Characterization of Cytoplasmically Oriented Golgi Proteins with a Monoclonal Antibody

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ABSTRACT BALB/c mice were repeatedly immunized with a galactosyl transferase-rich microsomal fraction of rat myeloma cells. Spleen cells were subsequently fused with Sp2/0 mouse myeloma cells, the resulting hybridomas were cloned, and their secreted Ig was screened for reactivity with antigens belonging to the Golgi complex. One such monoclonal antibody, 6F4C5, gave especially intense immunofluorescent staining of the Golgi area of myeloma cells and fibroblasts. It recognized two proteins bands on immunoblots of gel-fractionated cell lysates: a major one with an estimated M_r of 54,000 and a minor one at 86,000. Both proteins were concentrated in microsomal fractions isolated at low ionic strength. They were hydrophilic judging from partitioning of a Triton X-114 cell lysate. Both were cytoplasmically oriented as demonstrated by protease and high KCl treatments of postmitochondrial supernatants and microsomal fractions. Neither was retained by columns of insolubilized wheat germ agglutinin or concanavalin A, which suggests that they are not glycoproteins. Their more detailed location in the Golgi complex was studied by immunoelectron microscopy, using a saponin permeabilization procedure and peroxidase-conjugated reagents. The observed staining was restricted to two or three cisternae in the medial part of the stack. Nevertheless, differential centrifugation experiments indicated that the two antigens may be recovered in distinct subcellular fractions: this may be related to the unexpected observation that rather low salt concentrations strip the antigens from microsomal fraction.

The Golgi complex plays a central role in vesicular traffic in the cell cytoplasm. In addition to mediating intracellular transport, it is a site of maturation of oligosaccharides of glycoproteins and appears to accomplish the sorting of lysosomal enzymes from secretory and cell surface glycoproteins (1–3). It is composed of vesicles and a stack of flattened cisternae. Those near the rough endoplasmic reticulum (proximal cisternae) are thought to receive vesicle-enclosed quanta of proteins which exit from the rough endoplasmic reticulum. At the other (distal) face of the stack, vesicles or vacuoles that contain mature content (e.g., with complete oligosaccharide chains) bud off toward the cell surface. A cytochemical heterogeneity of the stack has been demonstrated in many cell types, although only a very small number of markers is available. The conventional markers are an unidentified osmiophilic material that is restricted to the proximal cisternae, nucleoside diphosphatase (thiamine pyrophosphatase) which is found in distal cisternae, and acid phosphatase which is found in the still more distal structure named GERL (4). Moreover, antibodies directed toward galactosyl transferase have been used to detect the enzyme in distal cisternae of HeLa cells (5). This result is in agreement with the observation that wheat germ agglutinin (WGA)[1] (which binds more mature oligosaccharides) stains the same Golgi region and a population of vesicles that are thought to be in transit to the cell surface of myeloma cells (6).

The existence of functionally distinct subcompartments in the Golgi complex is also indicated by biochemical studies. Enzyme activities involved in oligosaccharide processing can be partially separated by subfractionation of microsomal frac-

[1] *Abbreviations used in this paper:* Con A, concanavalin A: Mab, monoclonal antibody; NP-40, Nonidet P-40; PAGE-IB, PAGE followed by immunoblotting; PMS, postmitochondrial supernatant; 0.25 M STKM, a buffer solution containing 0.25 M sucrose, 50 mM Tris-HCl, pH 7.3, various concentrations of KCl, and 5 mM $MgCl_2$; TBS, Tris-buffered saline; TX-100 and TX-114, Triton X-100 and -114, respectively; WGA, wheat germ agglutinin.

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tions on isopycnic gradients (7–10). Studies of the maturation of biosynthetically labeled secretory proteins (11), and the study of the effects of ionophores (1, 12), also indicate functional specialization.

As a step toward further dissection of the Golgi complex, we and others (13–15) are developing an extensive new set of markers of this composite organelle: a family of monoclonal antibodies (MAbs). With such antibodies it should prove possible to have markers for at least as many subregions of the Golgi complex as are presently distinguished and possibly for structures for which no markers are available. It should also prove possible to establish the cytologic origin of available Golgi subfractions isolated by isopycnic sedimentation, to study the biosynthesis of Golgi proteins representative of the various subcompartments, and possibly (for antigens on the cytoplasmic face of Golgi elements) to purify the corresponding subfractions by immunoadsorption.

We report here the production and characterization of one such Mab (6F4C5) which is strikingly Golgi specific.

MATERIALS AND METHODS

Cells

IgM-secreting (IR202; reference 16) and nonsecreting (YB2/0; reference 17) rat myeloma cells were gifts from H. Bazin (University Catholique de Louvain, Brussels, Belgium) and C. Milstein (Medical Research Council Laboratory, Cambridge, England). The cells were recovered from ascitic fluid of pristane-primed Lou rats injected intraperitoneally 1 wk earlier with 10^7 cells. FR 3T3 rat fibroblasts (18) came from F. Cuzin (Université de Nice, France). They were maintained in Dulbecco's modified Eagle's medium which contained 5% fetal calf serum.

Subcellular Fractionation and Preparation of Cell Lysates

A total microsomal fraction was purified according to Tartakoff (unpublished). In brief, 2×10^8 IR202 cells in 0.5 ml hypotonic salt solution were homogenized at 4°C with a Dounce homogenizer, then adjusted to 0.25 M sucrose, 50 mM Tris-HCl, pH 7.3, 25 mM KCl, and 5 mM $MgCl_2$ (0.25 M $STK_{25}M$) in the presence of 10 μg/ml cycloheximide, DNAse (10 μg/ml), and phenylmethylsulfonyl fluoride (40 μg/ml). After a first centrifugation for 3 min at 2,200 *g* to eliminate nuclei and unbroken cells, 2-ml samples of the supernatant were centrifuged for 7.5 min at 6,500 g_{av} in a Ti50 rotor equipped with adapters for 2-ml tubes (Beckman Instruments Inc., Palo Alto, CA). The resulting "postmitochondrial" supernatant (PMS) was then centrifuged for 45 or 150 min (see figure legends) at 165,000 g_{av} in the Ti50 rotor. The pelleted total microsomal fraction was resuspended in 0.2 ml of 0.25 M $STK_{25}M$ and loaded onto a 20–45% linear mannose $TK_{25}M$ gradient, which was centrifuged overnight at 165,000 g_{av} in an SW 50.1 swinging bucket rotor (Beckman Instruments, Inc.). Fractions were collected with an Autodensiflo II (Buchler Instruments Inc., Fort Lee, NJ) and their density was determined by using an organic column of 1,2-dichlorobenzene and pet ether. Each fraction was assayed for galactosyl transferase activity (*vide infra*). Fractions rich in galactosyl transferase (between 1.14 and 1.16 g/ml) were pooled and will be referred to as the "Golgi preparation" (11).

Rat liver Golgi fractions were isolated according to the procedure of Leelevathi as modified by Tabas and Kornfeld (19). Cell lysates were obtained by resuspension of 10^8 cells in 1 ml of 1% Nonidet P-40 (NP-40) in Tris-buffered saline (TBS; see below) followed by 5-min sedimentation at 2,200 *g* to eliminate nuclei. Protein was determined according to Lowry et al. (20) using BSA as a standard.

