Two Integral Membrane Proteins Located in the *Cis*-Middle and *Trans*-Part of the Golgi System Acquire Sialylated N-Linked Carbohydrates and Display Different Turnovers and Sensitivity to cAMP-Dependent Phosphorylation

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Abstract. The localization and chemical characteristics of two Golgi integral membrane proteins (GIMPs) have been studied using monoclonal antibodies. The two proteins are segregated in different parts of the Golgi system and whereas $GIMP_c$(130 kD) is located in the *cis* and medial cisternae, $GIMP_t$ (100 kD) is confined in the *trans*-most cisterna and *trans*-tubular network. Both GIMPs are glycoproteins that contain N- and O-linked carbohydrates. The N-linked carbohydrates were exclusively of the complex type. Although excluded from the *trans*-side of the Golgi system, where sialylation is believed to occur, $GIMP_c$ acquires sialic acid in both its N- and O-linked carbohydrates. Sialic acid was also detected in the N-linked carbohydrates of $GIMP_t$. $GIMP_c$ is apparently phosphorylated in the luminal domain in vivo. Phosphorylation occurred exclusively on serine and was stimulated by dibutyryl cyclic AMP. $GIMP_c$ and $GIMP_t$ displayed half-lives of 20 and 9 h, respectively.

THERE is strong evidence indicating that the Golgi system plays pivotal roles in the posttranslational modification and distribution of proteins co-translationally sorted in the endoplasmic reticulum (i.e., secretory, plasma membrane, lysosomal, Golgi, and viral proteins) or internalized by endocytosis (i.e., plasma membrane proteins and ligands, organelle proteins transiting through the plasma membrane) (reviewed in 21, 25, 38, 42). These functions are performed by two morphologically distinct parts of the organelle, the stack of cisternae and the *trans*-tubular network. By acting in tandem, enzymes that are asymmetrically distributed through the cisternae and *trans*-tubular network introduce a variety of posttranslational modifications (i.e., glycosylation [27], sulfation [70], phosphorylation [14, 68], acylation [56, 57], proteolytic digestion [reviewed in 61]) in newly synthesized proteins and proteins internalized by endocytosis that need repair (44, 45, 59, 60, 69). In contrast, delivery of proteins to the sites where they function appears to occur exclusively in the network of tubules located in the *trans*-side of the organelle (7, 24, 26, 39; reviewed in 25; for a dissenting view see 11). In these tubules proteins processed in the cisternae or transported from the plasma membrane by endosomes are first sorted and then packed and delivered to the sites where they function. Antibodies against components of the cisternae and *trans*-tubular parts of the Golgi system could be useful tools to characterize the pathways of proteins through the Golgi system, study the molecular bases of the morphological and functional asymmetries of this organelle, examine its assembly and disassembly through the cell cycle, and determine its biogenesis and turnover. Also, characterization of the Golgi components reacting with the antibodies and study of their synthesis, processing, and turnover should be helpful to understand the function and organization of the Golgi system. We report here the development of monoclonal antibodies that specifically recognize two Golgi integral membrane proteins (GIMPs)[1] located, respectively, in the *cis* and medial cisternae ($GIMP_c$) and in the *trans*-cisternae and *trans*-tubular network ($GIMP_t$) of the Golgi system. Using these antibodies, we have characterized $GIMP_c$ and $GIMP_t$, studied their synthesis, glycosylation, phosphorylation and turnover, and compared their distribution in the Golgi system of interphase cells and Golgi-derived elements of cells in mitosis, or treated with taxol or colcemid.

Materials and Methods

Cell Culture

Normal rat kidney (NRK) and rat basophilic leukemia (RBL) cells were grown in 45% DME/45% Ham's F-12/10% FBS/2 mM glutamine, 150 U/ml penicillin, and 50 µg/ml streptomycin (regular medium). When required, the NRK cells were cultured with 10 µM colcemid (Gibco, Grand Island,

1. *Abbreviations used in this paper*: GIMP, Golgi integral membrane protein; $GIMP_c$ and $GIMP_t$, GIMPs located in the *cis* and medial cisternae, and in the *trans*-cisternae and *trans*-tubular network, respectively; NRK, normal rat kidney; RBL, rat basophilic leukemia.

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NY) for 3 h, or with 20 μM taxol (National Cancer Institute) for the same time.

Monoclonal Antibodies

6-wk-old BALB/c mice were immunized with integral membrane proteins from rat liver fractions enriched in Golgi cisternae (27) or lysosomes (32) treated with 0.1 M Na_2CO_3, pH 11.3 (22). The immunization schedule was as follows: On day 1, 300 μg of protein prepared in 0.2 ml of 0.25 M sucrose were mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously in the backs of the mice. Three similar injections in incomplete Freund's adjuvant were administered every other 30 d, starting 1 mo after the first injection. 15 d later, and 3 d before removal of the spleens, a final injection of 300 μg protein in 0.25 M sucrose was administered intraperitoneally. Spleens were removed from the mice under anesthesia, minced, and fused with SP2/O myeloma cells to obtain hybridomas. The fusion was performed using polyethylene glycol (23). The fused cells were suspended in regular medium containing 10% Hy-Clone FCS, 20 mM hypoxanthine, 3 μM thymidine, 80 mM aminopterin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 μg/ml selenious acid, and plated on forty 96-well trays for every two spleens, each well containing 3×10^3 rat macrophages seeded 24 h before the fusion. Positive clones were selected by immunofluorescence microscopy using NRK cells (3,9) and subcloned twice. Only clone-conditioned media, concentrated 10-fold by precipitation with 50% $(NH_4)_2SO_4$, pH 7.0, and dialyzed against PBS, was used in the studies of GIMPs. The antibodies used in this study were produced by clones 15C8, 18B11, and 5G6. The first two antibodies were IgGs and the third one was an IgM. The monoclonal 53FC3 reacting with the 135-kD membrane protein located in the Golgi cisternae (13) was a generous gift of Dr. Brian Burke (European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany).

Immunofluorescence Microscopy

NRK cells were grown attached to round glass coverslips in regular medium for 48 h before use. The cells were fixed and permeabilized with cold (−20°C) methanol for 2 min, washed briefly with PBS, and incubated for 1 h at 37°C with clone-conditioned medium, diluted 1:10 in PBS containing the antibody of choice. After washing for 15 min at room temperature with PBS to remove excess antibody, the cells were incubated for 45 min at 37°C with 80 μl of PBS containing 7.5 μg of either rhodamine-conjugated goat anti-mouse μ chain antibody, rhodamine or fluorescein-conjugated goat anti-mouse γ-chain antibody, or fluorescein-conjugated rabbit anti-rat IgG antibody mixed with 12.5 μg nonimmune goat IgG (all of the second antibodies were from CooperBiomedical, Inc., Malvern, PA). Excess second antibody was removed by washing the cells with PBS for 30 min and the cover glasses were mounted on glass slides using gelvatol (Montsanto Co., St. Louis, MO). Cells were studied by immunofluorescence microscopy using an ICM 405 inverted Zeiss microscope.

Electron Microscopy

The localization of GIMPs in the Golgi system was studied by electron microscopy using NRK cells grown for 2 d to 80% confluency in regular medium on 35-mm plastic dishes. Study of $GIMP_c$ was performed on cells fixed for 15 min at room temperature with 2% formaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (11, 63). Because the reactivity of $GIMP_t$ with antibody 18B11 was lost after glutaraldehyde fixation, its study was performed using cells fixed for 1 h at 4°C and then for 1 h at room temperature with 2% formaldehyde (Fisher Scientific Co., Pittsburgh, PA), 0.075 M lysine, 0.01 M $NaIO_4$ in 37 mM phosphate buffer, pH 6.2 (34). Fixed cells were permeabilized for 15 min at 37°C with 0.1% saponin (Sigma Chemical Co., St. Louis, MO)/0.1% BSA (Miles Scientific, Div. Miles Laboratories, Naperville, IL) in PBS (buffer A) and studied by the preembedding immunoperoxidase procedure (11, 63). Buffer A was used in all the incubations with antibodies and washes. Incubation with the monoclonal antibody of choice was performed for 1 h at 20°C. After washing to remove excess of antibody the cells were incubated for 1 h at 20°C with peroxidase conjugated $F(ab')_2$ goat anti-mouse (Miles Scientific), washed to remove excess antibody, and the peroxidase reaction developed by incubation for 1 h at 20°C with 0.2% diaminobenzidine hydrochloride (Sigma Chemical Co.), 0.01% H_2O_2 (Mallinckrodt, Inc., St. Louis, MO) in 0.1 M Tris-HCl, pH 7.4. Ferrocyanide-reduced OSO_4 (Polysciences, Inc., Warrington, PA) was used in postfixation before dehydrating and embedding the cells in Epon 812 (Tousimis Research, Inc.). Cellular thin sections (800 A) were stained with lead citrate (Polysciences, Inc.) and examined in a Philips 400 electron microscope.

