

# Antibodies to Rat Pancreas Golgi Subfractions: Identification of a 58-kD *cis*-Golgi Protein

Jaakko Saraste, George E. Palade, and Marilyn Gist Farquhar

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

**Abstract.** A 58-kD *cis*-Golgi protein has been identified by generating polyclonal antibodies against heavy (*cis*) Golgi subfractions. Total microsomes isolated from rat pancreatic homogenates were subfractionated to yield a rough microsomal fraction (B1) and three smooth membrane subfractions (B2–B4) enriched in *cis*-, middle, and *trans*-Golgi elements, respectively. The heavy (*cis*) subfraction, B2 ( $d = 1.17$  g/ml), was fractionated by Triton X-114 phase separation, and the proteins recovered in the detergent phase were used to immunize rabbits. One of the anti-B2 antibodies obtained gave a “Golgi” staining pattern when screened by immunofluorescence on normal rat kidney cells and mouse RPC 5.4 myeloma cells. In rat pancreatic exocrine cells the antibody reacted with the

plasmalemma as well as elements in the Golgi region. By immunoelectron microscopy, the antigen recognized by anti-B2 IgG was found to be restricted to *cis*-Golgi elements in myeloma cells where it was concentrated in the fenestrated *cis*-most cisterna and in some of the tubules and vesicles located along the *cis* face of the Golgi complex. By immunoprecipitation and immunoblotting, the anti-B2 IgG exclusively recognized a 58-kD protein in myeloma cells. The anti-B2 IgG reacted with several proteins in solubilized pancreatic B2 membranes, including a 58-kD protein, but affinity-purified anti-58-kD IgG reacted exclusively with the 58-kD protein. These results suggest that the 58-kD protein is a specific component of *cis*-Golgi membranes.

THERE is now widespread agreement that the Golgi complex consists of a series of distinct subcompartments arranged in polarized series (reviewed in reference 11). According to the prevailing stationary cisternae model, each Golgi cisterna or set of cisternae is assumed to represent a separate subcompartment with a distinctive membrane composition. Protein and lipid substrates are assumed to move sequentially across the Golgi stack and to be progressively modified in transit by resident Golgi enzymes. The exact number of Golgi subcompartments is still unknown. Three subcompartments have been defined, *cis*, middle, and *trans*, based on the location in Golgi subfractions of early, intermediate, and late-acting Golgi enzyme activities (1, 7, 8, 10, 16), but morphologic and cytochemical findings suggest that there may be many more than three (11, 12). Although many enzyme activities have been ascribed to the Golgi complex, the number of resident Golgi proteins that have been purified and localized *in situ* is limited to two *trans*-Golgi markers, galactosyl- (28) and sialyltransferase (29), and two middle Golgi markers, *N*-acetylglucosaminyltransferase I (9) and  $\alpha$ -mannosidase II (24). So far no *cis*-Golgi resident protein has been purified and localized.

Recently we succeeded in preparing subfractions enriched in *cis* (heavy), middle (intermediate), and *trans* (light) Golgi elements based on the distribution of enzyme and kinetic markers (32) from rat pancreatic microsomes. The fact that the heaviest (B2) Golgi subfraction was well separated from

*trans*-Golgi elements marked by galactosyltransferase activity suggested that this fraction might represent a suitable starting material to attempt to generate antibodies against *cis*-Golgi membrane proteins. The overall strategy that we devised (31) is as follows. (*Step 1*) Prepare heavy ( $d = 1.17$  g/ml) Golgi subfractions from the rat pancreas as done previously (32) and separate (by Triton X-114 phase-separation [3]) hydrophobic membrane proteins from hydrophilic membrane or secretory proteins (3, 27). (*Step 2*) Use the fractions enriched in hydrophobic membrane proteins to immunize rabbits. (*Step 3*) Screen for widely distributed Golgi antigens by immunofluorescence on several, nonpancreas cell types. (*Step 4*) Determine by immunoelectron microscopy the location within the Golgi complex of the Golgi-specific antigen(s). (*Step 5*) Characterize by immunoblotting and immunoprecipitation any *cis*-Golgi-specific antigens recognized.

Here we report that with this strategy we have identified a 58-kD protein that appears to represent a specific marker for the *cis*-Golgi cisternae.

## Materials and Methods

### Materials

Reagents and supplies were obtained from the following sources: [<sup>35</sup>S]methionine (1,375  $\mu$ Ci/mmol) and [<sup>35</sup>S]cysteine (>600  $\mu$ Ci/mmol) from Amersham Corp. (Arlington Heights, IL); <sup>125</sup>I-Na (14.8 mCi/ $\mu$ g) and <sup>125</sup>I-protein A (2–10  $\mu$ Ci/ $\mu$ g) from New England Nuclear (Boston, MA); Autofluor from

National Diagnostics Inc. (Somerville, NJ); and Iodobeads from Pierce Chemical Co. (Kansas City, MO). All cell culture media were from Gibco (Grand Island, NY); aprotinin was from Boehringer Mannheim (Indianapolis, IN), and the Sephadex G-25 columns (PD-10) from Pharmacia Fine Chemicals (Piscataway, NJ); phenylmethylsulphonyl fluoride (PMSF), Tween-20, Triton X-114, protein A-Sepharose CL-4B, benzamidine hydrochloride, diamidobenzidine, and anti-rabbit IgG coupled to alkaline phosphatase were from Sigma Chemical Co. (St. Louis, MO). The BCIP/NBT substrate system for alkaline phosphatase was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD), and rhodamine-conjugated goat anti-rabbit IgG and peroxidase-conjugated sheep Fab fragments against rabbit IgG were from Cooper Biomedical Inc. (Malvern, PA) and Biosys (Campiégne, France), respectively. Nitrocellulose filters (0.45- $\mu$ m-pore size) were purchased from Schleicher & Schuell, Inc. (Keene, NJ), Aquaside II from Calbiochem-Behring Corp. (San Diego, CA), and SDS-PAGE reagents from Bio-Rad Laboratories (Richmond, CA).

Male Sprague-Dawley rats (150–180 g) and female New Zealand White rabbits (~1.5 kg) were purchased from Camm Laboratory Animals (Wayne, NJ) and Montowese Rabbitry (Woodstock, CT), respectively.

### Preparation of Heavy Golgi Subfractions and Immunization of Rabbits

The procedure for the isolation and subfractionation of total microsomes (TM)<sup>1</sup> from rat pancreatic cell homogenates was described previously (32). Briefly, TM were subfractionated by flotation in a sucrose gradient yielding five bands (B1–B5) of decreasing density. B1 contains 85% of the total protein and practically all of the RNA and consists primarily of rough microsomes. B2–B4 consist primarily of smooth vesicles ( $d = 1.17, 1.15,$  and  $1.13$  g/ml, respectively). B2 represents the first post-endoplasmic reticulum (ER) compartment reached by secretory proteins and their site of transport arrest at 16°C (32). B4 has the highest specific activity for galactosyltransferase, a *trans*-Golgi marker. B2 fractions were collected from a total of 12 analytical sucrose gradients, diluted with an equal volume of 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, containing 100 U/ml aprotinin and 5 mM benzamidine, and concentrated by centrifugation at 100,000  $g_{av}$  for 60 min at 3°C in a SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA). The pellet was resuspended (~500  $\mu$ g/ml protein) in 1 ml of the above buffer (lacking protease inhibitors) containing 0.5% Triton X-114 and was incubated on ice for 15 min. Phase separation was carried out as described by Bordier (3). The detergent phase was diluted with PBS to give a final detergent concentration of ~0.5%, then divided into aliquots, and stored at –80°C.