Immunization and Cell Fusion

BALB/c mice were injected four times weekly intraperitoneally with samples of the Golgi preparation containing 100 μg of protein. The first sample was emulsified with complete Freund's adjuvant in a total volume of 0.5 ml, the latter ones with incomplete Freund's adjuvant. 3 d after a final intraperitoneal boost with 500 μg protein (of Golgi preparation) in PBS, spleens from three mice were teased and 10^8 cells from the resulting suspension were used for fusion with 3×10^7 Sp 2/0 Ag 14 mouse myeloma cells (21) according to the procedure described by Galfré et al. (22). After fusion, cells were suspended in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, hypoxanthine, aminopterin, and thymidine, plus streptomycin and penicillin, and they were distributed into five 96-well tissue culture trays that contained 3×10^3 mouse peritoneal cells per well. After 15–20 d, supernatants from wells with growing cells were screened by a dot immunobinding assay (23). Cells recovered from positive wells were cloned twice by limiting dilution in the presence of feeder peritoneal cells, and the clones were grown in mass culture or injected into pristane-primed BALB/c mice to obtain ascitic fluid.

Analysis of Monoclonal Antibodies

IMMUNOBINDING DOT ASSAY: The procedure was that of Hawkes et al. (23) using immobilized rat liver Golgi fractions. 1-μl samples of a 0.4 mg/ml rat liver Golgi fraction in 50 mM Tris-HCl, pH 7.3, 200 mM NaCl (TBS) were dotted onto small squares of nitrocellulose. They were saturated for 30 min at room temperature with agitation with 20% normal rabbit serum in TBS, incubated 2 h with 100 μl of undiluted culture supernatant, and washed three times with TBS, followed by incubation with peroxidase-conjugated rabbit anti-mouse Ig (Nordic Laboratory, Tillburg, The Netherlands) at 1/500 dilution in 20% normal rabbit serum/TBS. After three rinses, squares were incubated with 4 chloro-1-naphthol (Merck Chemical Div., Merck & Co., Rahway, NJ)/H_2O_2 reagent (23).

IMMUNOFLUORESCENCE: Cells (IR 202, YB 2/0, FR 3T3 rat fibroblasts) were grown on glass coverslips, fixed with formaldehyde as described by Ash et al. (24), and permeabilized with 0.2% Triton X-100 (TX-100) in 0.2% gelatin (Bio-Rad Laboratories, Richmond, CA)/PBS. The coverslips were then incubated with culture supernatants for 30 min (or with purified 6F4C5 Mab, intact or biotinylated), repeatedly washed with 0.2% gelatin/PBS, and then stained with rhodamine- or fluorescein-conjugated rabbit anti-mouse Ig or rhodamine-conjugated avidin (Vector Laboratories, Inc., Burlingame, CA). After further washings, cells were observed with an epifluorescence illumination photomicroscope (Zeiss).

TRANSFER AND IMMUNOBLOT STAINING: IR 202 cell lysates or subcellular fractions (2×10^6 cell equivalents) were electrophoresed after reduction on 7.5–20% gradient polyacrylamide SDS slab gels (25) and transferred to nitrocellulose (26). Transfers were stained with the hybridoma supernatants or with purified 6F4C5 Mab as in the dot immunobinding assay (see above), or with Amido black (Merck & Co.) (27) to detect all protein bands. In all cases, molecular weight standard proteins (Bio-Rad Laboratories) were run in parallel. The electrophoresis, transfer, and immunostaining procedure is referred to as PAGE-IB.

Localization of IR202 IgM on the transfer was accomplished with a rabbit anti-IR202 IgM antiserum (gift of H. Bazin) followed by a peroxidase-conjugated goat anti-rabbit Ig (Nordic Laboratory). In these cases, saturation and incubations were in 0.2% gelatin/TBS.

Analysis of Cell Fractions

PROTEASE TREATMENT OF PMS: PMS from 2×10^6 IR202 cells was incubated with trypsin *N*-tosyl-L-phenylalanine chloromethyl ketone (Millipore Corp., Bedford, MA) or pronase (Calbiochem-Behring, La Jolla, CA) at 0.5 and 5 μg/ml for 60 min at 4°C in the absence or presence of 0.5% TX-100. At the end of the incubation a mixture of inhibitors was added: 5 mM EGTA, 50 μg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride (final concentrations). Control experiments lacked proteases or had inhibitors added before the proteases. The incubations were terminated by addition of 10% trichloroacetic acid. The resulting precipitates were collected by sedimentation, dissolved in reducing sample buffer, and analyzed by PAGE-IB. In parallel, galactosyl transferase activity was assayed in each of these conditions before trichloroacetic acid precipitation.

HIGH SALT TREATMENT: IR 202 PMS or total microsomal fractions (obtained after 150 min of sedimentation) were adjusted to 25, 100, or 500 mM KCl in 0.25 M sucrose, 50 mM Tris-HCl, pH 7.3, 5 mM $MgCl_2$, incubated 15 min at 4°C, and centrifuged 150 min at 165,000 g_{av} in a Ti 50 rotor. Supernatants and pellets (2×10^6 cell equivalents) were analyzed by PAGE-IB. Pellets were also assayed for the latency of galactosyl transferase activity.

Lectin-binding Protein Analysis

IR 202 cell lysates were loaded onto 1-ml WGA-Sepharose or concanavalin A (ConA)-Sepharose (both from Pharmacia Fine Chemicals, Upsala, Sweden) Pasteur pipette columns equilibrated in 0.1% NP-40/TBS, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride (column buffer). After 1 h at room temperature, the columns were washed with 5 ml of column buffer (pool A) and eluted with 5 ml of 0.5 M α-methyl mannoside or

0.5 M *N*-acetyl glucosamine (both from Sigma Chemical Co., St. Louis, MO) in column buffer, for Con A- and WGA-Sepharose columns, respectively (pool B). Pools A and B were precipitated with 7 vol of acetone at 0°C, reduced, and prepared for PAGE-IB.

Immunoelectron Microscopy

IR 202 cells in suspension or adherent to tissue culture dishes were fixed with 4% paraformaldehyde for 1 h at room temperature and processed in the presence of 0.05% saponin as in reference 28 using biotinylated 6F4C5 protein (30 $\mu g/10^7$ cells) followed by peroxidase-conjugated avidin (Vector Laboratories, Inc.) at 60 $\mu g/10^7$ cells. After several washes including saponin, the cells were refixed with glutaraldehyde and then incubated 30 min at room temperature with peroxidase substrates in the presence of imidazole at pH 7.3 (29), postfixed with 1% OsO_4 in 0.1 M cacodylate, pH 7.3, for 60 min at 4°C, dehydrated in graded ethanols, and embedded in Epon. Unstained sections were examined in a Philips 300 electron microscope. In some experiments, this staining was combined with that of thiamine pyrophosphatase (30) which came after the refixation.

Miscellaneous Procedures

Extraction of membrane proteins with Triton X-114 (TX-114) was as described by Bordier (31). Galactosyl transferase activity assay was performed according to Bretz, Bretz, and Palade (32) with ovalbumin as acceptor. For latency studies, assays were performed in the presence or absence of Triton. Biotinylation was as recommended by Guesdon et al. (33), with a biotinyl *N*-hydroxy-succinimide ester (Calbiochem-Behring Corp.) to amino group ratio of 1:1. (This procedure yields 42% blocking of the amino groups in sheep Ig [33]). Test of Ig class of the Mab by immunodiffusion was with Miles rabbit anti-mouse subclasses (Miles Laboratories Inc., Elkhart, IN).