Radiolabeling of GIMPs

Iodination. Crude membrane fractions from NRK cells, RBL cells, rat pituitary, and rat pancreas were prepared as described (9). A rat liver fraction enriched in Golgi cisternae was prepared by the method of Howell and Palade (27). All membranes were treated with 0.1 M Na_2CO_3, pH 11.3 to obtain integral membrane proteins (22), collected by centrifugation for 30 min at 100,000 *g*, 4°C, resuspended in 1% Triton X-100/PBS with a Dounce homogenizer (Kontes Glass Co., Vineland, NJ), and incubated for 30 min at 4°C. Detergent-insoluble material was removed by centrifugation for 10 min at 30 psi, 4°C, using an Airfuge (Beckman Instruments, Inc., Palo Alto, CA) and the supernatants used for radioiodination. Radioiodination of the integral membrane proteins was performed with 1 mCi Na ^{125}I (33 μCi/μg, Amersham Corp., Arlington Heights, IL) using enzymobeads (Bio-Rad Laboratories, Richmond, CA) (33).

Metabolic Labeling. 2×10^7 NRK cells, grown to 90% confluency on plastic dishes were labeled with [^{35}S]methionine, [2-^{3}H]-D mannose, [6-^{3}H(*N*)]-*N*-acetylmannosamine, or [^{32}P]orthophosphate as follows. (*a*) Methionine labeling: cells were incubated for 30 min at 37°C in 6 ml of methionine-free DME/0.2 mM glutamine and pulse labeled for 15 min with 1 mCi [^{35}S]methionine (1,000 Ci/mmol; Amersham Corp.). (*b*) Mannose labeling: cells were incubated for 30 min at 37°C with 10 ml MEM/50 μM glucose/0.2 mM glutamine/2% dialyzed FCS and pulsed for 15 min or 2 h with 1 mCi 2 [^{3}H]-D-mannose (24 Ci/mmol; ICN Biochemicals, Inc., Irvine, CA). (*c*) Sialic acid labeling: cells were preincubated for 30 min at 37°C in glucose-free MEM/0.2% BSA/1 mM sodium pyruvate/0.2 mM glutamine and labeled for 5 h with 5 mCi [6-^{3}H(*N*)]-*N*-acetylmannosamine (28.6 Ci/mmol; New England Nuclear, Boston, MA) (5). (*d*) Phosphate labeling: cells were incubated for 1 h at 37°C with 15 ml phosphate-free DME medium/2% dialyzed FCS/0.2 mM glutamine and then for 2.5 h in 15 ml of the same medium with 2.5 mCi [^{32}P]orthophosphate (carrier-free; Amersham Corp.) in the absence or presence of 0.2 mM dibutyryl 3'-5'-cAMP. To study the effect of Ca^{++} levels on the phosphorylation of GIMPs, untreated cells were incubated for the last 30 min with 1 μg/ml of the calcium ionophore A23187 (Sigma Chemical Co.).

When required, the label incorporated into GIMPs was chased for the periods of time indicated with complete regular medium. Labeled cells were extracted with 1% Triton X-100/2 mM EDTA/10 μg/ml leupeptin (Boehringer Mannheim Diagnostics, Inc., Houston, TX)/0.1 mM PMSF (Sigma Chemical Co.)/PBS (extraction buffer) for 30 min at 4°C. An antiphosphatase cocktail, consisting of 0.4 mM sodium vanadate, 0.4 mM EDTA, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate was added to the extraction buffer to prevent protein dephosphorylation and obtain a maximal phosphorylation signal (52). This cocktail was omitted in studies of the topology of the phosphorylation sites. Insoluble material was removed by centrifugation for 1 h at 100,000 *g*, 4°C.

Immunoprecipitation

Detergent-solubilized radiolabeled integral membrane proteins were passed through 0.22-μm filters (Millex-GV; Millipore/Continental Water Systems, Bedford, MA) and used in immunoprecipitation studies. Immunoprecipitations were performed using the monoclonal antibody of choice bound to protein A-Sepharose beads (Pharmacia Fine Chemicals, Div. Pharmacia, Inc., Piscataway, NJ). IgGs, 15C8, and 18B11 antibodies were directly bound to protein A-Sepharose, whereas the IgM 5G6 antibody was bound to rabbit anti-mouse IgM (kindly supplied by Dr. Edmundo Lamoyi; Universidad Nacional Autonoma de Mexico, Mexico) linked to protein A-Sepharose. For this purpose, 50 μl of protein A-Sepharose or rabbit anti-mouse IgM-protein A-Sepharose, in extraction buffer (wt/vol) were incubated for 4 h at 4°C, with continuous rotation, with 100 μl of conditioned medium concentrated 10 times containing ~50 μg antibody. The beads were washed twice with 1 ml extraction buffer and incubated for four additional hours under the same conditions with radiolabeled integral membrane proteins (5×10^6 dpm) preadsorbed with protein A-Sepharose. After incubation the beads were collected by low speed centrifugation, washed twice with 1 ml of 10 mM Tris-HCl buffer, pH 7.4/0.5% Triton X-100/0.2 M NaCl/2 mM EDTA/0.1% BSA/10 μg/ml leupeptin/0.1 mM PMSF (washing buffer) and once with 1 ml of the same buffer containing 0.2% SDS. For direct analysis of samples by SDS-12.5% PAGE, the beads were washed with 1 ml of 20 mM Tris-HCl buffer, pH 7.4. Samples treated enzymatically before the electrophoresis were washed with 0.1 M sodium phosphate buffer, pH 6.1.

Glycosidase Digestions

Digestion with Endo-β*N*-acetylglucosaminidase F (Endo F; New England Nuclear) (20), Endo-β*N*-acetylglucosaminidase H (Endo H, Streptomyces

griseus; Boehringer Mannheim Diagnostics, Inc.) (36), and O-Glycanase (Diplococcus pneumonae; Genzyme Corp., Boston, MA) (8) were performed as described. For digestion of sialic acid, samples were boiled for 5 min in 0.1 M sodium phosphate buffer, pH 6.1, containing 50 mM EDTA and 1% NP-40, and incubated for 1 h at 37°C with 5 μm of neuraminidase (*Arthrobacter ureafaciens*; Calbiochem-Behring Corp., La Jolla, CA) (8).

Binding of GIMPs to Limulus Polyphemus Lectin

[^{125}I]GIMPs were immunoprecipitated from rat liver Golgi membranes, incubated in the absence or presence of neuraminidase or Endo F, resolved from the antibodies by SDS PAGE, visualized by autoradiography, cut, eluted from the gels, and incubated (2×10^3 dpm) for 2 h at 4°C with 75 μl limulus polyphemus lectin-Sepharose (E.Y. Laboratories, Inc., San Mateo, CA) (wt/vol) in 1 ml PBS/0.05% BSA/1% NP40/5 mM $CaCl_2$. The beads were washed twice with the same buffer and GIMPs bound to the lectin specifically eluted with 0.02 M sialic acid (Sigma Chemical Co.) in 0.5 M NaCl/0.1% EDTA/0.5% NP-40, pH 8.0, counted for ^{125}I, run on SDS PAGE, and visualized by autoradiography.

Epitope Localization

The epitope recognized by antibody 18B11 was studied by incubating intact and Triton X-100 (0.2%)-treated Golgi elements obtained from rat liver, with and without proteinase K (100 μg/ml, 1 h at 4°C) (Boehringer Mannheim Diagnostics, Inc.) in PBS. The protease digestion was stopped with 0.1 mM PMSF and the intact Golgi vesicles solubilized with 0.2% Triton X-100 before iodination of the proteins with ^{125}I. 2×10^6 cpm of each ^{125}I-protein preparation was incubated with antibody 18B11 and the resultant immunoprecipitates analyzed by SDS PAGE. The topology of the epitope recognized by antibody 15C8 was similarly studied using Golgi elements from NRK cells labeled in vivo with [^{32}P]orthophosphate.