Two rabbits were immunized with B2 membrane proteins (derived from the Triton X-114 detergent phase) according to the procedure of Louvard et al. (21). Each rabbit received a total of ~300  $\mu$ g B2 protein. Rabbits were bled at 2-wk intervals. From the antisera obtained, referred to as anti-B2, IgG was prepared using a protein A-Sepharose CL-4B column.

### Cell Culture and Biosynthetic Labeling

Normal rat kidney (NRK) cells were grown in DME supplemented with 14.6 mg/liter L-glutamine, 5% FBS, penicillin (10,000 U/liter), and streptomycin (100 mg/liter), and were used at ~50% confluency. Mouse myeloma (RPC 5.4) cells were grown in DME supplemented with nonessential amino acids containing 4,500 mg/liter glucose and 10% FBS and penicillin/streptomycin as above, and harvested in the exponential growth phase.

For radiolabeling, cells were incubated (30 min at 37°C) in methionine-free medium containing 10% dialyzed FBS followed by incubation in [<sup>35</sup>S]methionine (100  $\mu$ Ci/ml) for 4–5 h. After labeling, the cells were washed and incubated for 15 min on ice in 0.5 ml lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, containing 1% Triton X-100, 100 U/ml aprotinin, and 1 mM PMSF). Lysates were cleared by centrifugation (11,500  $g$  for 15 min in a microfuge), and the supernatants were used for immunoprecipitation.

### Radioiodination of Pancreatic B2 Proteins

B2 membrane proteins (100–200  $\mu$ g) harvested from the detergent phase after Triton X-114 separation were suspended in 0.5 ml of 0.1 M phosphate buffer, pH 7.4, and radioiodinated with 0.5 mCi <sup>125</sup>I-Na using Iodobeads according to the instructions of the manufacturer (Pierce Chemical Co.). After iodination (10 min at 0°C) the labeled proteins were separated from

1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; NRK, normal rat kidney; TM, total microsomes.

free <sup>125</sup>I by gel filtration on a 9-ml PD-10 column equilibrated with PBS containing 0.5% Triton X-100.

### Immunoprecipitation

Aliquots (10  $\mu$ l) of radioiodinated B2 proteins or biosynthetically labeled NRK or myeloma cell extracts were solubilized in 1 ml NET buffer (400 mM NaCl, 5 mM EDTA, 20 mM Tris, pH 8.0, 1% Triton X-100, containing 100 U/ml aprotinin and 1 mM PMSF), and cleared by incubation at 4°C in 10  $\mu$ l preimmune serum (overnight) and protein A-Sepharose CL-4B beads preswollen in NET buffer (60 min). After centrifugation (30 s at 11,500  $g$  in a microfuge), 50  $\mu$ g anti-B2 IgG was added to the supernatants, incubated overnight, and bound to protein A-Sepharose. The beads were washed five times with NET buffer and once with 10 mM Tris-HCl (pH 7.4). The precipitated proteins were solubilized by boiling in SDS-PAGE sample buffer (3.65% SDS, 18 mM dithiothreitol, 4.5 mM EDTA, 40 mM Tris-phosphate, pH 6.8, 10% glycerol) for 3 min and separated on 10% SDS-gels (22). The gels were soaked in Autofluor, dried, and exposed to preflashed Kodak X-Omat film.

### Preparation of TM from Myeloma Cells

RPC 5.4 myeloma cells ( $5-8 \times 10^8$ ) were swollen in 10 ml ice-cold 10 mM Tris (pH 7.4), 10 mM KCl, 1.5 mM MgCl<sub>2</sub> for 5 min and then homogenized with five passes through a ball bearing homogenizer (2). The homogenate was made 0.25 M in sucrose, nuclei and cell debris were sedimented (600  $g_{av}$  for 10 min), and the pellet was washed with 5 ml buffer containing 0.25 M sucrose. The first supernatant and the wash were combined and centrifuged at 10,000  $g_{av}$  for 20 min, yielding a supernatant which was decanted and then centrifuged at 100,000  $g_{av}$  for 60 min in a SW50.1 rotor (Beckman Instruments, Inc.) to sediment TM. All the above steps were carried out at 0–4°C. The TM pellets were solubilized in SDS-PAGE sample buffer, and their proteins (5–10 mg) were separated by electrophoresis in 2-mm-thick preparative 10% slab gels and transferred to nitrocellulose.

### Immunoblotting

Electrophoretic transfer of proteins to nitrocellulose paper (0.44  $\mu$ m, pore size) was carried out at 4°C for 12–18 h at 160–200 mA according to Towbin et al. (37). The transferred proteins were visualized by staining with 1% Ponceau S in 5% TCA (6) followed by destaining in three brief rinses of 10% acetic acid. The nitrocellulose strips were quenched for 2 h in buffer A (50 mM Tris, pH 7.6, 200 mM NaCl, 1% Tween-20, 5% nonfat dry milk), and then reacted for 2–12 h with anti-B2 IgG (20  $\mu$ g/ml) diluted in the same buffer. After washing with buffer B (buffer A containing only 0.1% Tween-20), the strips were reacted with either <sup>125</sup>I-protein A or anti-rabbit IgG coupled to alkaline phosphatase, diluted 1:1,000 in buffer B, washed, and then either exposed to x-ray film or reacted with alkaline phosphatase substrate (0.1 M Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub> containing 5-bromo-4-chloro-3-indolyl-phosphate) for 2–10 min.

### Affinity Purification of Anti-58-kD Antibodies

Antibodies against the 58-kD protein were purified using a modification of the procedure described by Talian et al. (34). Briefly, proteins from a TM fraction of myeloma cell proteins and separated by SDS-PAGE were transferred to nitrocellulose, and the location of the 58-kD protein was determined on marker strips cut from both sides of the transfer by the alkaline phosphatase-immunostaining procedure. The nitrocellulose region containing the 58-kD protein was excised, quenched (10% FBS, 1% BSA in PBS for 2 h), and reacted overnight with anti-B2 antiserum diluted 1:4 in PBS. After three rinses with PBS, the bound antibodies were eluted from the nitrocellulose by two successive treatments (15 s each) with 3 ml 0.2 M glycine-HCl buffer (pH 2.8) containing 0.1% gelatin. Elution was carried out in 5 ml syringes as described (34), and the eluates were immediately neutralized with 1 M NaOH, dialyzed overnight against PBS, and concentrated (5–10 $\times$ ) using Aquaside (Calbiochem-Behring Corp.).

### Immunofluorescence

NRK cells grown on glass coverslips in 35-mm culture dishes were fixed in 3% formaldehyde in 0.1 M Na-phosphate buffer, pH 7.2, for 60 min as described previously (4). Mouse myeloma cells were similarly fixed in suspension and attached to polylysine-coated glass slides (39). Both NRK and mouse myeloma cells were then permeabilized (in 0.05% Triton X-100) and incubated (30 min to 12 h) with rabbit protein A-purified (50–100  $\mu$ g/ml)

or affinity-purified anti-B2 IgG followed by TRITC-anti-rabbit IgG (60 min). They were then examined by epifluorescence and photographed using a Zeiss Photomicroscope III and Kodak Tri-X Pan (400 ASA) film.