Purified 6F4C5 Mab was obtained from ascitic fluid or culture supernatant by a first precipitation with $(NH_4)_2SO_4$ at 50% saturation. After sedimentation, the pellet was resuspended in PBS and dialyzed 24 h at 4°C against PBS. The retained fraction was loaded onto a column of rabbit anti-mouse Ig-Sepharose, washed with 0.5 M NaCl in PBS, and eluted with 0.2 M glycine, pH 2.8. The resulting Ig fraction was concentrated by pressure dialysis in PBS for 24 h and then diluted 1:1 in glycerol and stored at −20°C.

Elution of the 6F4C5 Mab from immunoblots was effected by a 30-min incubation of the transfer in 0.2 M glycine, pH 2.8, at room temperature (14). The eluate was removed and adjusted to pH 7.3 with 2 M Tris-HCl, pH 7.3, and used at once. Coupling of proteins to Sepharose was as recommended by Pharmacia Fine Chemicals with CNBr-activated Sepharose. Fluorescent probes were conjugated as in reference 34.

RESULTS

The four hybridization experiments performed yielded 32 positive wells, as judged by the dot test using rat liver Golgi fractions. Each population was cloned twice. Among these a further selection by immunofluorescent staining of FR 3T3 rat fibroblasts allowed us to select eight clones. Supernatants of four of them stained a restricted perinuclear region corresponding to the Golgi area, as judged by staining with WGA-rhodamine (not shown), whereas the others stained both the Golgi area and other sites in the cell cytoplasm. None were obviously reactive with the cell surface.

6F4C5, the most strikingly positive clone as judged by both tests, is the subject of this report. By immunodiffusion, we have determined that this antibody is a $\gamma 1$.

Immunofluorescence

Fig. 1 shows the intracellular staining observed with 6F4C5 Mab which reveals a perinuclear reticulated structure typical of the Golgi area. A similar pattern was observed with fibroblasts (Fig. 1, *a* and *b*) and myeloma cells (Fig. 1, *c* and *d*).

Immunoblot Staining

A cell lysate was reduced and submitted to PAGE-IB (Fig. 2*a*). Two bands were consistently detected, of approximate M_r 54,000 (major band) and 86,000 (minor band).

Antibodies that banded to the 54,000- and 86,000-mol-wt regions of the transfer were eluted separately (see Materials and Methods). Both preparations stained a cell lysate transferred to nitrocellulose with a pattern indistinguishable from that of Fig. 2*a*. Thus, these two proteins share a common antigenic determinant.

Localization of 6F4C5 Antigen to the Total Microsomal Fraction

Various cell fractions were then analyzed by PAGE-IB (Fig. 2, *b–e*). When the PMS was sedimented for 45 min at 165,000 g_{av}, the 86,000-mol-wt species was recovered in supernatant and the 54,000-mol-wt protein was found primarily in the pellet. When the centrifugation was extended to 150 min both proteins were detected in the pellet and little or no 86,000-mol-wt protein was left in the supernatant (Fig. 2). These observations show that the 54,000-mol-wt species is associated with a particle that is more readily sedimented than that of the 86,000-mol-wt species. Relatively little 54,000- and almost no 86,000-mol-wt protein were detected in the mitochondrial fraction (not shown).

Studies on Lectin Binding

The lectins Con A and WGA were then used to explore the possible presence of carbohydrate in the antigens recognized by 6F4C5. An NP-40 cell lysate was loaded onto either a Con A- or a WGA-Sepharose column and specifically bound glycoproteins were eluted as indicated in Materials and Methods. The presence of the antigens in the flow-through or in the specifically eluted fractions was monitored by PAGE-IB. Results are shown in Fig. 3. The 54,000- and 86,000-mol-wt proteins were detected only in the flow-through fractions of both columns, where their staining intensity was comparable to that of the sample loaded. As a control, it was observed that when the nitrocellulose strips were incubated with Con A peroxidase, the only stained bands were in the specifically eluted glycoproteins (not shown). Hence, the 54,000- and 86,000-mol-wt proteins are probably not glycoproteins, but we cannot exclude that they contain O-linked oligosaccharides.

Since the proteins recognized by 6F4C5 Mab appear to be membrane associated, it is important to know (*a*) whether they are intrinsic or extrinsic membrane proteins, and (*b*) on which side of the membrane their antigenic sites are exposed. To this end, we treated cell fractions with detergent, high KCl concentration, or proteolytic enzymes.

Localization of the Antigens

TX-114 is useful for rapid and quantitative partitioning of integral membrane proteins with a hydrophobic domain and soluble, hydrophilic proteins (31). After extraction of IR202 myeloma cells with TX-114, aqueous and detergent phases were analyzed by PAGE-IB. The validity of the extraction was monitored in two ways. (*a*) The activity of galactosyl transferase, a Golgi membrane protein that requires detergent for its solubilization (32), was assayed in both fractions: 86% of the recovered activity was found in the detergent extract. (*b*) IgM of the myeloma was stained by immunoblotting: all Ig was detected in the aqueous phase, none in the detergent phase (Fig. 4). Immunoblots that had been stained with 6F4C5 showed that both proteins were recovered in the aqueous phase, indicating that they are probably *not* intrinsic mem-