Cellular Levels of $GIMP_c$ and $GIMP_t$

The number of copies of $GIMP_c$ and $GIMP_t$ per cell was calculated by Scatchard analysis (55) from assays of antibody binding to cellular membranes. Membranes were obtained from NRK cells (9). The cells were lysed in 50 vol of cold water using 50 strokes with a Dounce homogenizer (Kontes Glass Co.), and membranes from a postnuclear supernatant collected by centrifugation (150,000 *g*, 40 min, 4°C), and resuspended in 0.2% saponin in 130 mM NaCl, 20 mM Tris-HCl, pH 7.4 (solubilization buffer). At the concentration used, saponin produced the permeabilization of the membranes, allowing their penetration by the antibodies (as shown by immunoelectron microscopy) without causing significant solubilization of the antigens. Binding of ^{125}I-15C8 and ^{125}I-18B11 antibodies (1.7 Ci/μmol, 2.3×10^6 cpm/assay) to $GIMP_c$ and $GIMP_t$, respectively, was studied at equilibrium, after overnight incubation at 4°C, using different concentrations of the corresponding cold antibody (15C8, 0.006–3.6 pmoles; 18B11, 0.09–3.4 pmoles), and a fixed amount of membranes (14 or 70 μg membrane protein) in 0.2 ml solubilization buffer. Nonspecific binding was measured in the presence of 600 pmoles of the corresponding cold antibodies. Bound was separated from free antibody by pelleting the membranes at 30 psi for 15 min at 4°C using a Beckman Airfuge (Beckman Instruments, Inc.). Membrane pellets were counted for ^{125}I using a counter (Beckman 5500 counter; Beckman Instruments, Inc.).

Other Methods

SDS-12% PAGE (31) and phosphoamino acid analysis (17) were performed as described. Removal of the phosphate incorporated into $GIMP_c$ was performed using alkaline phosphatase from calf intestine (Boehringer Mannheim Diagnostics, Inc.).

Results

Antibodies to GIMPs

To prepare antibodies against GIMPs two cellular fractions from rat liver enriched in Golgi cisternae (27) and lysosomes (32), respectively, were used as source of the antigens. Surprisingly, the preparation of lysosomal integral membrane proteins was more effective in inducing the production of anti-GIMP antibodies (10% positive clones) than the fraction enriched in GIMPs (1% positive clones). Of the 22 clones initially positive, only six continued producing anti-GIMP antibodies during the expansion of these cell lines. Four of the positive clones produced IgG class antibodies and two IgMs. Antibodies 15C8 and 5G6 recognized an integral membrane protein located in the *cis* and medial Golgi cisternae, whereas antibody 18B11 reacted with an integral membrane protein contained in the *trans*-cisternae and *trans*-network of tubules of the Golgi system (see below). Henceforth, the protein located in the *cis* and medial cisternae will be called $GIMP_c$ and the protein contained in the *trans*-part of the Golgi system $GIMP_t$.

Cellular Localization of GIMPs

Indirect immunofluorescence microscopy studies of NRK cells, fixed-permeabilized with methanol and stained with the monoclonal anti-GIMP antibodies 15C8, 5G6, or 18B11, revealed identical fluorescence patterns. The three antibodies specifically stained a reticular organelle (Fig. 1, *A* and *B*) located in the area of cytoplasm housing the microtubule organizing center in the vicinity of the nucleus (Fig. 1, *C* and *D*). Both the morphology and cellular localization of the organelle were those previously described for the Golgi system (48). Also supporting this identification were the changes in the localization and organization of the organelle during mitosis and in response to colcemid and taxol. As previously described for the Golgi system, the organelle stained by anti-GIMP antibodies was fragmented during mitosis (46), with the fragments being clustered around the poles of the mitotic spindle throughout metaphase (Fig. 1, *M* and *N*) and encircling the nuclei of daughter cells in late telophase (Fig. 1, *K* and *L*). Also, like the Golgi system, the organelle was fragmented into pieces randomly scattered throughout the cytoplasm in cells treated with colcemid (Fig. 1, *E* and *F*) (35, 65, 66) and broken into parts associated with microtubule bundles in cells treated with taxol (Fig. 1, *G–J*) (66). Electron microscopy studies confirmed that the antigens recognized by the anti-GIMP antibodies were exclusively located in the Golgi system (Fig. 2, *A* and *D*). Furthermore, $GIMP_c$ and $GIMP_t$ were displayed in different sites of the organelle. $GIMP_c$ was localized in the first three, *cis*-medial cisternae (Fig. 2, *A*, *B*, *I–K* lateral view, and *C*, front view) located in the vicinity of transitional elements of the endoplasmic reticulum and clusters of transitional vesicles (i.e., Golgi vesicles) (Fig. 2, *B*, *J*, and *K*). In contrast, $GIMP_t$ was contained in elements of the *trans*-Golgi, including the *trans*-most cisternae (Fig. 2, *O*) (47), coated tubules (Fig. 2, *G*, *H*, *N*, and *P*) (40, 41), coated vesicles (Fig. 2, *E*, *O*, and *Q*) (40, 41), and condensing vacuoles (Fig. 2, *P* and *Q*) (62). It is interesting that individual stacks of Golgi cisternae and corresponding *trans*-tubular elements displayed different orientations of the *cis*- and *trans*-sides with respect to the centrosome (Fig. 3).

$GIMP_c$ and $GIMP_t$ Antigens

To characterize the antigens recognized by antibodies 15C8, 5G6, and 18B11, detergent-solubilized integral membrane proteins from NRK cells were labeled with [^{125}I]iodine and immunoprecipitated with the antibody bound to protein A-Sepharose beads. Analysis of the immunoprecipitates by SDS PAGE revealed that antibodies 15C8 (Fig. 4, lane *2*) and 5G6 (not shown) immunoprecipitated a protein, $GIMP_c$,