Rat pancreas was fixed for 4 h in paraformaldehyde-lysine-periodate fixative (23), cryoprotected in sucrose, and frozen in liquid N<sub>2</sub> (36). Semithin (~0.5 μm) frozen sections were cut on an ultramicrotome (Reichert Scientific Instruments, Div. Warner-Lambert Technologies Inc., Buffalo, NY) equipped with a cryoattachment (36), quenched in 50 mM NH<sub>4</sub>Cl, and stained for immunofluorescence with anti-B2 antibodies as described above for cultured cells except that the permeabilization step was omitted.

Controls consisted of incubations in which preimmune serum was substituted for immune serum or incubation in the first antibody was eliminated.

### Immunoperoxidase

Staining of mouse myeloma (RPC 5.4) cells by immunoperoxidase and processing for electron microscopy was carried out as previously described (39). Briefly, cells were fixed in paraformaldehyde-lysine-periodate fixative (4–12 h), attached to polylysine-coated glass slides, incubated in anti-B2 IgG (100 μg/ml) overnight, and peroxidase-conjugated Fab fragments of sheep anti-rabbit IgG (60 min), followed by reaction with diaminobenzidine, and Epon embedding. Thin sections were stained with lead citrate and examined and photographed using either a Philips 410 or a JEOL 100CX electron microscope operated at 60 kV.

## Results

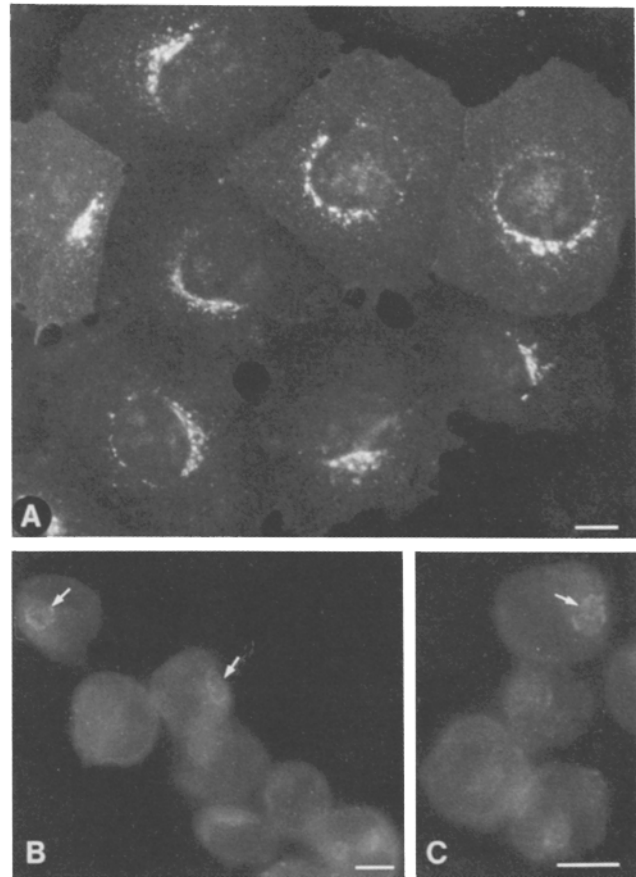
### Preparation and Characterization of the Antigen (Steps 1 and 2)

SDS-PAGE analysis of the detergent and aqueous phases of heavy pancreatic Golgi subfractions (B2), prepared according to Bordier (3), showed that this procedure efficiently (but not completely) separated membrane proteins from the content and adsorbed secretory proteins that fractionated to the aqueous phase (data not shown). The proteins in the detergent phase were used to immunize two rabbits, which generated two antisera referred to as anti-B2.

### Screening by Immunofluorescence on NRK and Mouse Myeloma Cells (Step 3)

Since the rabbits were immunized with a mixture of proteins derived from B2 fractions, we expected that the antibodies generated would recognize specific pancreas antigens (including contaminating secretory antigens) as well as Golgi-specific antigens. We screened several, nonpancreas cultured cell lines by immunofluorescence to detect general Golgi-specific antigens. With one of the anti-B2 IgGs, a prominent juxtannuclear immunofluorescence pattern corresponding to the location of the Golgi region was seen in both NRK (Fig. 1 A) and mouse myeloma cells (Fig. 1, B and C). In addition to Golgi staining, however, there was also staining of the plasma membrane and of small vesicles in the perinuclear region in NRK cells, but no staining of the ER or the nuclear envelope was observed in either cell type. We concluded that this antiserum was worthy of further characterization. The second antiserum gave a similar but considerably weaker staining pattern and was not studied further.

When the anti-B2 IgG that gave a strong Golgi signal on NRK and myeloma cells was used to stain semithin sections of the exocrine pancreas, it also gave a strong staining of the Golgi region (Fig. 2, A–B). However, it reacted with the apical and basolateral plasma membranes of pancreatic cells as well. These results indicated that in pancreatic exocrine cells, anti-B2 recognizes not only one or more Golgi antigens, but it also recognizes other, non-Golgi antigens.

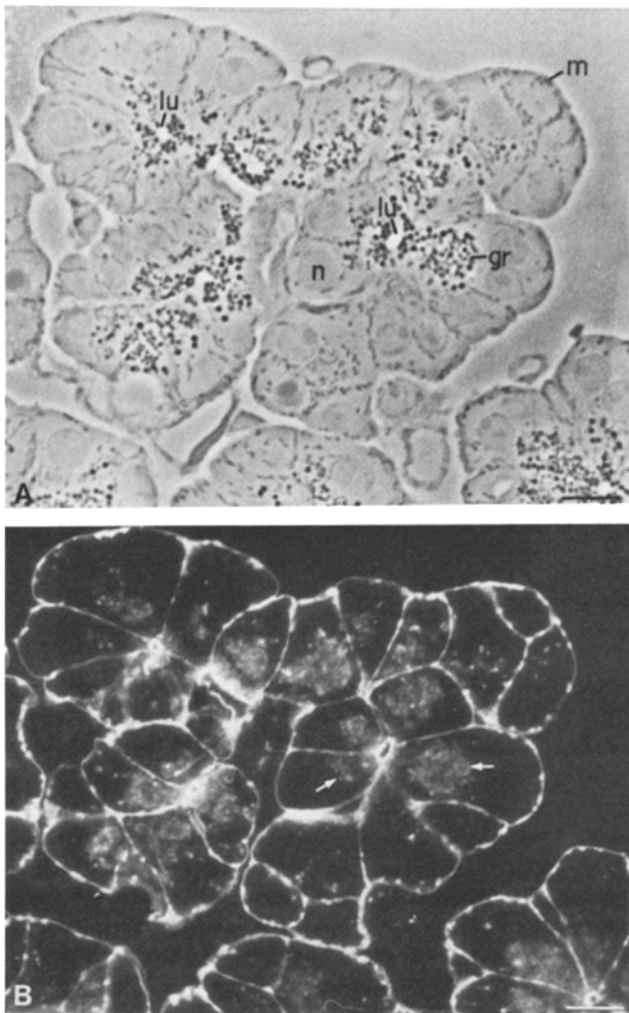


**Figure 1.** Immunofluorescence microscopy of aldehyde-fixed NRK cells (A) and mouse myeloma cells (B and C) stained with anti-B2 IgG. In both cell types there is a strong fluorescent signal in the Golgi region. In NRK cells it is seen as an intensely stained cap located to one side of the nucleus. In myeloma cells it appears as a pale ringlike structure. There is also vesicular staining in the perinuclear region in NRK cells. No staining of the nuclear membrane or ER is seen in either cell type. The difference in fluorescence intensity and apparent resolution in the two cell types is due to a marked difference in cell thickness. Bar, 10 μm.