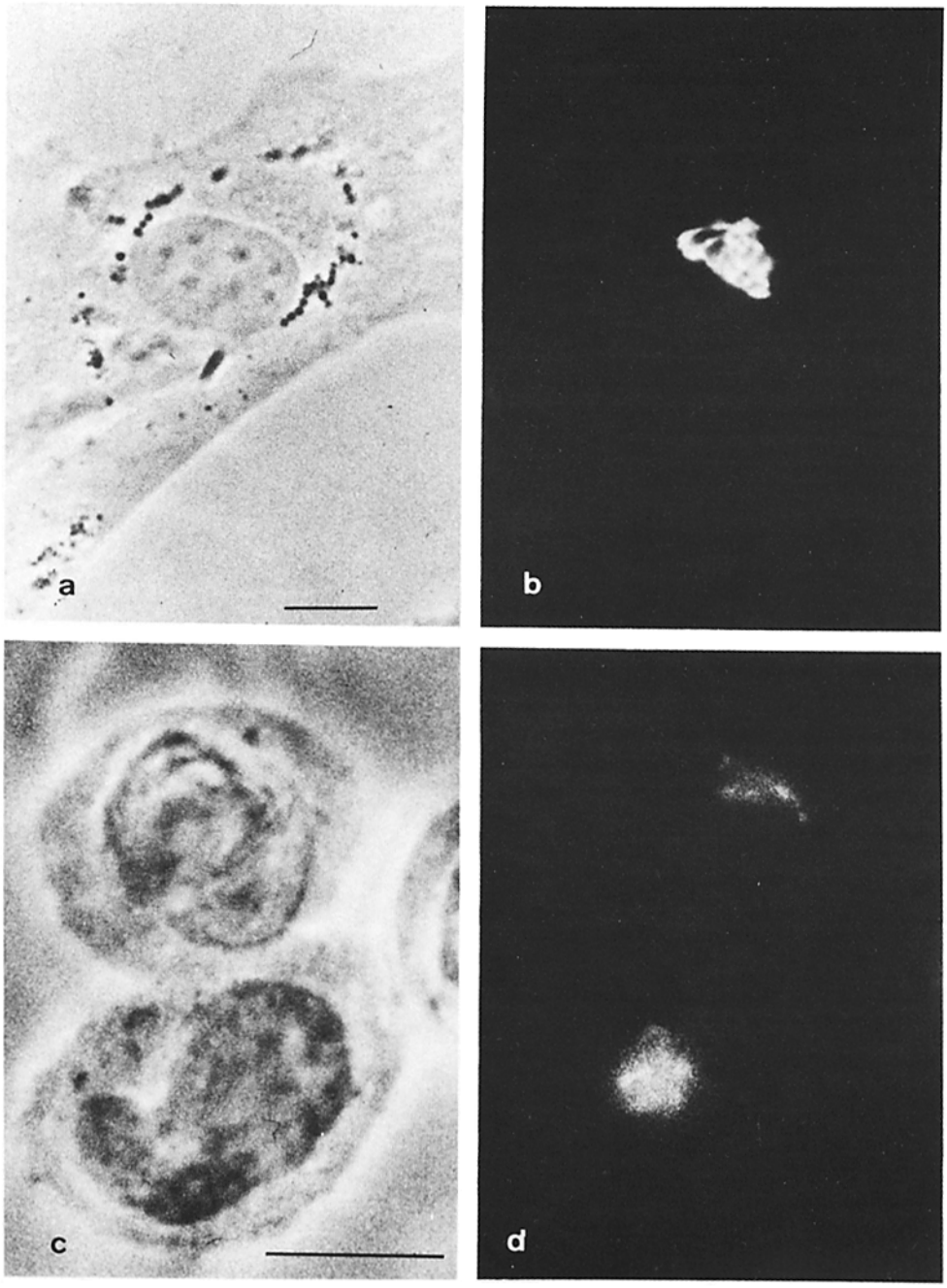


FIGURE 1 Immunofluorescent staining by 6F4C5 Mab of fibroblasts FR3T3 (*a* and *b*) and myeloma cells YB 2/0 (*c* and *d*). Cells were grown on glass coverslips, then fixed and stained with culture supernatants as described in Materials and Methods. (*a* and *c*) Phase-contrast micrographs. (*b* and *d*) Immunofluorescence of the same fields. Note the intense staining in the perinuclear cytoplasm. In the fibroblasts a reticular pattern is visible. YB2/0 cells have been chosen instead of IR202 cells because IgM of the latter cross-reacts with mouse Ig. Bar, 10 μM. (*a* and *b*) × 1,085. (*c* and *d*) × 2,320.

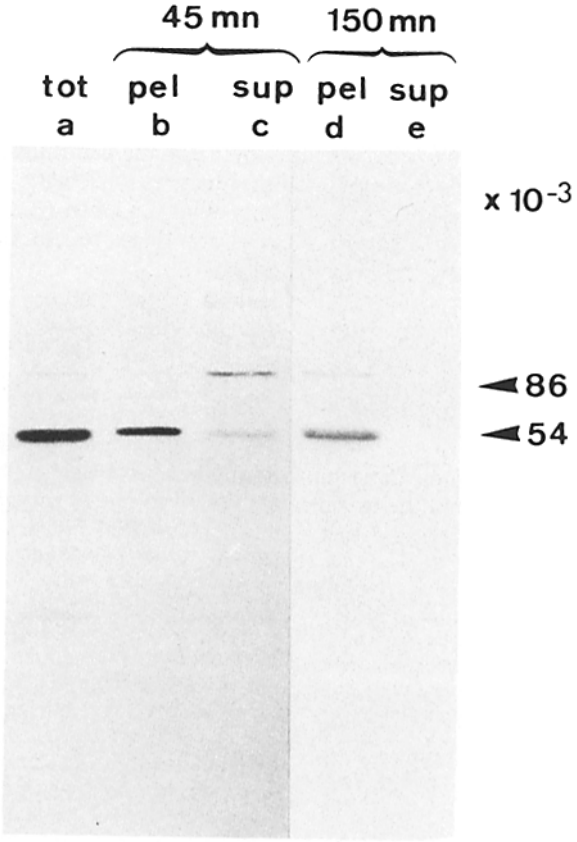
brane proteins (Fig. 4).

To further explore the localization of these antigens, we treated subcellular fractions with high KCl concentrations which should release adlumenal extrinsic proteins, but not extrinsic proteins on the lumenal face of the microsomal membrane (35). PMS and total microsomal fractions (150-min centrifugation) were adjusted to 0.25 M STKM at 25, 100, and 500 mM KCl and sedimented for 150 min at 165,000 g_{av}. The presence of 6F4C5-specific proteins in the sedimented fractions or in the supernatants was monitored by PAGE-IB (Fig. 5). Galactosyl transferase activity and latency in the pellets were determined in parallel, as indications of the intactness of the vesicles at different concentrations of KCl. As can be seen (Fig. 5), both proteins became nonsedimentable at 100 or 500 mM KCl and some 86,000-mol-wt species was even released at 25 mM KCl. This extraction was not due to a permeabilization of microsomal vesicles since 84–94% of the total galactosyl transferase activity remained latent. Thus, the 54,000- and the 86,000-mol-wt molecules appear to be associated with the cytoplasmic face of the microsomal membrane.

To confirm this hypothesis, we explored the protease-sensitivity of the antigens. Samples of PMS were incubated with a range of concentrations of pronase or trypsin in the absence or presence of TX-100. Even at the lowest doses of proteases in the absence of detergent, the 54,000- and 86,000-mol-wt proteins were susceptible as judged by immunoblot analysis. In contrast, galactosyl transferase activity was abolished by such treatments only in presence of detergent. Fig. 6 shows the result of trypsin treatment.

Localization to Submicrosomal Fractions

At 25 mM KCl, both proteins were recovered in microsomal fractions. Nevertheless, it was not possible to establish