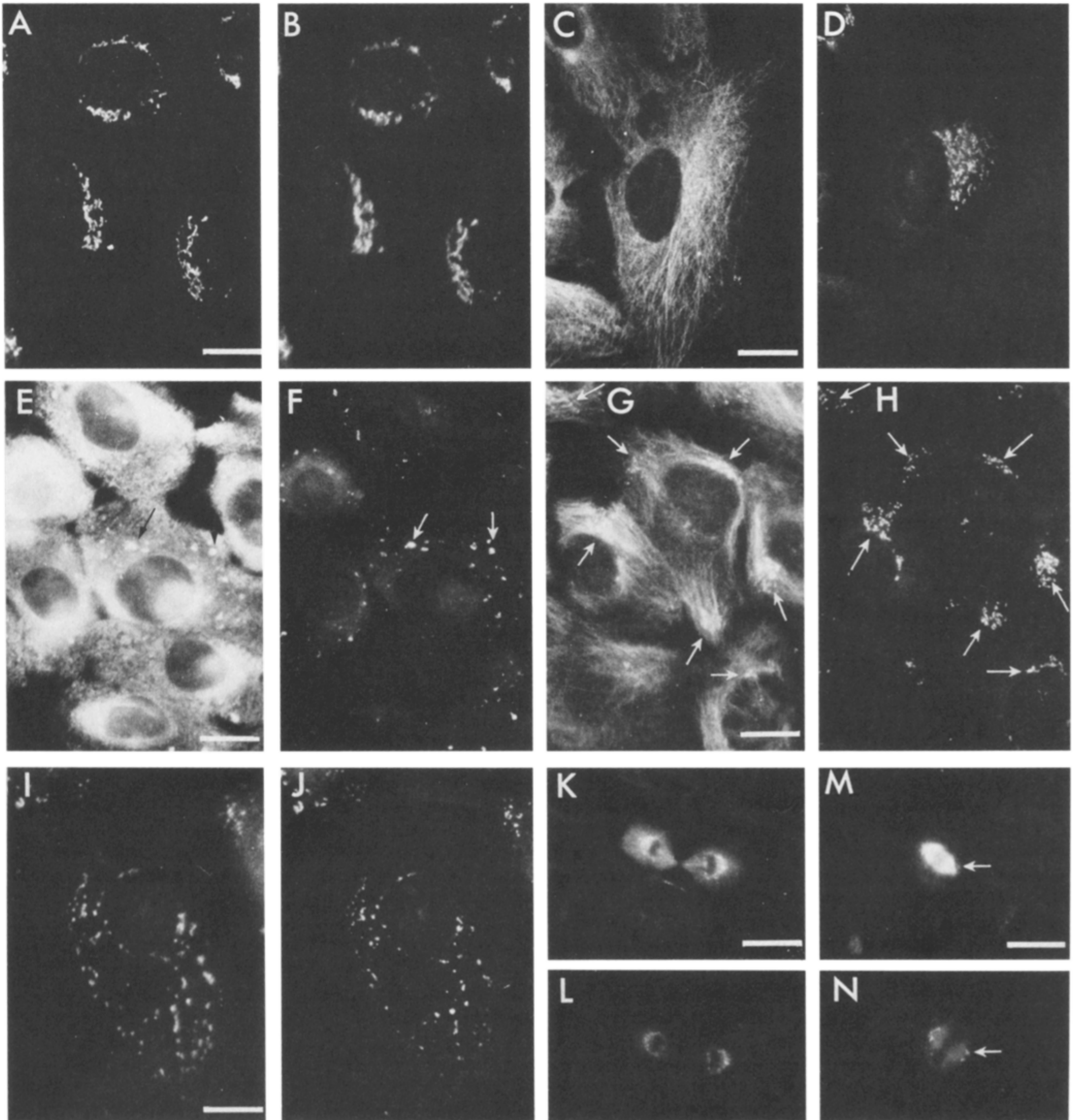


Figure 1. Immunofluorescence microscopy of NRK cells stained with anti-GIMP antibodies. Interphase cells simultaneously stained with antibodies 18B11 (*A*, rhodamine channel) and 5G6 (*B*, fluorescein channel). Note the staining of the same reticular organelle by both anti-GIMP antibodies. Interphase cells simultaneously stained with the anti-tubulin YOL 1/2 antibody (*C*, fluorescein channel) and antibody 18B11 (*D*, rhodamine channel). Note the location of the reticular organelle stained by the anti-$GIMP_t$ antibody in the area of cytoplasm housing the microtubule organizing center from which microtubules radiate to the cell periphery. Interphase cells treated with 10 μM colcemid for 3 h and simultaneously stained with antibodies 5G6, YOL 1/2 (*E*, fluorescein channel), and 18B11 (*F*, rhodamine channel). Note the disappearance of microtubules and uniform distribution of tubulin throughout the cytoplasm (*E*). Observe the fragmentation of the Golgi system into $GIMP_c$- and $GIMP_t$-containing vesicles randomly distributed throughout the cytoplasm (compare *E* with *F*, *arrows*). Control cells incubated without antibody 5G6 did not display Golgi-derived vesicles in the fluorescein channel (not shown). Interphase cells treated with 20 μM taxol for 3 h and stained with antibodies 5G6, YOL 1/2 (*G*, fluorescein channel), and 18B11 (*H*, rhodamine channel). Note the rupture of the Golgi system and the association of fragments containing $GIMP_c$ and $GIMP_t$ (*arrows*) with microtubule bundles. Cells incubated without antibody 5G6 did not display Golgi fragments in the fluorescein channel (not shown). Interphase cells treated with taxol displaying extensive alteration of the normal microtubule organization. Observe the codistribution of the Golgi pieces stained with antibodies 5G6 (*I*, fluorescein channel) and 18B11 (*J*, rhodamine channel), with the microtubule bundles. Mitotic cells simultaneously stained with antibodies 5G6, YOL 1/2 (*K* and *M*, fluorescein channel) and 18B11 (*L* and *N*, rhodamine channel). Note the accumulation

that migrated at 130 kD. Sequential immunoprecipitation studies, analysis of the pattern of glycosylation, and study of the phosphorylation revealed that both antibodies reacted with the same protein (data not shown). In contrast, antibody 18B11 precipitated a major protein, $GIMP_t$, with an apparent molecular mass of 100 kD (Fig. 3, lane *1*). Occasionally, this antibody also immunoprecipitated a 90-kD polypeptide (Fig. 3, lane *5*). This was probably a species of $GIMP_t$ with a different carbohydrate content, as indicated by the co-migration of both proteins on SDS PAGE after their treatment with Endo F. A protein with the same apparent molecular mass of $GIMP_c$ isolated from NRK cells was also immunoprecipitated from crude membranes from RBL cells, rat pituitary, rat pancreas, and from membranes of a rat liver fraction enriched in Golgi cisternae (Fig. 4, *even lanes*). Those extracts, with the exception of pancreas, also contained the 100-kD $GIMP_t$ (Fig. 4, *odd lanes*). Rat pancreas contained species of $GIMP_t$ that migrated at 120 kD (Fig. 3, lane *7*). These results indicated that $GIMP_c$ and $GIMP_t$ were widespread among different rat cell lines and tissues, and that a different species of $GIMP_t$ existed in pancreas. It is noteworthy that antibody 53FC3 (generously provided by Dr. B. Burke), when incubated with NRK cell extracts, immunoprecipitated a 135-kD protein (12) that migrated more slowly than $GIMP_c$ on SDS PAGE (data not shown).

Treatment of intact Golgi elements with proteinase K had different effects on $GIMP_c$ and $GIMP_t$ (Fig. 5). The protease treatment of $GIMP_t$ produced a 87-kD fragment, still reactive with antibody 18B11. In contrast, proteinase K had no apparent effect on the molecular mass of $GIMP_c$. These results suggested that $GIMP_t$ possessed a relatively long cytoplasmic tail and that the epitope recognized by antibody 18B11 was located in the lumen of *trans*-Golgi elements. The apparent insensitivity of $GIMP_c$ to the protease could be compatible with either the cleavage of a very short cytoplasmic tail and location of the epitope in the lumen of the cisternae, or with a protease-resistant cytoplasmic domain and a cytoplasmic or lumenal epitope location.

Scatchard analysis of the binding of ^{125}I-labeled 15C8 and 18B11 antibodies to NRK cells permeabilized with 0.2% saponin revealed the existence of 7.5×10^4 and 5×10^5 copies/cell of $GIMP_c$ and $GIMP_t$, respectively.

Glycosylation of GIMPs

To study the carbohydrate content of GIMPs, we examined the sensitivity of the mature forms of $GIMP_c$ and $GIMP_t$ obtained from NRK cells to Endo H, Endo F, O-glycanase, and neuraminidase digestions (Fig. 6). Neither GIMP was sensitive to Endo H digestion (Fig. 6, compare lanes *1* and *2*), indicating the absence of N-linked high-mannose oligosaccharides in the two proteins. However, both were effectively digested by Endo F, and removal of their N-linked complex oligosaccharides by the enzyme resulted in species of $GIMP_c$ and $GIMP_t$ that migrated at 118 and 70 kD, respectively (Fig. 6, compare lanes *1* and *3*). In addition, the two GIMPs were sensitive to digestion with neuraminidase (Fig. 6, compare lanes *1* and *4*), the sialic acid–free form displaying mobilities at 118 ($GIMP_c$) and 70-kD ($GIMP_t$). The presence of sialic acid in both proteins was also confirmed by their labeling with the sialic acid precursor [6-^{3}H(*N*)]-*N*-acetylmannosamine (data not shown). Incubation of the sialic acid-free GIMPs with O-glycanase resulted in further decrease of their apparent molecular masses to 110 ($GIMP_c$) and 63 kD ($GIMP_t$) (Fig. 6, compare lanes *1* and *5*), indicating the presence of *O*-linked oligosaccharide chains in both proteins. Finally, complete digestion of GIMPs with a mixture of Endo F, neuraminidase, and O-glycanase produced peptides with apparent molecular masses of 105 ($GIMP_c$) and 47 kD ($GIMP_t$) (Fig. 6, lane *6*).

Characterization of Sialic Acid–containing Carbohydrates of GIMPs

Sialic acid is a frequent component of the N- and O-linked carbohydrates acquired by proteins in the Golgi system. There is evidence that several different sialyltransferases are involved in the sialylation of the two types of carbohydrates (5, 6, 49, 64, 67). Sialylation of N-linked carbohydrates is believed to occur exclusively in the *trans*-part of the Golgi system (6, 49) and it has been used as a criteria to trace proteins transiting through that part of the organelle (51). The presence of sialic acid in both GIMPs (see above), the exclusive localization of $GIMP_c$ in the *cis* and medial-Golgi cisternae, and the confinement of $GIMP_t$ in the *trans*-part of the organelle provided a system to test that localization. For this purpose, the presence of sialic acid in the N-linked carbohydrates acquired by $GIMP_c$ and $GIMP_t$ was studied. The study was performed by comparing the binding of the two proteins with the sialic acid–specific lectin Limulus polyphemus, before and after digestion with Endo-F (Fig. 7 *B*). We found that digestion with Endo F not only reduced the binding of $GIMP_t$ to the lectin by fourfold but that it also produced a fivefold decrease in the binding of $GIMP_c$. The latter result indicated the presence of sialic acid in the N-linked carbohydrates of $GIMP_c$, strongly suggesting that proteins confined in the *cis* and medial cisternae of the Golgi system could incorporate sialic acid into N-linked carbohydrates (see Discussion). In parallel experiments it was observed that after treatment with neuraminidase the binding of $GIMP_c$ and $GIMP_t$ to the lectin was reduced by 11- and 5-fold, respectively (Fig. 7 *A*). The two-fold difference between the binding of the Endo F- and neuraminidase-digested species of $GIMP_c$ to the lectin suggested that the protein contained sialic acid in O-linked carbohydrates. This possibility was directly examined by studying the effect of neuraminidase on the electrophoretic mobility of $GIMP_c$ isolated from tunicamycin-treated cells. The increase in the mobility of $GIMP_c$ after treatment with the enzyme (data not shown) was consistent with that suggestion. A similar study of $GIMP_t$ was hindered by the failure to metabolically label the polypeptide part of the protein.

of Golgi elements in the two poles of the mitotic spindle of cells in metaphase. Although the elements stained by antibody 5G6 are greatly eclipsed by the strong fluorescence of microtubules, the colocalization with elements stained by antibody 18B11 can still be observed at the poles of the spindle (*M* and *N*, *arrows*). This is more clearly seen in telophase cells where the fragments of Golgi surround the newly formed nuclei of cells in telophase (*K* and *L*). Control cells, incubated without antibody 5G6, did not display Golgi elements in the fluorescein channel. Bars: (*A* and *B*) 13 μm; (*C–H*) 15 μm; (*I* and *J*) 11.5 μm; (*K–N*) 14 μm.