### Immunoelectron Microscopic Localization of the Anti-B2 IgG to *cis*-Golgi Cisternae of Mouse Myeloma Cells (Step 4)

To obtain detailed information on the localization of the Golgi-specific antigens recognized by anti-B2 within the Golgi complex, we carried out immunocytochemistry in mouse myeloma (RPC 5.4) cells at the electron microscope level using an indirect immunoperoxidase technique. Myeloma cells were used for this purpose because: (a) the anti-B2 IgG exclusively gave a Golgi signal by immunofluorescence on this cell type (Fig. 1 B), and (b) these cells are particularly suitable for localization of antigens among Golgi subcompartments since the Golgi complex is highly polarized and its sidedness (*cis* vs. *trans*) can usually be determined unequivocally by the presence of transitional elements of the ER on the *cis*-side of the stack (25, 39).

After immunolabeling myeloma cells with anti-B2 IgG, peroxidase reaction product was found within the Golgi complex of virtually every cell. Within the Golgi complex it was seen to be restricted entirely to *cis* elements. In the vast



**Figure 2.** Indirect immunofluorescence staining of a semithin (0.5  $\mu\text{m}$ ) cryosection prepared from aldehyde-fixed rat pancreas. The section was reacted with anti-B2 IgG and photographed by phase contrast (A) or fluorescence (B) microscopy. Intracellular staining is almost completely restricted to the apical or Golgi region (arrows). In addition, both the apical and basolateral domains of the plasmalemma of the acinar cells are intensely labeled. The contents of the acinar lumen (lu) and the rough ER at the base of the cell are not reactive. Fig. 4 A demonstrates the location of the nuclei (n), apical zymogen granules (gr), and mitochondria (m). Bar, 1  $\mu\text{m}$ .

majority of cells reaction product was typically confined to the lumina of the fenestrated, *cis*-most cisterna and some of the tubules and vesicles associated with it (Figs. 3–5), but in some cells partial labeling of the next (penultimate) *cis* element was also seen (Fig. 4). No staining of *trans* or middle cisternae was observed. The fenestrated nature of the reactive *cis* cisterna was particularly evident in grazing sections (Fig. 3 B), which showed that some of the reactive tubules and vesicles on the *cis* face of the Golgi complex were in continuity with the *cis*-most Golgi cisterna (Figs. 3 and 4). Fig. 5 shows a panel of labeled *cis* cisternae, vesicles, and tubules in fields in which the *cis* side of the Golgi complex is clearly marked by the presence of transitional elements of

the rough ER. Although usually the ER cisternae, including the perinuclear cisternae and the transitional elements of the rough ER, were not immunoreactive, in 20–25% of the cells some rough ER and transitional elements showed a weak reaction (e.g., Fig. 5). It was concluded that anti-B2 recognizes one or more antigens that are largely restricted to *cis*-Golgi elements in myeloma cells. Similar results were obtained on anterior pituitary cells (micrographs not shown).

#### **Anti-B2 Recognizes Primarily a 58-kD Protein by Immunoprecipitation of Biosynthetically Labeled NRK and Mouse Myeloma Cells (Step 5)**

The next step was to attempt to identify the *cis*-Golgi-specific antigen recognized by the anti-B2 IgG. When immunoprecipitations were carried out on [ $^{35}\text{S}$ ]methionine-labeled NRK cell lysates with anti-B2, a 58-kD protein was the major antigen recognized, but the antibody also reacted with two minor proteins ( $\sim 35$  and 85 kD). When similar immunoprecipitations were done on detergent lysates of TM from biosynthetically labeled myeloma cells, these antibodies reacted with only a single major protein of 58 kD (Fig. 6 A, lanes 3–4). The specific reaction of anti-B2 with the 58-kD protein could also be demonstrated by immunoblotting on myeloma cell microsomes (Fig. 6 B, lane 5). These results indicated that the *cis*-Golgi-staining seen with anti-B2 was related to its recognition of a 58-kD protein.

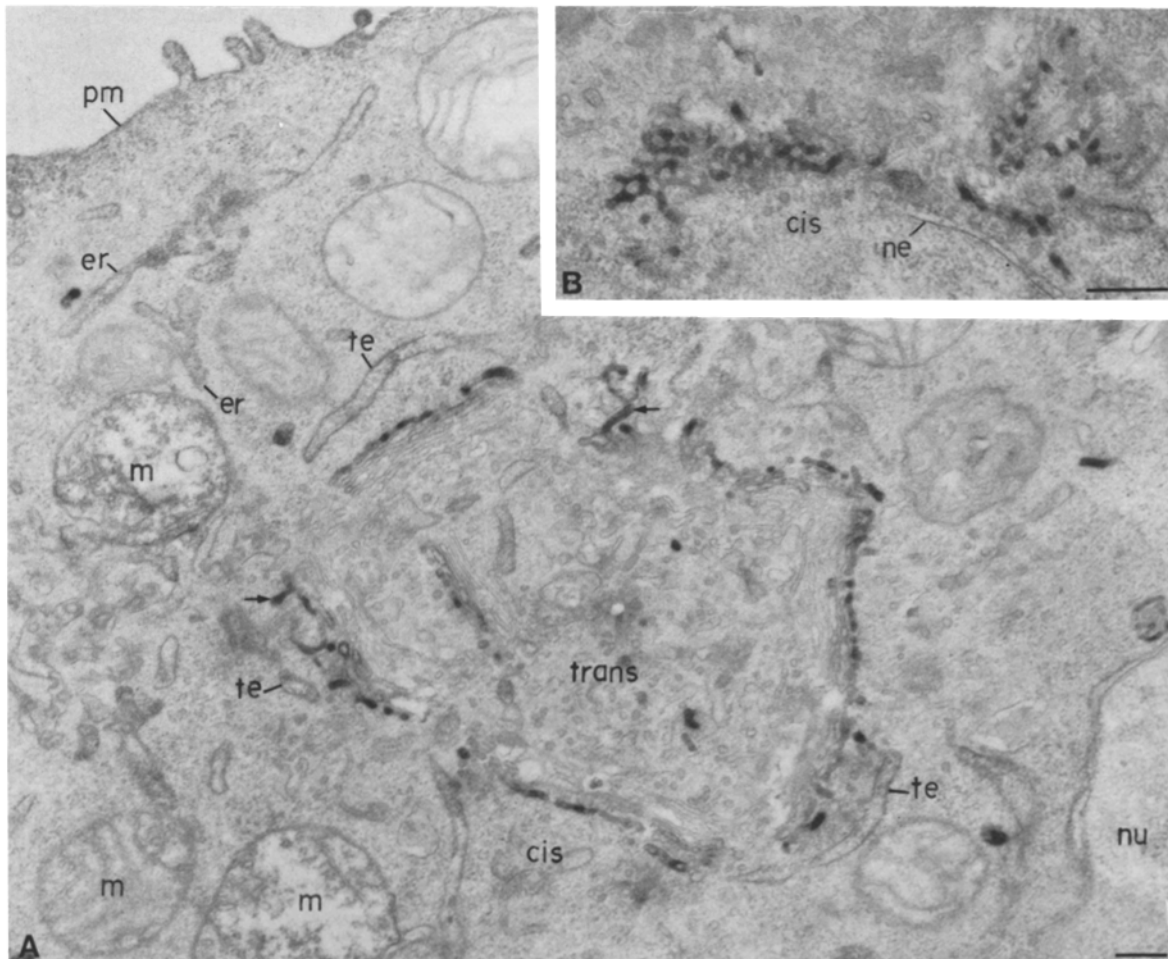
#### **Immunoprecipitation of Pancreatic B2 Proteins with Anti-B2**

To determine whether a band corresponding in mobility to the 58-kD protein was recognized in pancreatic B2 membranes, immunoprecipitations were carried out with radioiodinated B2 proteins recovered in the detergent phase after Triton X-114 separation. The results (Fig. 6 A, lanes 1–2) showed that the antibodies recognize a 58-kD band as well as two other major and three minor proteins.