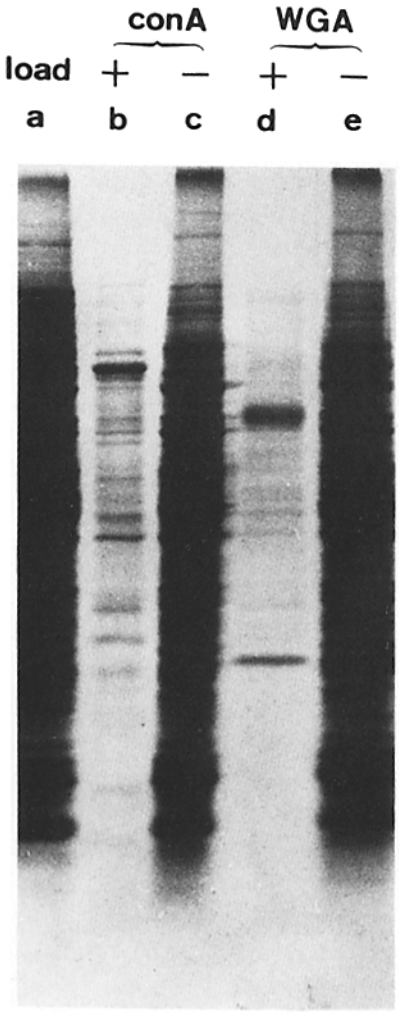


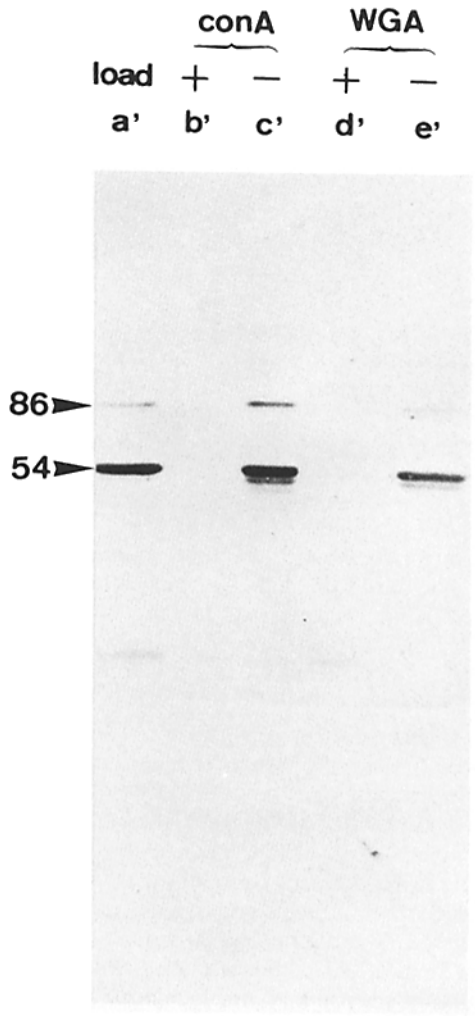
their submicrosomal origin by isopycnic subfractionation of microsomes because subfractionation in the presence of 25 mM KCl resulted in extensive pelleting of the antigens (along with a considerable amount of galactosyl transferase) with rough microsomes (ρ>1.22 g/ml), whereas at KCl concentrations >100 mM, the antigens were no longer membrane associated.

Immunoelectron Microscopy

Since immunofluorescence clearly showed the staining of the Golgi area by the 6F4C5 Mab, we used ultrastructural immunocytochemistry to examine the more detailed localization of this staining of the Golgi complex. Although immunoblotting can be performed after glutaraldehyde treatment of the nitrocellulose strip (not shown), the use of even small amounts (0.05%) of glutaraldehyde is not compatible with an immunocytochemical staining by the 6F4C5 Mab, despite the saponin treatment (unpublished observations). Cells were therefore fixed only with paraformaldehyde. Fig. 7*a* shows

FIGURE 2 Identification by immunoblotting of the antigen recognized by 6F4C5 Mab. IR202 PMS were centrifuged 45 min (lanes *b* and *c*) or 150 min (lanes *d* and *e*) at 165,000 g_{av}. The respective pellets (lanes *b* and *d*) and supernatants (lanes *c* and *e*) and an IR-202 NP-40 cell lysate (lane *a*) were reduced and analyzed by PAGE-IB. Arrowheads indicate the specifically stained proteins. Each of the three samples (*a*, *b* and *c*, and *d* and *e*) is derived from 2 × 10^6 cells.





the overall distribution of staining in the IR202 cells: peroxidase staining is almost completely restricted to the Golgi complex. The only other structures that are stained are the focal portions of the rough endoplasmic reticulum which are lightly positive. At higher magnification, the strong staining is seen to be restricted to the Golgi cisternae (Fig. 7*b*); small vesicles budding off or in the vicinity of the Golgi cisternae may also be stained (Fig. 8). In most cases the staining clearly outlines the membranes of the cisternae (cf. Fig. 7, *c*). In other images (Figs. 7*b* and 8) it appears to cover the whole width of a cisterna probably as a result of a diffusion of the enzymatic reaction product (36), which may be facilitated by the exposure to saponin. The cytochemical marker of distal cisternae, thiamine pyrophosphatase, was used to further establish the exact location of the stained structures. As can be seen (Fig. 8), thiamine pyrophosphatase was restricted to the two most distal cisternae, the peroxidase reaction product was found in medial cisternae, and the most proximal cisternae were unstained.

DISCUSSION

The monoclonal antibody described in this paper was raised by immunization of mice with a smooth microsomal fraction of rat myeloma cells, obtained by isopycnic centrifugation and selected for its high galactosyl transferase activity. It is one of a series of Mab that clearly stain the Golgi complex and was chosen for this study because its fluorescent localization is especially striking.

We will discuss first the nature and orientation of the proteins recognized by this Mab, then their localization in situ in the Golgi complex as revealed by immunoelectronmicroscopy.

The Mab 6F4C5 recognizes two proteins, a major one of 54,000 mol wt, and a minor one of 86,000 mol wt, which share a common antigenic determinant, as proven by the observation that the Ig molecules bound to one of them can be eluted and bound to the other. Many Mabs have been reported to recognize several proteins (37–39). In the present case, it is difficult to decide whether these two proteins only

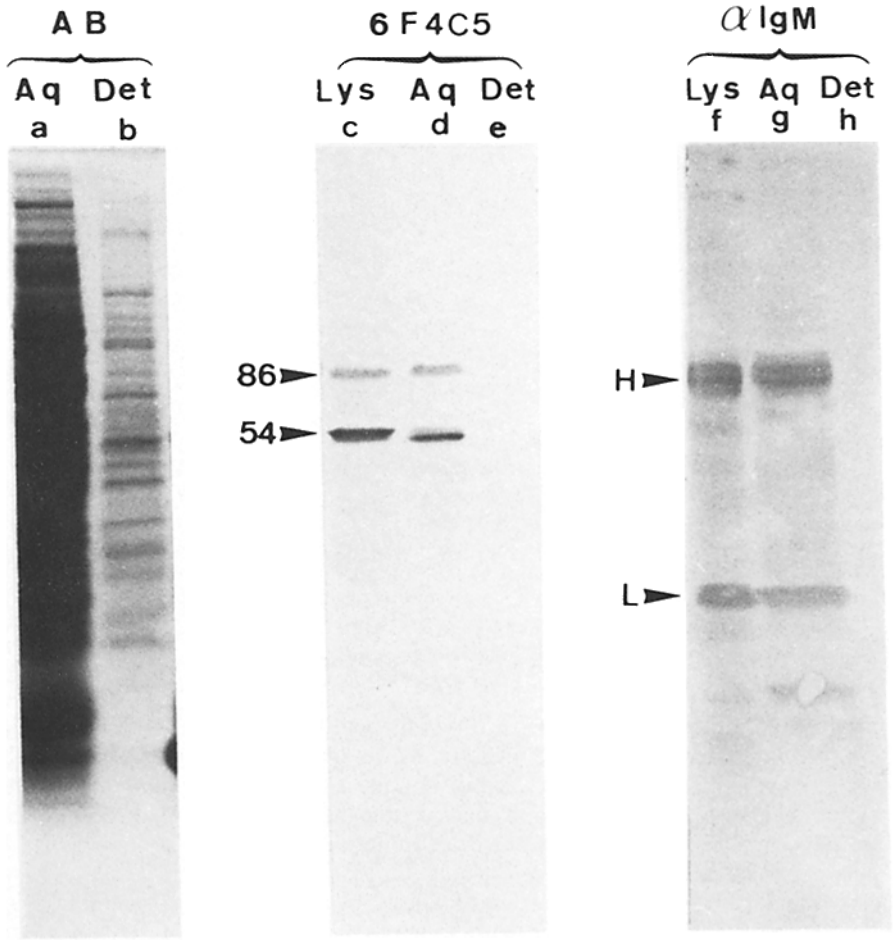


FIGURE 4 Extraction with TX-114 of IR-202 myeloma cells. IR 202 cells were lysed with TX-114 at 4°C and clarified by a 5-min centrifugation (2,200 *g* at 4°C). The resulting supernatant was partitioned with TX-114 (31). The aqueous and detergent phases were reduced and analysed by PAGE-IB. (lanes *a* and *b*) Amido black staining of aqueous and detergent phases, respectively. (lanes *c–e*) Immunostaining by 6F4C5 of the total lysate, aqueous, and detergent phases, respectively. (lanes *f–h*) Immunostaining (by an antiserum anti-rat) of IgM of the total lysate, aqueous, and detergent phases, respectively.