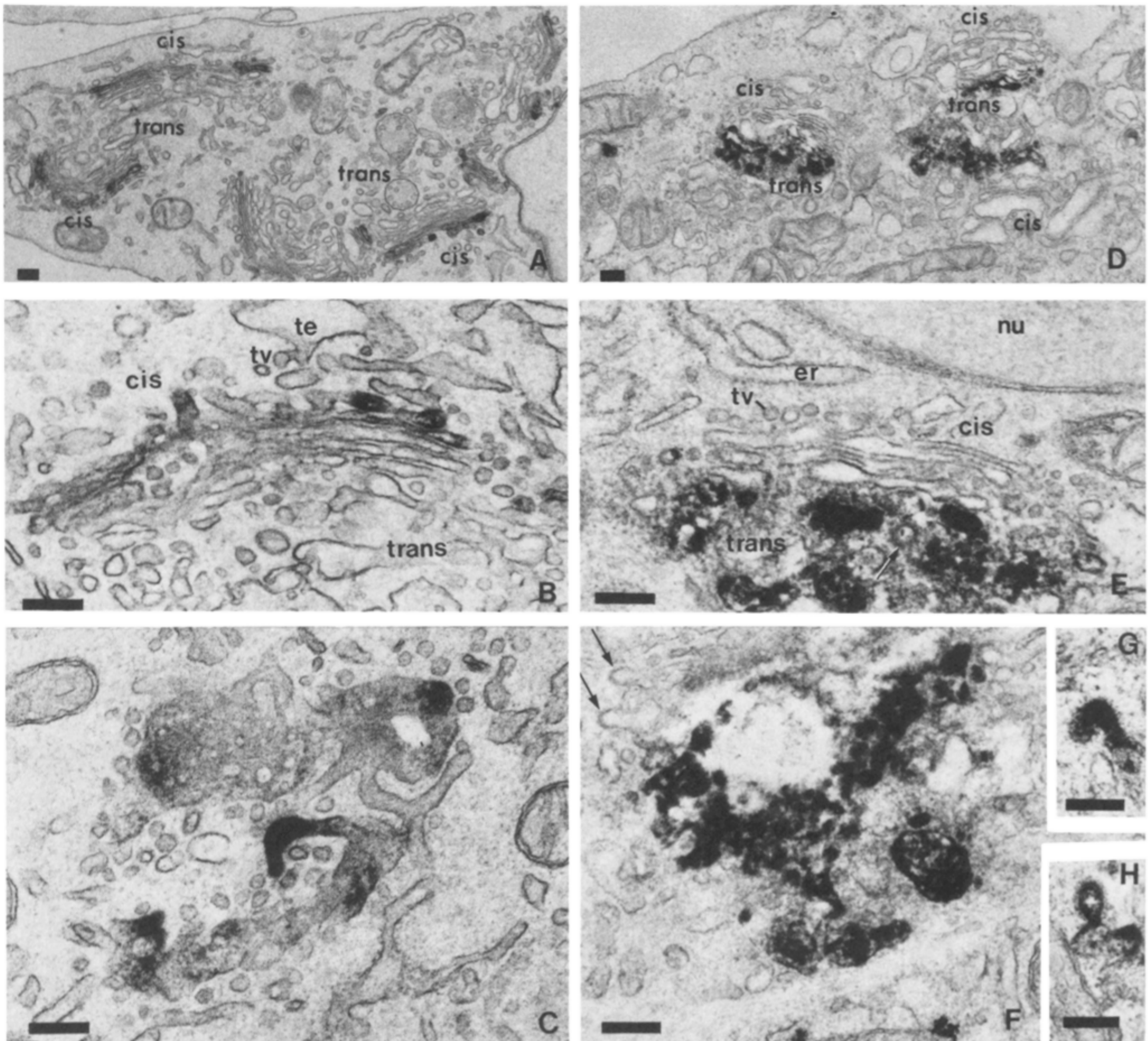
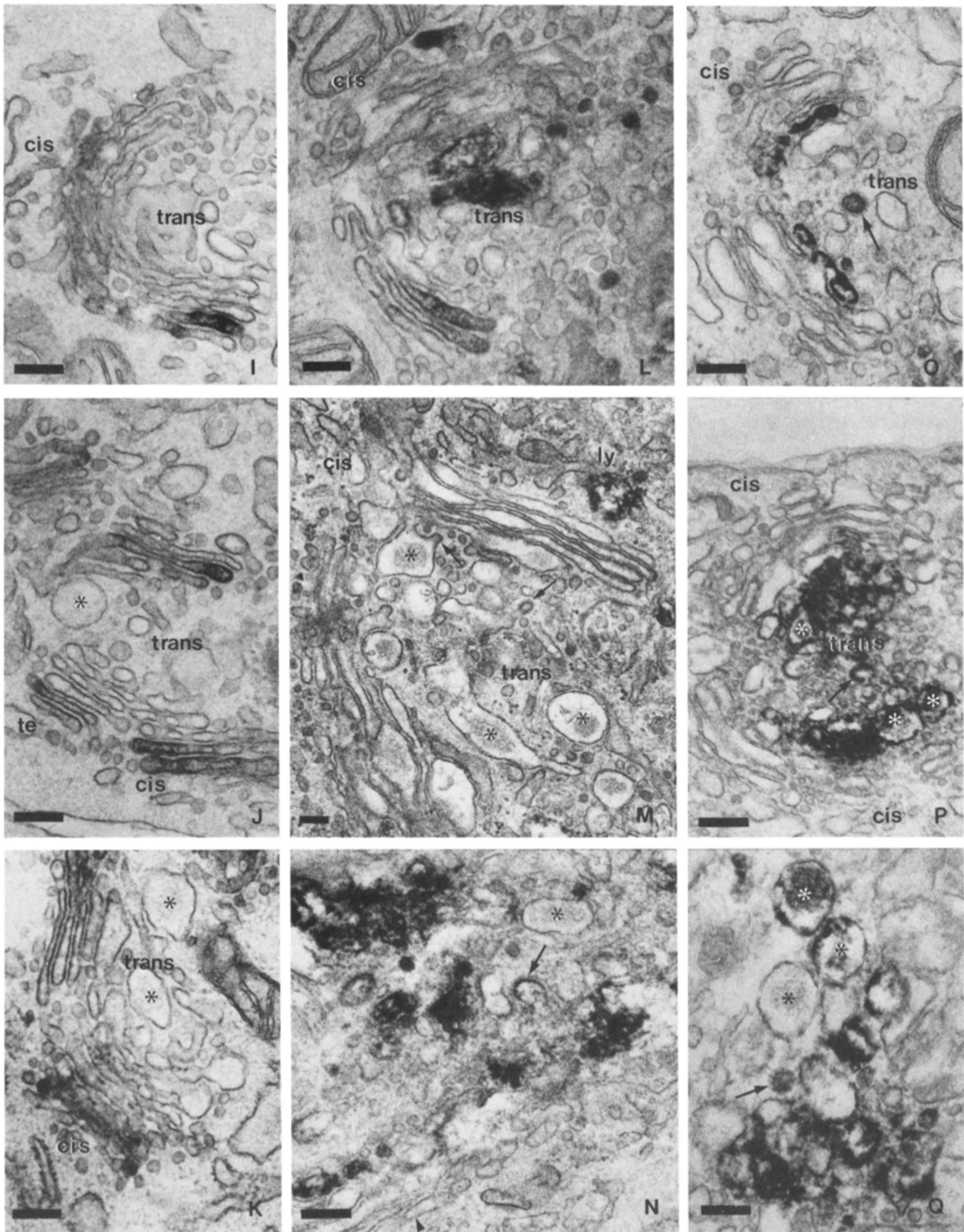