#### **Affinity Purification of Anti-58-kD IgG**

To verify that the observed reaction of the anti-B2 IgG with the Golgi complex was due to the recognition of the 58-kD protein, antibodies specifically directed against this protein were affinity purified from the polyclonal antiserum by binding them to the 58-kD myeloma cell protein which had been electrophoretically transferred to nitrocellulose (34). When the antibodies were eluted and concentrated, they were found to react specifically with only the 58-kD protein in immunoblots of myeloma cell TM (Fig. 6 B, lane 6) and pancreas B2 proteins (Fig. 6 B, lane 7). These results indicate that the anti-58-kD IgG recognizes the same 58-kD Golgi protein in both mouse myeloma and rat exocrine pancreatic cells, and that the 58-kD protein is enriched in heavy (*cis*) pancreatic Golgi subfractions.

By immunofluorescence the affinity-purified anti-58-kD IgG gave a typical Golgi-type signal in aldehyde-fixed NRK cells and mouse myeloma cells. Moreover, they also gave a weak staining pattern restricted to the apical or Golgi region in cryostat sections of rat pancreas. Attempts were also made to use the affinity-purified anti-58-kD IgG to label mouse myeloma cells and pancreatic acinar cells by immunoelectron microscopy, but so far these attempts have not been suc-



**Figure 3.** Immunoperoxidase localization of anti-B2 antibodies in mouse myeloma RPC 5.4 cells showing that peroxidase reaction product is predominantly confined to the lumen of the fenestrated *cis*-most Golgi cisterna and to vesicles or tubules in continuity with this cisterna (arrows). Occasionally partial staining of the penultimate cisterna is also observed. In addition, sometimes a tubule or vesicle located at some distance from the Golgi complex is also labeled. Weak peroxidase reaction is also seen in the ER (*er*) and in the transitional elements (*te*) (part rough, part smooth) of the ER. The inset (**B**) shows an oblique section through a labeled *cis* element (to the left and upper right), demonstrating its fenestrated nature. *pm*, plasma membrane; *nu*, nucleus; *ne*, portion of the nuclear envelope; *m*, mitochondria; *te*, transitional elements. Bar, 0.5  $\mu$ m.

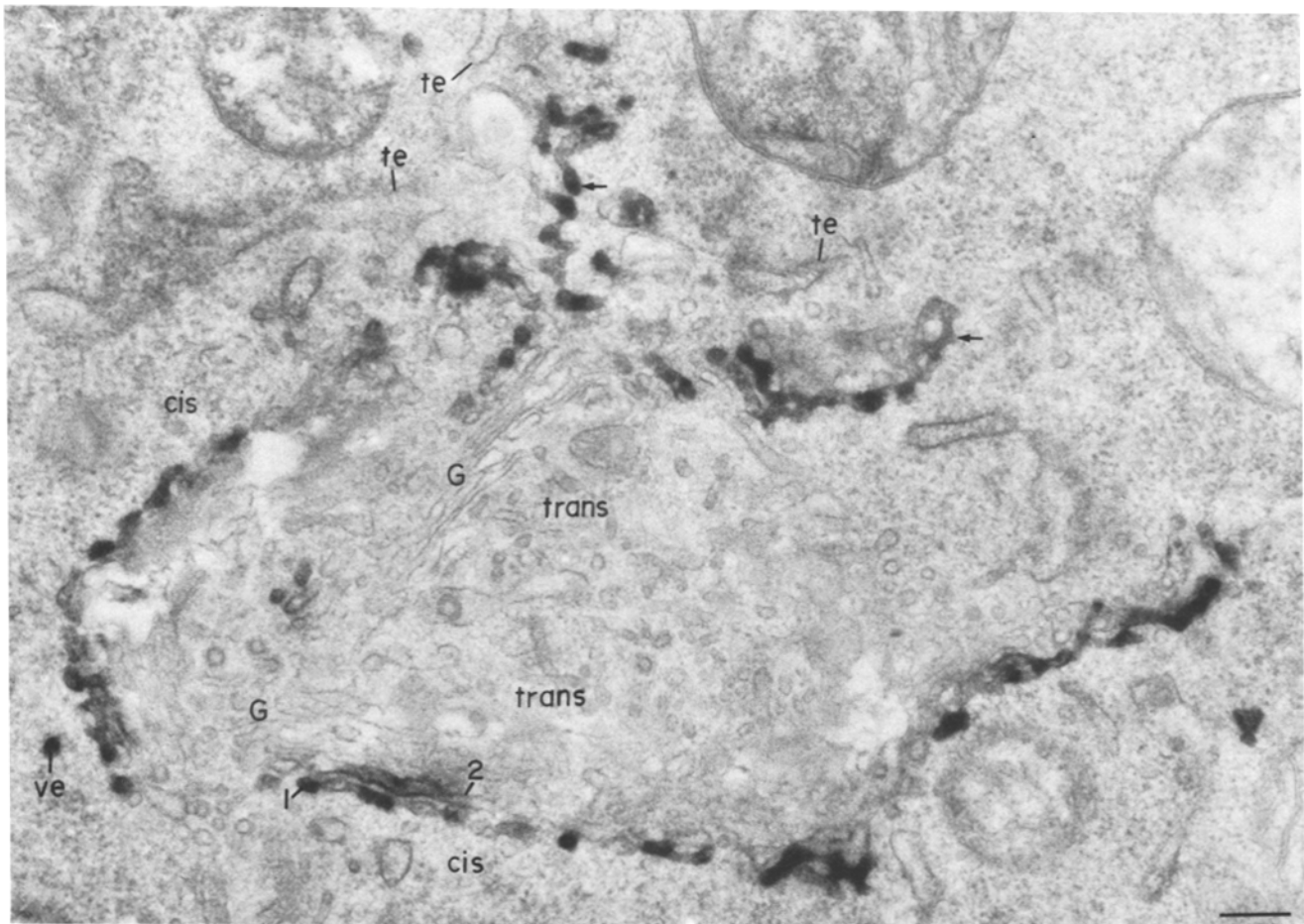
cessful. Although the affinity-purified IgG reacted strongly by immunoblotting, it yielded only a weak signal on aldehyde-fixed tissues by immunofluorescence and was too weak to be useful for immunoelectron microscopy.