FIGURE 3 Immunoblot staining by 6F4C5 of fractions eluted from WGA- and Con A-Sepharose columns. NP-40 lysates of IR202 myeloma (lanes *a* and *a'*) were loaded onto Con A- and WGA-Sepharose columns. After 1 h at room temperature, columns were rinsed and glycoproteins eluted specifically (see Materials and Methods). Con A-Sepharose flow-through (lanes *c* and *c'*) and specifically eluted (lanes *b* and *b'*) fractions, and WGA-Sepharose flow-through (lanes *e* and *e'*) and eluted (lanes *d* and *d'*) fractions, were run on SDS gels and transferred to nitrocellulose. The paper was cut and one half (lanes *a–e*) was stained with amido black and the other (lanes *a'–e'*) was immunostained with 6F4C5 as in Fig. 2. Although a range of proteins is retained by the lectin columns (lane *b* and *d*), the antigens of interest are not (lanes *b'* and *d'*). In *d*, the proteins specifically eluted from the WGA column are mainly heavy and light chains of IR202 IgM.

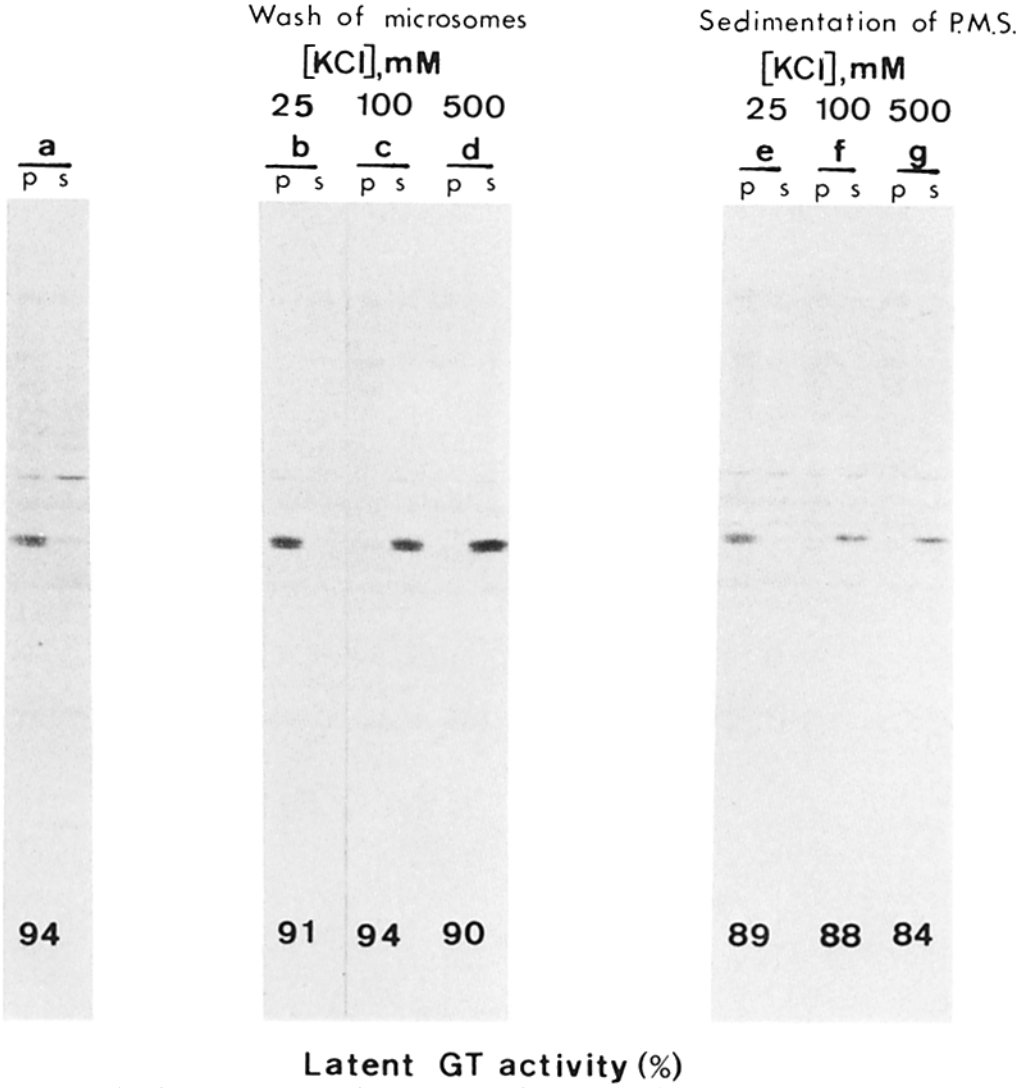


FIGURE 5 Influence of KCl concentration on the association of 6F4C5 specific proteins to microsomal vesicles. Analysis by PAGE-IB. (lane *a*) Sample of sedimented PMS (150′ at 165,000 g_{av}). (lanes *b–d*) Microsomal fractions collected by sedimentation of PMS (150 min at 165,000 g_{av}) were resuspended by hand (Dounce, two to three strokes) in 0.25 M STKM containing 25, 100, or 500 mM KCl. Each was then resedimented (150 min at 165,000 g_{av}) and the resulting pellets (*p*) and supernatants (*s*) were analyzed. (lanes *e–g*) Samples of PMS were diluted with an equal volume of 0.25 M STKM to yield 25, 100, and 500 mM KCl. They were sedimented and analyzed as for lanes *b–d*. In parallel, pellets were assayed for galactosyl transferase activity in the presence or absence of TX-100 and the percent latency (activity without detergent/activity with detergent) was determined. By this measure, it is evident that the KCl treatments did not change the degree of intactness of the microsomes.

share a common antigenic determinant or whether they are more directly related, for instance by a precursor product relationship. On the one hand, both proteins share similar characteristics in their relation with the smooth membranes: (*a*) They are extrinsic proteins localized on the cytoplasmic side of the membranes (as shown by their release at high KCl concentration); (*b*) they are present in the hydrophilic phase after extraction of the membranes with TX-114; and (*c*) they are similarly sensitive to protease treatment of microsomes in the absence of detergent. Furthermore, both the 54,000- and the 86,000-mol-wt molecules appear to be free of N-linked oligosaccharides. On the other hand, membranes bearing the 54,000-mol-wt proteins present in the PMS sediment faster (entirely present in the pellet after 45 min at 165,000 g_{av}) than the structure bearing the 86,000-mol-wt protein (Fig. 2). The evidence thus suggests that these proteins are recovered on different-sized particles after cell homogenization. If the site of synthesis of one or both of them is on the rough endoplasmic reticulum, they must move very quickly to the Golgi complex judging from the immunocytochemical data. On the other hand, considering the orientation of these proteins, which is like cytochrome b_5 and NADH cytochrome b_5 reductase (40–42), it is more logical to anticipate synthesis on free ribosomes followed by posttranslational membrane association. Furthermore, the 86,000-mol-wt protein is still detectable in very small amounts in the PMS, even after long centrifugation times, when most of the membranes and all the 54,000-mol-wt antigens have pelleted. If the 86,000-mol-wt protein were indeed synthesized on free ribosomes, it is conceivable that after rapid membrane binding, it is cleaved and transported to another site (possibly still part of the same cisternae), thus being the precursor of the major 54,000-mol-wt protein. A partial analogy may be found in the synthesis of certain reticulocyte proteins which are associated with the