Figure 2. Localization of $GIMP_c$ and $GIMP_t$ in the Golgi system. NRK cells were processed, stained with antibodies 15C8 or 18B11, and studied by electron microscopy using the modification of the pre-embedding immunoperoxidase method described in Materials and Methods. Note that staining is exclusively observed in the Golgi-apparatus (*A* and *D*). The polarity of the cisternae stacks of the Golgi system is clear because it faces transitional elements (*te*) of the endoplasmic reticulum and clusters of transitional vesicles (i.e., Golgi vesicles) (*tv*) (*B, J,* and *K*) on the *cis*-side, and coated tubules (*G, H, N,* and *P*), coated vesicles (*E, O,* and *Q*), and condensing vacuoles (*cv*) (*P* and *Q*) on the *trans*-side. Cisternae 1, 2, and 3, representing the *cis*-most and middle cisternae, are clearly labeled with the anti-$GIMP_c$ antibody 15C8 (*A, B, I–K*). *Trans*-most cisternae (*O*), coated vesicles (*E, O,* and *Q*), coated tubules (*G, H, N,* and *P*) and condensing vacuoles (cv, *P* and *Q*), cell elements of the trans-tubular Golgi are stained with the anti-$GIMP_t$ antibody, 18B11. *Cis*, medial-cisternae,

Precursors, Intermediate Forms, and Half-Lives of GIMPs

Both the study of precursors and turnover of $GIMP_c$ and $GIMP_t$ was performed by pulse–chase experiments. $GIMP_c$ was easily labeled metabolically with [^{35}S]methionine. In contrast, for unknown reasons, $GIMP_t$ could not be labeled with methionine, cysteine, or leucine, but it was labeled with [2-^{3}H]-D-mannose. These studies revealed a precursor form of $GIMP_c$ that migrated at 120 kD (Fig. 8, *A*). An intermediate form of $GIMP_c$ with an apparent molecular mass of 127 kD was recorded after a 1-h chase, and it could be observed that it was very slowly processed to mature $GIMP_c$ (Fig. 8 *A*). In similar studies, one precursor and one intermediate form of $GIMP_t$, migrating at 68 and 89 kD, respectively, were recorded (Fig. 8 *B*). As in the case of $GIMP_c$, the intermediate form of $GIMP_t$ was also very slowly processed. In longer chase experiments the half-lives of $GIMP_c$ and $GIMP_t$ were measured. $GIMP_c$ displayed a much longer half-life (20 h) than $GIMP_t$ (9 h) (Fig. 8 *C*). It was considered unlikely that label of $GIMP_t$ with mannose could explain the shorter half-life of this protein, as $GIMP_c$ displayed the same half-life when labeled with methionine or the sugar.



and elements of the *trans*-tubular Golgi are stained in cells incubated simultaneously with anti-GIMP$_c$ and anti-GIMP$_t$ antibodies (*L*). Neither the cisternae nor elements of the *trans*-tubular Golgi were stained with the anti-lysosomal integral membrane protein antibody 38C7 (*M*) (3) that stained two small primary lysosomes. (*te*) Transitional element of the endoplasmic reticulum; (*tv*) transitional vesicle (i.e., Golgi vesicle); (*er*), endoplasmic reticulum; (*nu*) nucleus; (*arrows*), coated tubules, coated vesicles; (*stars*) condensing vacuoles. Bars, 0.2 μm.

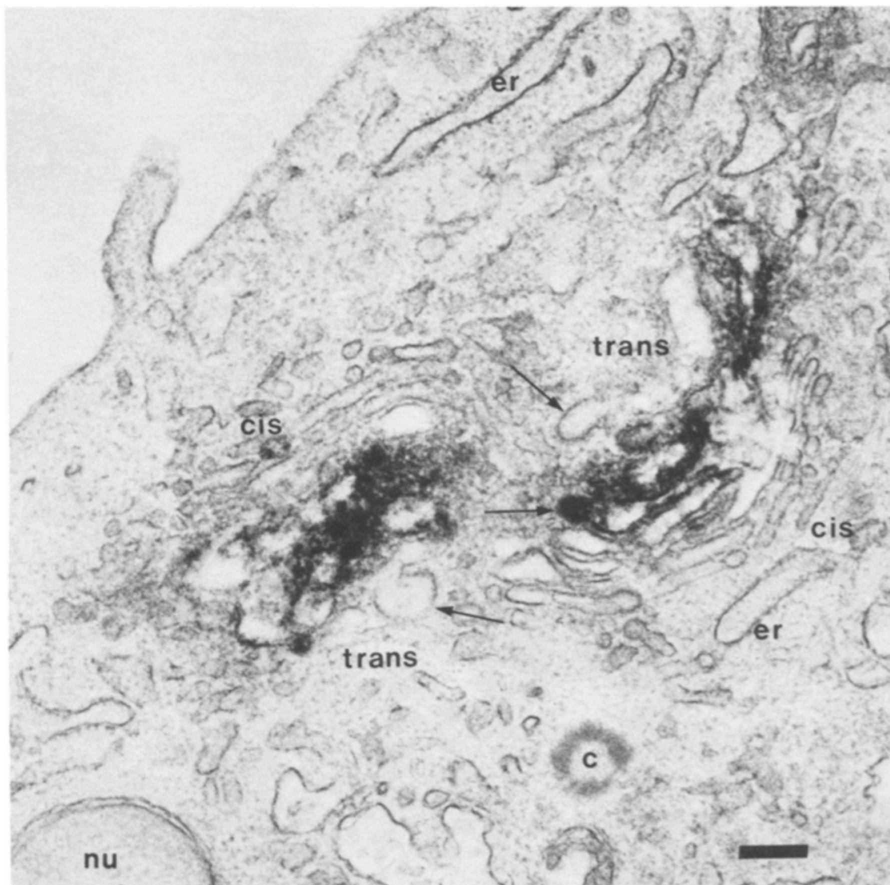


Figure 3. Opposite orientations of the *cis* and *trans*-parts of the Golgi system with respect to the centrosome. NRK cell stained with the anti-GIMP$_t$ antibody, 18B11. Two stacks of Golgi cisternae and corresponding *trans*-tubular elements are seen to be separated from each other. Note, the different orientations of the stained *trans*-tubular Golgi with respect to the centrosome (*c*). It is noteworthy that the separation of the two Golgi apparatus displayed on the picture could be more apparent than real: they could be segments of the same Golgi system, linked by a third segment out of the plane of section. (*er*) Endoplasmic reticulum; (*nu*) nucleus; (*arrows*) coated tubules. Bar, 0.2 μm.

Phosphorylation of GIMPs

To study whether GIMPs were phosphorylated in vivo, immunoprecipitation studies were performed using NRK cells metabolically labeled with [^{32}P]orthophosphate. The results of these studies showed that only GIMP$_c$ was phosphorylated. The failure of proteinase K and alkaline phosphatase, incubated with intact Golgi vesicles, to remove the phosphate from GIMP$_c$ (Fig. 9 *A*, compare lanes *1*, *3*, and *5*), and the hydrolysis of the phosphate after the incubation of Triton X-100–treated Golgi vesicles with alkaline phosphatase (Fig. 9 *A*, compare lanes *5* and *6*), suggested that the lumenal domain of the protein was phosphorylated. However, these results did not rule out the possibility that phosphorylation could occur in a site on the cytoplasmic domain of the protein, resistent to the protease and unaccessible to the phosphatase. Furthermore, phosphate was exclusively incorporated into the peptide core of the protein, as shown by the failure of a mixture of Endo F, neuraminidase, and O-glycanase to release it from the protein (Fig. 9 *C*). Phosphorylation of GIMP$_c$ was significantly stimulated by incubating the cells with dibutyryl-cAMP, indicating that a cAMP-dependent kinase was involved in the process of GIMP$_c$ phosphorylation (Fig. 9 *B*). In contrast, the Ca^{++} ionophore A23187 had no effect on the levels of phosphate incorporation (Fig. 9 *B*). Phosphoaminoacid analysis of GIMP$_c$ revealed that only serine was phosphorylated (Fig. 9 *D*).