We also analyzed the distribution of the 58-kD protein in pancreatic subfractions by immunoblotting and found that although the 58-kD protein was detected in all subfractions (B2–B4), it was much more enriched in heavy (B2) as compared with light (B4) Golgi subfractions. We therefore assume that the subfractions are more extensively contaminated by *cis*- than by *trans*-Golgi elements.

### Discussion

We have produced antibodies to membrane proteins prepared from a heavy Golgi subfraction enriched in *cis*-Golgi elements. The antibodies recognize a 58-kD protein by immunoprecipitation and immunoblotting and by immunocyto-

chemistry we have localized the 58-kD protein to *cis*-Golgi elements. This Golgi protein appears to be widely distributed: our collective immunochemical and immunocytochemical results indicate that it is found in cells of diverse tissue origin (i.e., mouse myeloma cells and rat kidney, exocrine pancreatic, and anterior pituitary cells). The 58-kD protein is in all likelihood an integral membrane protein since it separates virtually completely with the detergent phase by the Triton X-114 procedure (3). Moreover, preliminary lectin-binding and enzyme-digestion studies (17) suggest that it is a glycoprotein that carries both N- and O-linked oligosaccharides. The epitopes of the 58-kD protein recognized by anti-B2 are located largely on the ectodomain of the molecule, because when the antibody was localized by immunoperoxidase at the EM level reaction product was confined to the lumen of the *cis*-cisternae. Further information on the nature and function of the 58-kD protein should be of great interest since to date no *cis*-Golgi resident protein



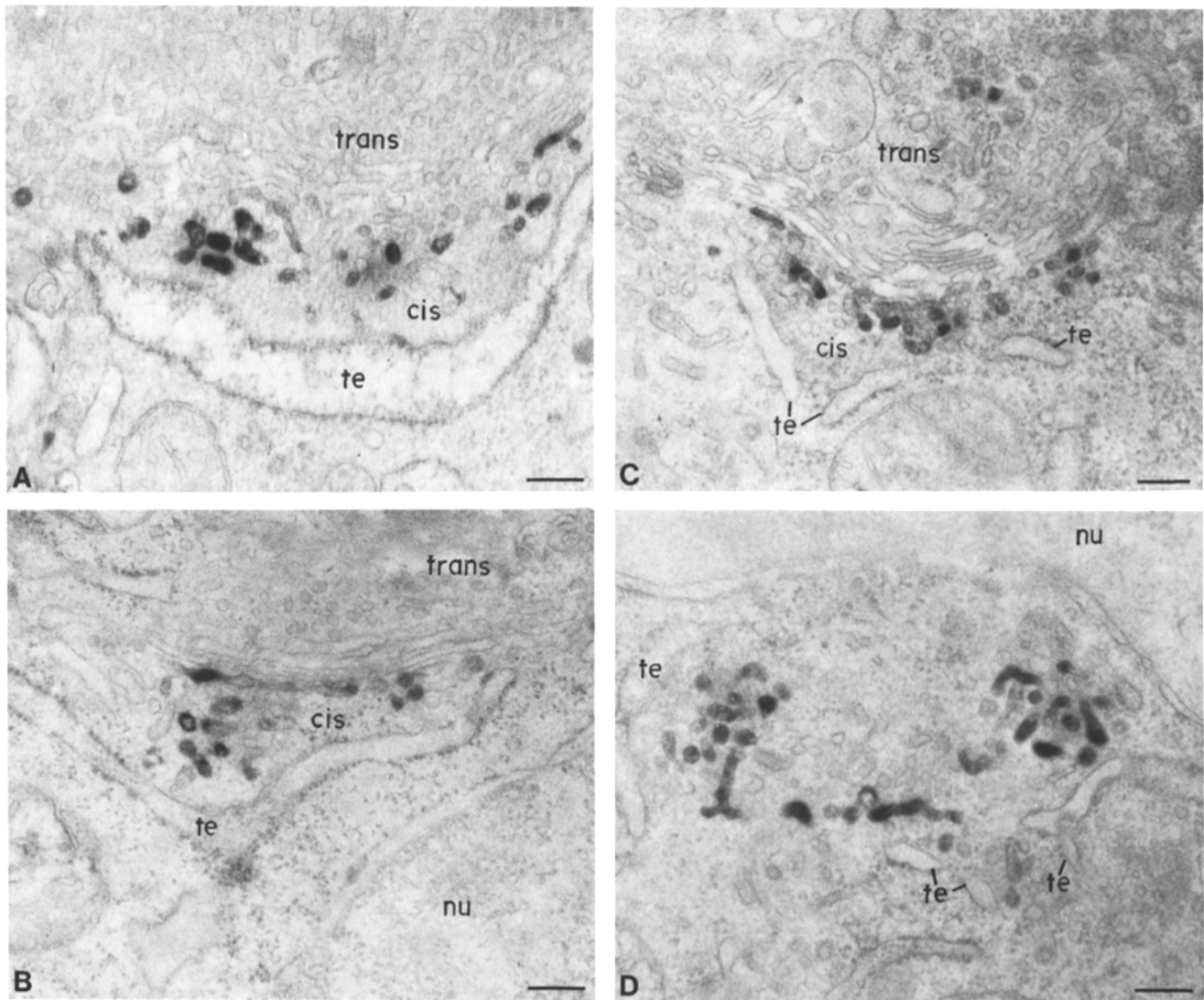
**Figure 4.** Immunoperoxidase labeling of the Golgi complex of a myeloma cell with anti-B2 IgG shown at higher magnification which demonstrates that typically the staining is concentrated within the fenestrated *cis*-most Golgi cisterna (1) of the Golgi stack (G) or within vesicles (ve) closely associated with the *cis* cisterna. Partial staining of the penultimate cisterna (2) is also observed. The reactive *cis* cisternae are cut in normal section below and in grazing section above (arrows). Bar, 0.25  $\mu$ m.

has been characterized, and no reliable marker exists for this subcompartment of the Golgi complex.

The Golgi complex is known to be the exclusive intracellular site of a number of posttranslational modifications that secretory, membrane, and lysosomal proteins undergo during their movement along the exocytic pathway. These modifications include trimming and addition of terminal sugars (galactose, sialic acid) to N-linked oligosaccharides, addition of mannose-6-phosphate (Man-6-P) residues to lysosomal enzymes, sulfation, and proteolytic processing of some secretory proteins (8, 11, 12, 15, 19, 35). Several enzymes involved in these processing events have been assumed to be present in *cis*-Golgi elements, because the corresponding activities are most concentrated in heavy Golgi subfractions. The list includes: *N*-acetylglucosaminylphosphotransferase and *N*-acetylglucosamine-1-phosphodiester  $\alpha$ -*N*-acetylglucosaminidase (14, 19, 26) (the two enzymes that generate the Man-6-P recognition marker on lysosomal enzymes), as well as Golgi  $\alpha$ -mannosidase I (19), and perhaps the enzyme(s) involved in fatty acid acylation (7, 10). However, none of the above enzymes has yet been purified or localized by immunocytochemistry. The only successful purification of a putative *cis*-Golgi protein is that of Tulsiani

and Touster (38), who reported in a recent abstract the isolation of Golgi  $\alpha$ -mannosidase IA which, interestingly, appears on SDS gels as a doublet at 57 and 58 kD. The osmium reduction impregnation method (13) was used extensively in the past to label *cis*-Golgi cisternae, but the chemistry of the reactions involved remains unknown and the distribution of the reaction product varies somewhat with cell type and reaction conditions.