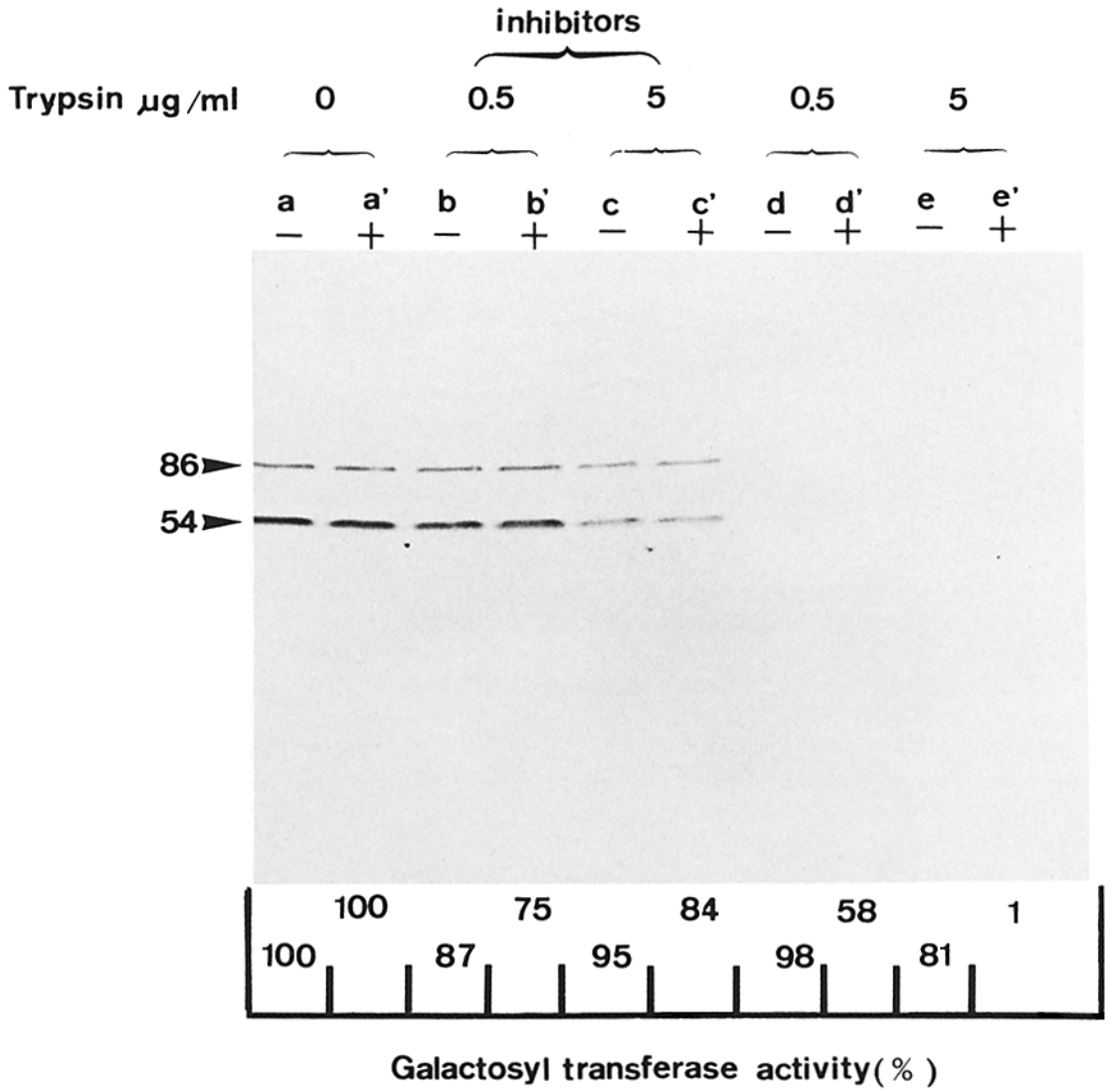


FIGURE 6 Susceptibility to proteases of 6F4C5 specific proteins in an IR202 PMS. Samples or IR202 PMS were incubated with trypsin at 0 (lanes *a* and *a'*), 0.5 (lanes *b*, *b'*, *d*, and *d'*) and 5 μg/ml (lanes c, *c'*, e, and e') for 60 min at 4°C in the absence (lanes a–e) or presence (lanes a'–e') of TX-100. Inhibitors were added at the end of the incubation. In control experiments, samples were incubated with inhibitors before and during protease treatment (lanes b, c, b', and c'). Samples were then assayed for transferase activity, reduced, and submitted to PAGE-IB. It is clear that both antigens are cleaved in the absence of detergent, in conditions that maintain microsomal integrity.

inner surface of the plasma membrane proteins (43).

The release of the antigens from microsomal membranes at subphysiological KCl concentrations (~150 mM; see reference 44) is surprising. This might cause some problems in the purification of Golgi subfractions by immunoadsorption. Several considerations bear on the weakness of the interaction: (*a*) In the cell sap a variety of ions, metabolites, and soluble macromolecules may influence the membrane affinity of the antigens in question. Indeed, when subcellular fractionation is conducted in the presence of 100 mM KCl, the amount of the antigens recovered in the microsomal pellet is inversely proportional to dilution of the PMS (unpublished observation); (*b*) the mechanical trauma of homogenization may labilize the adhesion of such components. Moreover, it is possible that homogenization fragments Golgi cisternae into domains of distinctive composition, size, and/or density. Such considerations may explain the apparent nonidentity of the sedimentation properties of the two antigens. Cytochemical and morphological heterogeneity in single cisternae have been described (45).

Whatever their possible interrelationship, the two proteins recognized by 6F4C5 Mab are the first identified Golgi proteins localized to the cytoplasmic face of Golgi membranes. To date, four other Mabs against Golgi proteins have been described. One detects a 110,000-mol-wt protein in the Golgi region (13), and the others recognize Golgi intrinsic membrane proteins of 135,000 (14), 103,000–108,000, and 180,000 mol wt (15). Since the present Mab selectively reacts with at least three rat cell types, namely myeloma cells, fibroblasts, and liver, it is likely that the corresponding proteins are basic elements of the Golgi membranes, and possibly are important for effecting transport.