Discussion

Two of the most relevant characteristics of the Golgi system are its morphological and functional asymmetries. The former is clearly manifested in the different structure of the cisternae (43) and the piling of these into a stack clearly distinguishable from the adjacent network of tubules prolonging the *trans*-most cisternae (49, reviewed in 25). The functional asymmetry of the organelle is exhibited in the segregation of the enzymes distributed throughout its *cis*-, medial and *trans*-parts (reviewed in 19), and in the likely exclusive involvement of the *trans*-network of tubules in the packing and sorting of proteins (7, 24–26, 54). Both asymmetries appear to result from the membrane-based compartmentalization of the organelle. Implicit in this compartmentalization is the existence of distinct membrane domains or separated membranes containing specific components and displaying different properties. Studies of model membranes such as that of the RBC (reviewed in 4) have revealed that integral membrane proteins play a pivotal role in the organization and function of membranes. These proteins, at the same time playing other functions, constitute the pillars around which partially embedded and peripheral membrane proteins are organized and membrane organization is created. Therefore, characterization of the GIMPs is of great importance in understanding the organization and function of this multimembrane-bound organelle. The lack of methods to isolate and

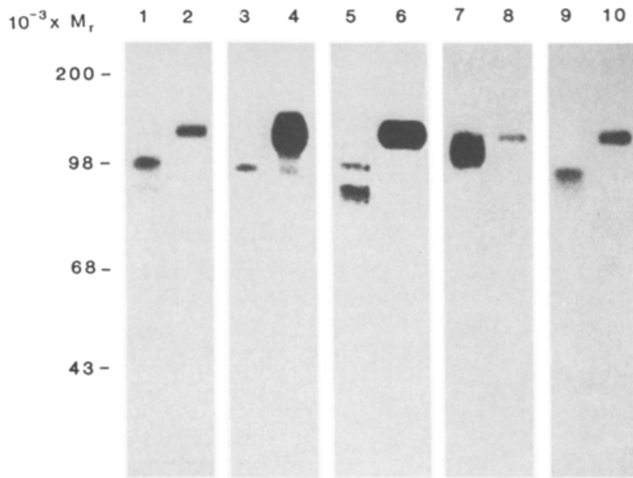


Figure 4. GIMP antigens. GIMP$_c$ (*even lanes*) and GIMP$_t$ (*odd lanes*) were immunoprecipitated from ^{125}I-integral membrane proteins of NRK cells (lanes *1* and *2*), RBL cells (lanes *3* and *4*), rat pituitary (lanes *5* and *6*), rat pancreas (lanes *7* and *8*), and a fraction of rat liver enriched in Golgi cisternae (lanes *9* and *10*) (see Materials and Methods) using antibodies 18B11 and 15C8 bound to protein A-Sepharose beads. The immunoprecipitates were resolved by SDS-12.5% PAGE and visualized by autoradiography. Note that GIMP$_c$ displayed the same apparent molecular mass, 130 kD, in all of the cell lines and tissues studied. GIMP$_t$ appeared as a major 100-kD species, except in pancreas where it migrated at 120 kD (lane *7*). A prominent species of 90 kD was observed in pituitary cells (lane *5*). Both the conversion of the 100- and 90-kD polypeptides treated with Endo F to a unique 80-kD species, and the display of the 90-kD polypeptide in pulse-labeling experiments suggested (see Fig. 6) that this was an incompletely glycosylated form of GIMP$_t$.

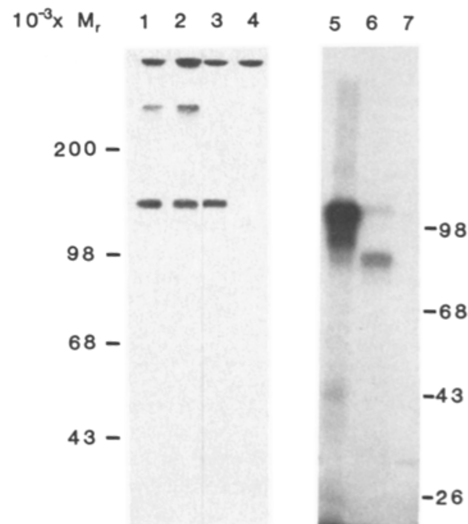


Figure 5. Studies on epitopes localization. (*1–4*) SDS PAGE autoradiograms of ^{32}P-GIMP$_c$ immunoprecipitated from Golgi elements of NRK cells using antibody 15C8. The Golgi elements were incubated without (*1* and *2*) or with proteinase K (100 μg/ml, 1 h at 4°C) (*3* and *4*), in the absence (*1* and *3*) or presence of 0.2% Triton X-100 (*2* and *4*) before incubation with the antibody. (*5–7*) SDS PAGE autoradiograms of ^{125}I-GIMP$_t$ immunoprecipitates obtained from rat liver Golgi elements incubated without (*5*) or with proteinase K (*6* and *7*) before incubation with antibody 18B11 (for more experimental details see Materials and Methods). Note: first, the destruction of the GIMP$_c$ and GIMP$_t$ epitopes when Golgi elements were incubated with proteinase K in the presence of detergent; second, the apparent insensitivity of GIMP$_c$ to the protease in the absence of detergent; third, the proteolytic cleavage and digestion of a 13-kD GIMP$_t$ fragment in the absence of detergent.

purify fractions of the Golgi system has hindered the study of those proteins. To bypass this problem, we have developed monoclonal antibodies against GIMPs. Five monoclonal antibodies reacting with Golgi membrane proteins have been already reported in the literature: one reacts with a 110-kD protein of undetermined location (28); a second and a third recognize one protein migrating at 103–108 kD and two at 103–108 and 180 kD, respectively, located in the *trans*-cisternae (58); a fourth antibody reacts with a 135-kD protein also localized in the *trans*-cisternae (13); finally, a fifth recognizes a protein migrating at 53 kD confined in the medial cisternae (16). Not one of these proteins has been further characterized.

The three anti-GIMP antibodies here developed react with two proteins, GIMP$_c$ and GIMP$_t$, located, respectively, in two of the most distinct parts of the Golgi system, the *cis*-medial cisternae and the *trans*-most cisternae and network of tubules expanding from it (49). This localization has been defined in lateral views of Golgi systems displaying a clear topography, with the *cis*-side marked by the presence of multiple transitional elements (69) and the *trans*-side by the display of numerous condensing vacuoles and spinny coated tubules and vesicles (40, 41, 62). Front views of the Golgi elements housing GIMP$_c$ and GIMP$_t$ are also consistent with the location of the former in cisternae and the latter in tubular and vesicular structures (43). It is noteworthy that whereas some Golgi stacks display a *trans*-orientation with respect to the centrosome, others show a *cis*-orientation. This indicates that in NRK cells the centrosome is not a good marker to define the topography of the Golgi system (11).

It is interesting that, although segregated in different parts of the Golgi system of cells in interphase, GIMP$_c$ and GIMP$_t$ are colocalized in the same cellular areas after the organelle fragmentation in mitosis, or when cells are treated with colcemid or taxol. Electron microscopy studies using double immunolabeling will be necessary to determine whether this colocalization results from the relocation of the two proteins in the same membrane compartment or if they remain separated in their original compartments and the fragments of these are maintained in register. The distinction between these two possible different distributions can have important implications in the understanding of the organization and compartmentalization of the Golgi system. It could help to distinguish whether the compartmentalization of the organelle is created within a continuous membrane, by segregation of proteins with different properties and functions into distinct domains, or as recent results suggest (1, 2, 10, 51, reviewed in 19), is established on the basis of independent membrane-bound compartments. Colocalization of the two proteins in the same compartment after fragmentation would be more consistent with compartmentalization resulting

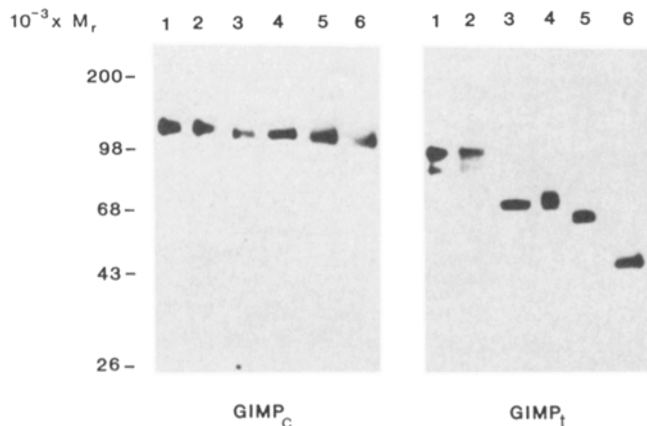


Figure 6. Characterization of the carbohydrate chains of $GIMP_c$ and $GIMP_t$. $GIMP_c$ and $GIMP_t$ immunoprecipitated from ^{125}I-labeled integral membrane proteins of NRK cells using antibodies 15C8 and 18B11, respectively, were incubated without (lanes *1*) or with Endo H (lanes *2*), Endo F (lanes *3*), neuraminidase (lanes *4*), neuraminidase plus O-glycanase (lanes *5*), or a mixture of neuraminidase, O-glycanase, and Endo F (lanes *6*). Note the resistance of $GIMP_c$ and $GIMP_t$ to Endo H digestion (compare lanes *1* and 2), suggesting the absence of N-linked high-mannose carbohydrates in both proteins; their sensitivity to Endo F (compare lanes *1* and *3*) resulting from the presence of N-linked complex carbohydrates; their content of neuraminidase-sensitive sialic acid (compare lanes *1* and *4*), and the presence of O-linked carbohydrates removed by O-glycanase after neuraminidase digestion (compare lanes *4* and *5*). Numbers on the left indicate the electrophoretic mobility of molecular mass markers in kilodaltons.