The present observations, as well as previous morphological and cytochemical studies (reviewed in 11, 12), point to the existence of considerable heterogeneity among *cis*-Golgi elements. In practically all myeloma cells there is a specific and preferential concentration of the 58-kD protein in the *cis*-most cisterna of the stack, and the antigen is also regularly detected in some of the tubules and vesicles located along the *cis* face of the Golgi complex. Such elements are known (18, 30) to play a role in the transport of biosynthetic products between the transitional elements of the ER and the *cis*-Golgi cisterna. In the majority of cells no reaction product was detected in ER cisternae and transitional elements of the ER; however, weak labeling of some of these elements was detected in 20–25% of the cells. Although the anti-B2 IgG recognizes only the 58-kD protein by immunoprecipitation



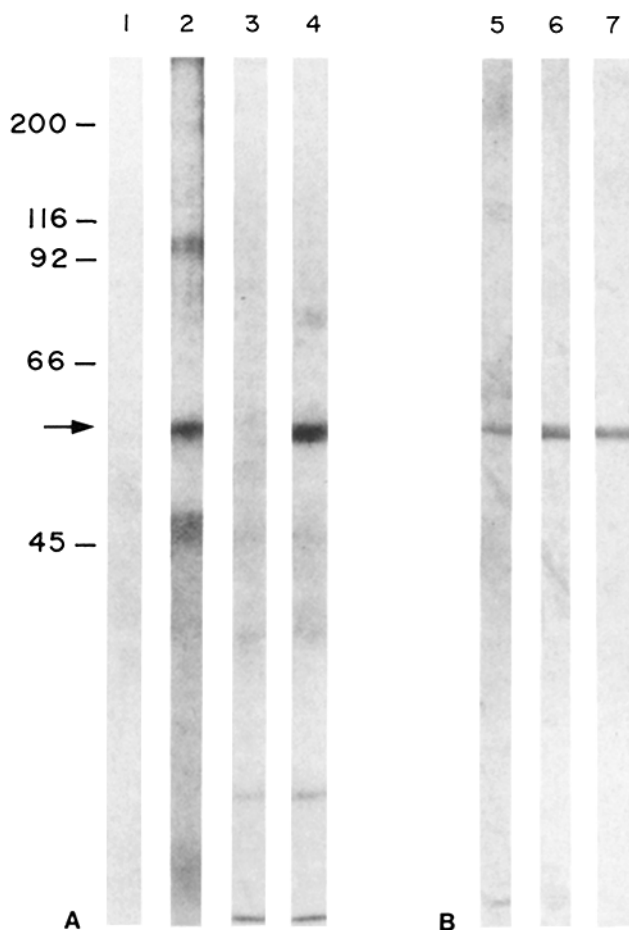
**Figure 5.** Four micrographs demonstrating the immunoreactivity of the *cis*-Golgi cisternae and of tubules and vesicles located between the transitional elements of the ER (*te*) and the *cis*-most Golgi cisterna with anti-B2 IgG. The stacked cisternae of the Golgi complex are cut normally in *A*, *B*, and *C* but are missed entirely in *D* which reveals only peripheral (*cis*) tubular and vesicular Golgi elements. Note that some of the latter are not labeled. Bar, 0.25  $\mu$ m.

and immunoblotting, it cannot be determined with certainty whether the weak labeling of a minority of the transitional ER elements is due to the presence of another antigenically related minor protein or to the occurrence of the 58-kD protein in these elements at a low concentration. The 58-kD protein is expected to transit from the ER to *cis*-Golgi cisternae during its biosynthesis. Further, more detailed localization of the 58-kD protein among *cis*-Golgi elements awaits the generation (now in progress) of specific monoclonal or polyclonal antibodies against the purified 58-kD protein.

There have been a few previous attempts to produce antibodies against Golgi proteins. The first successful generation of Golgi-specific antibodies was that of Louvard et al. (21) who immunized rabbits with light Golgi subfractions prepared from rat liver and removed unwanted antibodies (e.g., those reacting with plasma membrane and ER) by absorption with the appropriate membrane fractions. With this approach they generated an antibody against a 135-kD mem-

brane glycoprotein that specifically stains the Golgi region by immunofluorescence and is broadly distributed across the cisternal stacks by immunoelectron microscopy. More recently, Chicheportiche et al. (5) produced a monoclonal antibody against a Golgi fraction derived from myeloma cells that recognizes a 54-kD peripheral membrane protein localized primarily on middle Golgi cisternae. Two other monoclonals that stain Golgi elements have been generated: one recognizes a 110-kD heat shock protein present in the nucleus as well as the Golgi complex (20), and the other recognizes a 103–108-kD protein present in *trans*-Golgi elements (33). Both of the latter antibodies are IgMs and thus are of limited usefulness for immunocytochemical studies. None of the Golgi antibodies generated to date specifically recognizes *cis*-Golgi elements.

In summary, we have identified a 58-kD protein that appears to provide a suitable marker for *cis*-Golgi elements. Further studies now in progress are aimed at establishing the



**Figure 6.** (A) Immunoprecipitates obtained with anti-B2 antiserum from radiiodinated pancreas B2 proteins (lanes 1 and 2) or biosynthetically labeled myeloma cell extracts (lanes 3 and 4) showing that the antiserum recognizes three major bands including a 58-kD protein in B2 membranes (lane 2), whereas in myeloma cell extracts (lane 4) it recognizes only a 58-kD protein. Lanes 1 and 3 are preimmune controls. B2 fractions collected from four gradients were separated into membrane and content subfractions by the Triton X-114 procedure. The proteins recovered in the detergent phase (~200  $\mu$ g) were radioiodinated, aliquots of the labeled proteins ( $\sim 1 \times 10^6$  cpm) were subjected to immunoprecipitation as described in Materials and Methods, and the immunoprecipitates were processed for SDS-PAGE and fluorography. Mouse myeloma RPC 5.4 cells, labeled for 4 h with 100  $\mu$ Ci/ml of [ $^{35}$ S]methionine, were solubilized in lysis buffer containing 1% Triton X-100. Aliquots of the cleared lysates were used in immunoprecipitation, and the immunoprecipitates were analyzed by SDS-PAGE. (B) Immuneoverlays of myeloma TM or pancreas B2 proteins with anti-B2 or affinity-purified anti-58-kD IgG. Myeloma TM proteins and pancreas B2 proteins were separated by SDS-PAGE ( $\sim 100$   $\mu$ g protein/lane), the proteins were transferred to nitrocellulose, and strips of the nitrocellulose were reacted with anti-B2 (lane 5) or affinity-purified anti-58-kD IgG (lanes 6 and 7). In myeloma cells, both the affinity-purified anti-58-kD IgG and the whole anti-B2 IgG fraction specifically detect only the 58-kD protein. In pancreas B2 membranes, the affinity-purified anti-58-kD IgG recognizes only the 58-kD protein (lane 7), whereas the whole IgG reacts with several additional bands (see lane 2).

properties of the 58-kD protein, especially its function, the steps involved in its biosynthesis, the nature of its oligosaccharide moieties, and the kinetics of its incorporation into the Golgi complex.

We would like to thank Dr. John Woods for advice on the techniques for application of immunoperoxidase procedures to myeloma cells, Myriam Willenz and Mary Bronson for technical assistance and M. Lynne Wootton for expert wordprocessing.