At the ultrastructural level, the staining with 6F4C5 is primarily of Golgi cisternae, as opposed to vesicles. When a cross section of the Golgi stack was examined, only some of the cisternae were stained and these tended to be centrally

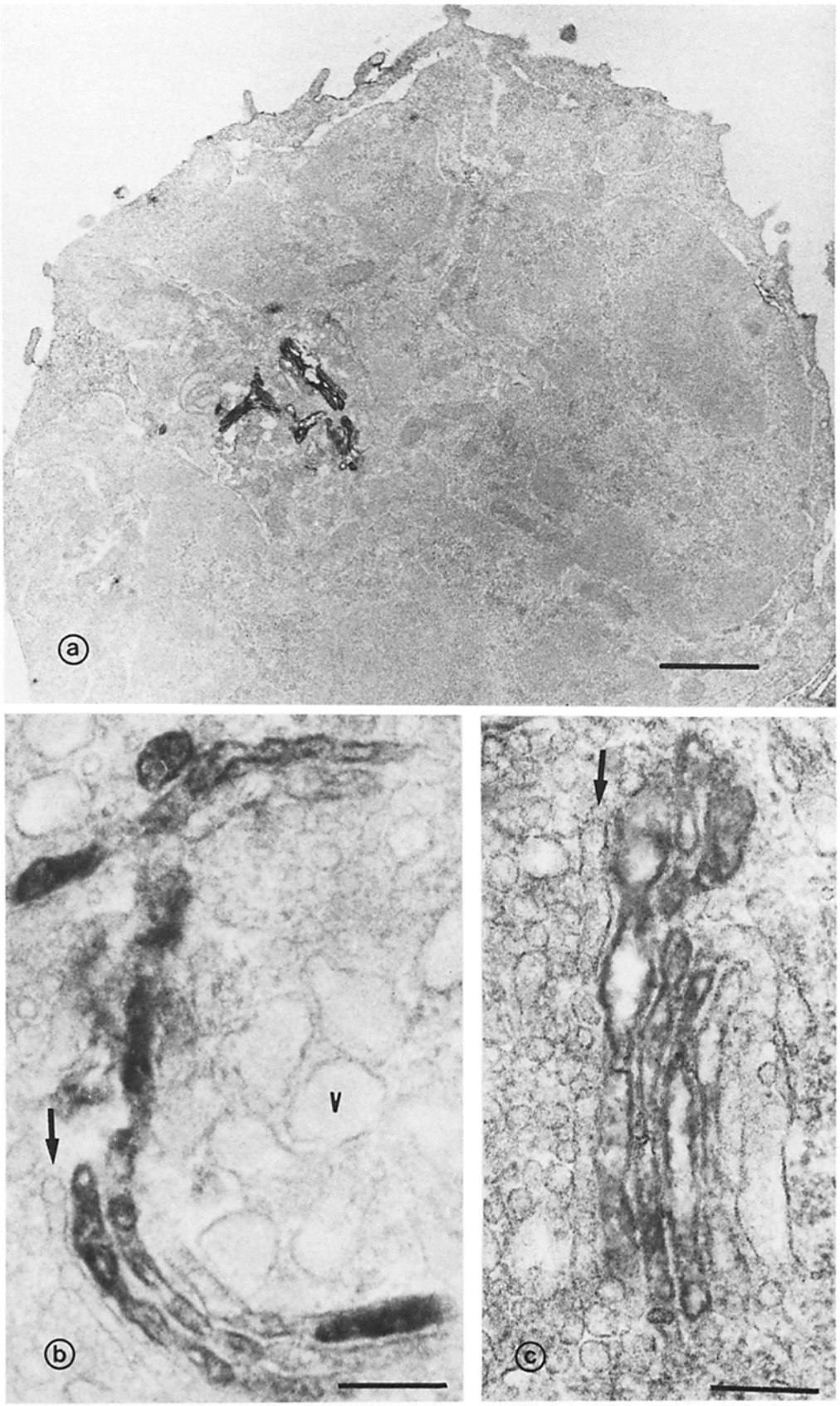


FIGURE 7 Ultrastructural localization of antigens recognized by 6F4C5. IR202 cells in suspension were fixed for 1 h with 4% paraformaldehyde, permeabilized with 0.05% saponin, and incubated with biotinylated 6F4C5 followed by avidin-peroxidase. The cells were then washed, refixed with 4% glutaraldehyde, and incubated with peroxidase substrates. After a postfixation with 1% OsO4, the cells were dehydrated and embedded in Epon. Unstained thin sections were examined in a Philips 300 electron microscope. (*V*) Vesicles similar to those that are thought to mediate Ig transport to the cell surface (6). Arrows indicate unstained cisternae. (*a*) Bar, 2 μm; × 7,800. (*b* and *c*) Bar, 0.2 μm; × 86,500.

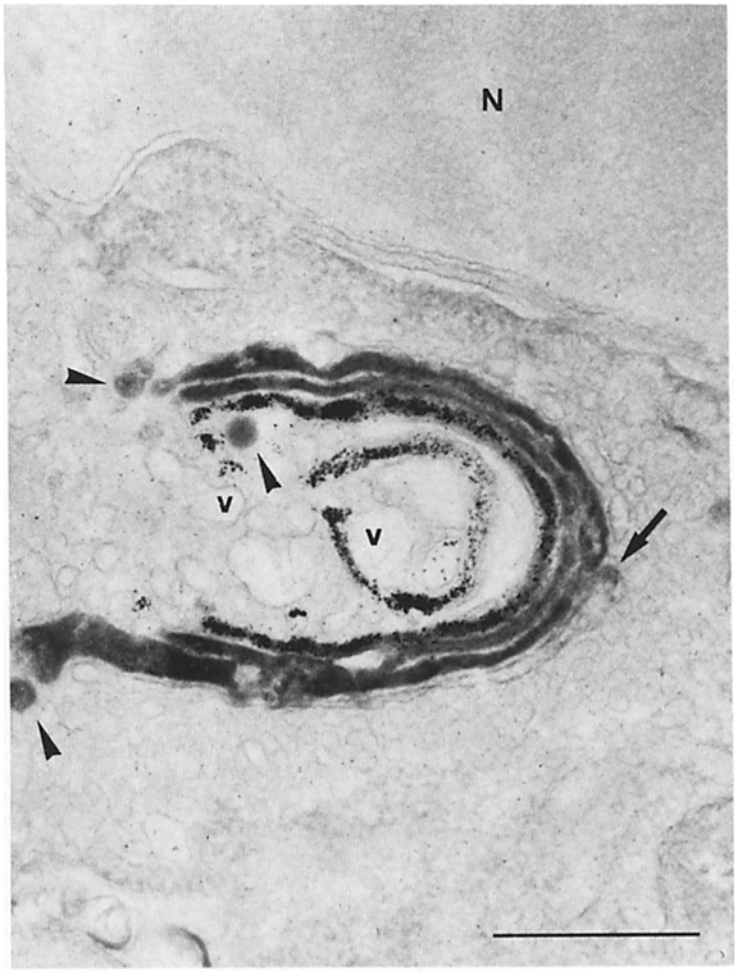


FIGURE 8 Double staining for thiamine pyrophosphatase activity and antigens recognized by 6F4C5. IR202 cells were processed as in Fig. 7, but were refixed in 0.2% glutaraldehyde/2% paraformaldehyde and stained for thiamine pyrophosphatase activity (30) 40 min at 37°C before the addition of peroxidase substrates. (*N*) Nucleus. (*V*) Ig transport vesicles. Arrows indicate unstained cisternae; arrowheads indicate peroxidase-stained small vesicles. Bar, 0.5 μm. × 54,000.

located (i.e., medial). Double staining experiments with nucleoside diphosphatase (thiamine pyrophosphatase), a marker of distal cisternae, confirmed this assignment. No distinctive functions are known to be associated with medial cisternae; however, in selected cells these cisternae are histochemically positive for nicotinamide adenine dinucleotide phosphatase activity (46, 47).

What is of particular interest in this restricted localization on medial Golgi cisternae is that it may be the expression of the dynamic membrane-membrane interactions which are characteristic of Golgi function. It is likely that these interactions require a considerable specificity, and that such specificity is mediated by membrane determinants on the cytoplasmic (adlumenal) surface of these membranes. For example, altogether uncharacterized determinants are thought to be responsible for the close apposition of adjacent Golgi cisternae (48–49), an association that persists even after considerable disorganization of Golgi structure (12). Other cytoplasmic determinants may be responsible for accurate targeting of vesicles to and from the Golgi stack.

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