 50 mM HEPES buffer pH 7.0 containing 0, 30, 300, 3000 μ g/ml TPCK-trypsin (Sigma) for 60 min at 25°C in the presence or absence of Triton X-100 at a final concentration of 0.4% (w/v). One set of samples was treated with 2 μ l 25 mg/ml ovomucoid (Sigma) and the entire sample assayed for galactosyl transferase activity using the method of Bretz and Stäubli (1977) as modified by Bretz et al. (1980). The other set of samples was precipitated with 10% (w/v) TCA, prepared for gel electrophoresis (Quinn et al., 1982) and fractionated on a 7.5% gel (Maizel, 1969). Immunoblotting was performed as described above using the undiluted affinity-purified rabbit anti-135 K antibody (above).

Immunoelectron microscopy

Immunoperoxidase labelling. This was carried out as described by Ohtsuki et al. (1978) and Tougard et al. (1980) with the modifications described by Louvard et al. (1982).

Immunolabelling of thin frozen sections. Thin frozen sections were prepared and labelled with specific antibody and protein A-gold using the methods first described by Tokuyasu (1973) with the modifications of Griffiths et al. (1982c).

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