This research was supported by National Institutes of Health Research Grants DK 17780 (to M. G. Farquhar) and GM 27303 (to G. E. Palade). Dr. Saraste was supported (1984–1986) by an EMBO long term fellowship, ALTF 270-1983.

Received for publication 27 April 1987, and in revised form 23 July 1987.

#### References

- Balch, W. E., and D. S. Keller. 1986. ATP-coupled transport of vesicular stomatitis virus G protein. *J. Biol. Chem.* 261:14690–14696.
- Balch, W. E., and J. E. Rothman. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of donor and acceptor activities in a cell-free system. *Arch. Biochem. Biophys.* 240:413–425.
- Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604–1607.
- Brown, W. J., E. Constantinescu, and M. G. Farquhar. 1984. Redistribution of mannose-6-phosphate receptors induced by tunicamycin and chloroquine treatments. *J. Cell Biol.* 99:320–326.
- Chicheportiche, Y., P. Vassalli, and A. M. Tartakoff. 1984. Characterization of cytoplasmically oriented Golgi protein with a monoclonal antibody. *J. Cell Biol.* 99:2200–2210.
- Courdier, E., H. Reggio, and D. Louvard. 1983. Characterization of an integral membrane glycoprotein associated with the microfilaments of pig intestinal microvilli. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:469–475.
- Dunphy, W. G., and J. E. Rothman. 1983. Compartmentation of asparagine-linked oligosaccharide processing in the Golgi apparatus. *J. Cell Biol.* 97:270–275.
- Dunphy, W. G., and J. E. Rothman. 1985. Compartmental organization of the Golgi stack. *Cell.* 42:13–21.
- Dunphy, W. G., R. Brands, and J. E. Rothman. 1985. Attachment of terminal N-acetylglucosamine to asparagine-linked oligosaccharides occurs in central cisternae of the Golgi stack. *Cell.* 40:463–472.
- Dunphy, W. G., E. Fries, L. J. Urbani, and J. E. Rothman. 1981. Early and late functions associated with the Golgi apparatus reside in distinct compartments. *Proc. Natl. Acad. Sci. USA.* 78:7453–7457.
- Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:447–488.
- Farquhar, M. G., and G. E. Palade. 1981. The Golgi apparatus (complex)—(1954–1981)—from artifact to center stage. *J. Cell Biol.* 91(3, Pt. 2):77s–103s.
- Friend, D. S., and M. J. Murray. 1965. Osmium impregnation of the Golgi apparatus. *Am. J. Anat.* 117:135–150.
- Goldberg, D. E., and S. Kornfeld. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J. Biol. Chem.* 258:3159–3165.
- Griffiths, G., and K. Simons. 1986. The trans Golgi network: Sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438–443.
- Griffiths, G., P. Quinn, and G. Warren. 1983. Dissection of the Golgi complex. I. Monensin inhibits the transport of viral membrane proteins from medial to trans-Golgi cisternae in baby hamster kidney cells infected with Semliki forest virus. *J. Cell Biol.* 96:835–850.
- Hendricks, L. C., J. Saraste, G. E. Palade, and M. G. Farquhar. 1987. A 58-kD cis-Golgi resident protein contains O-linked terminal GalNAc and N-linked high mannose residues. *J. Cell Biol.* 105(4, Pt. 2):267a. (Abstr.)
- Jamieson, J. D., and G. E. Palade. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. *J. Cell Biol.* 34:577–596.
- Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631–664.
- Lin, J. J.-C., and S. A. Queally. 1982. A monoclonal antibody that recognizes Golgi-associated protein of cultured fibroblast cells. *J. Cell Biol.* 92:108–112.
- Louvard, D., H. Reggio, and G. Warren. 1982. Antibodies to the Golgi complex and the rough endoplasmic reticulum. *J. Cell Biol.* 92:92–107.
- Maizel, J. V. 1969. Acrylamide gel electrophoresis of proteins and nucleic acids. In *Fundamental Techniques of Virology*. K. Habel, and N. P. Salzman, editors. Academic Press, Inc., New York. 334–362.
- McLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde



- hyde fixative: a new fixative for immunoelectron microscopy. *J. Histochem Cytochem.* 22:1077-1083.
24. Novikoff, P. M., D. R. P. Tulsiani, O. Touster, A. Yam, and A. B. Novikoff. 1983. Immunocytochemical localization of  $\alpha$ -D-mannosidase II in the Golgi apparatus of the rat liver. *Proc. Natl. Acad. Sci. USA.* 80: 4364-4368.
  25. Ottosen, P. D., P. J. Courtoy, and M. G. Farquhar. 1980. Pathways followed by membrane recovered from the surface of plasma cells and myeloma cells. *J. Exp. Med.* 152:1-19.
  26. Pohlmann, R., A. Waheed, A. Hasilik, and K. von Figura. 1982. Synthesis of phosphorylated recognition marker in lysosomal enzymes is located in the cis part of Golgi apparatus. *J. Biol. Chem.* 257:5323-5325.
  27. Pryde, J. G., and J. H. Phillips. 1986. Fractionation of membrane proteins by temperature-induced phase separation in Triton X-114. *Biochem. J.* 233:525-533.
  28. Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyltransferase in HeLa cells: codistribution with thiamine pyrophosphatase in *trans*-Golgi cisternae. *J. Cell Biol.* 92:223-229.
  29. Roth, J., D. J. Taatjes, J. M. Lucocq, J. Weinstein, and J. C. Paulson. 1985. Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. *Cell.* 43:287-295.
  30. Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535-549.
  31. Saraste, J., M. Bronson, G. E. Palade, and M. G. Farquhar. 1987. Organelle-specific antibodies: Production of antibodies to Golgi subcompartments. In *Cell Free Analysis of Membrane Traffic*, J. Morré, editor. Alan R. Liss, Inc., New York, In press.
  32. Saraste, J., G. E. Palade, and M. G. Farquhar. 1986. Temperature-sensitive steps in the transport of secretory proteins through the Golgi complex in exocrine pancreatic cells. *Proc. Natl. Acad. Sci. USA.* 83: 6425-6429.
  33. Smith, A. D. J., F. D'Eugenio-Gumkowski, K. Yanagisawa, and J. D. Jamieson. 1984. Endogenous and monoclonal antibodies to the rat pancreatic acinar cell Golgi complex. *J. Cell Biol.* 98:2035-2046.
  34. Talian, J. C., J. B. Olmsted, and R. D. Goldman. 1983. A rapid procedure for preparing fluorescein-labeled antibodies from whole antiserum: its use in analyzing cytoskeletal architecture. *J. Cell Biol.* 97:1277-1282.
  35. Tartakoff, A. M. 1983. The confined function model of the Golgi complex: Center for ordered processing of biosynthetic products of the rough endoplasmic reticulum. *Int. Rev. Cytol.* 85:221-252.
  36. Tokuyasu, K. T. 1986. Application of cryoultramicrotomy to immunocytochemistry. *J. Microsc. (Oxf.)* 143:139-149.
  37. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
  38. Tulsiani, D. R. P., and O. Touster. 1986. Purification of  $\alpha$  1,2-specific mannosidase from rat liver Golgi membranes. *Fed. Proc.* 45:1680.
  39. Woods, J. W., M. Doriaux, and M. G. Farquhar. 1986. Transferrin receptors recycle to cis and middle as well as trans Golgi cisternae in Ig-secreting myeloma cells. *J. Cell Biol.* 103:277-286.