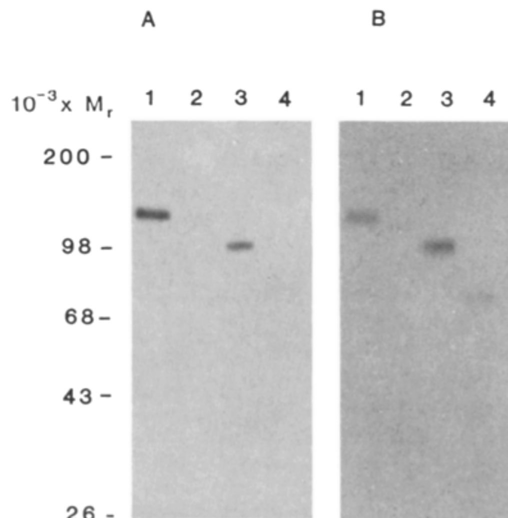


Figure 7. Binding of $GIMP_c$ and $GIMP_t$ to the sialic acid specific lectin Limulus polyphemus. ^{125}I-labeled $GIMP_c$ and $GIMP_t$ run and cut from SDS PAGE were treated without (*A* and *B*, lanes *1* and *3*) or with neuraminidase (*A*, lanes 2 and *4*), or Endo F (*B*, lanes 2 and *4*), incubated with Limulus polyphemus-Sepharose (see Materials and Methods), eluted specifically from the lectin with sialic acid and analyzed by SDS PAGE. Untreated $GIMP_c$ and $GIMP_t$ were bound 53–97% and 33–51% to the lectin in two separate experiments. Note the decrease in the binding of $GIMP_c$ and $GIMP_t$ to the lectin after the treatment with neuraminidase or Endo F.

from segregation of distinct protein domains within a continuous membrane. Localization of the proteins in separated registered compartments would tend to agree more with compartmentalization based on independent membrane-bound parts. This would also suggest that the distribution and registered position of the different parts of the Golgi system and elements derived from them result from their interaction with the same class of cellular elements. With respect to this, it is important to note that the Golgi system and derived elements are always found in areas rich in microtubules surrounding the centers that organize these cytoskeletal elements. Also relevant are the changes in the localization and organization of the Golgi system following the altered polymerization of microtubules in vivo (53, 65), as well as the association of its fragments with taxol-induced microtubule bundles (66). All this data point to a role of microtubules in the localization and organization of the Golgi system and probably in keeping the different parts of the organelle in register.

Although knowing the function of $GIMP_c$ and $GIMP_t$ would have been of great interest, characterization of some of the properties of the two proteins has revealed important aspects that could be relevant in the organization and function of the Golgi system. For example, the properties of the carbohydrates acquired by $GIMP_c$ and $GIMP_t$ tell us some interesting functional details of the organelle. The presence of sialic acid in the N-linked complex carbohydrates acquired by $GIMP_c$, the exclusion of the protein from the *trans*-most cisternae and *trans*-tubular network, and the localization of the Galβ1→4GlcNAcα2→6 sialyltransferase, believed to be exclusively responsible for eliciting the sequence NeuAcα2→6Galβ1→4GlcNAc in N-linked oligosaccharides of glycoproteins (67) in the *trans*-Golgi elements (49), suggests that this enzyme is not the only sialyltransferase that incorporates sialic acid into N-linked carbohydrates in the Golgi system. There is evidence indicating the existence of different sialyltransferases (5, 6, 49, 64, 67), including one transferring sialic acid to O-linked carbohydrates (6) in the cell. Probably one or some of them have access to proteins that, like $GIMP_c$, are confined in the *cis* or medial cisternae. Exploration of this possibility is of great importance because acquisition of sialic acid by N-complex carbohydrates has been used as a marker of proteins reaching or passing through the *trans*-side of the Golgi system. Furthermore, the model of vectorial-vesicle-mediated transport of proteins through the Golgi system is based on the assumption that proteins acquired sialic acid in N-linked carbohydrates exclusively in the *trans*-Golgi. However, the data does not rule out other alternatives: for example, leaks in the vesicular intercompartmental transport coupled to a counter-flow mechanism that would correct those leaks, or a continuity between Golgi compartments allowing proteins located in different ones to drift slowly through the common membrane and interact. With respect to this it should be noted that the immunocytochemistry data only tells where the bulk of the protein is located and that minor quantities could pass undetected.

Among the properties of $GIMP_c$ studied here, of particular relevance is its phosphorylation in vivo. Our studies on the topology of the phosphorylation site of $GIMP_c$, suggest that it is located in the lumenal domain of the protein. However, the results of these studies do not rule out its location

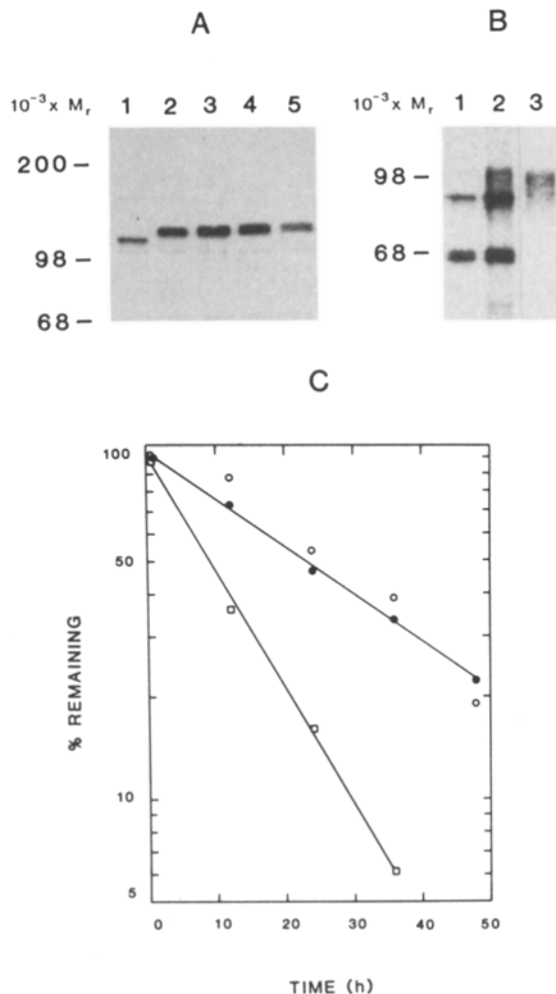


Figure 8. Precursors, intermediate forms, and half-lives of GIMPs. *A* and *B* describe the precursor, intermediate, and mature forms of $GIMP_c$ and $GIMP_t$, respectively. $GIMP_c$ was pulse labeled for 15 min and immunoprecipitated immediately (lane *1*), or after 1 (lane *2*), 2 (lane *3*), 4 (lane *4*) or 8 h (lane *5*) of chase. $GIMP_t$ was pulse labeled for 15 min and immunoprecipitated immediately (lane *1*) or labeled for 2 h and chased for 30 min (lane *2*) or 2 h (lane *3*). *C* records the half-lives of $GIMP_c$ labeled for 2 h with [^{35}S]methionine (●) or 2[^{3}H]-D-mannose (○), and $GIMP_t$ labeled for 2 h with [2-^{3}H]-D-mannose (□). Chase of the labeling was performed for the times indicated in the abscissa. In this experiment, the two proteins labeled with [^{35}S]methionine were immunoprecipitated from the same cell extracts. Points correspond to the average of two parallel immunoprecipitations (see Materials and Methods).

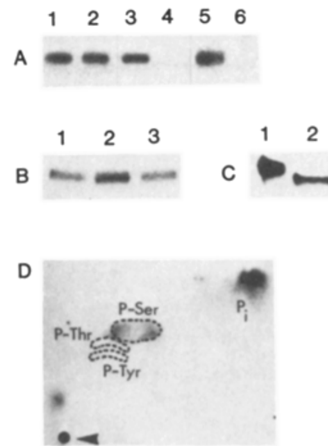


Figure 9. Phosphorylation of $GIMP_c$ in vivo. NRK cells were incubated for 1 h with 15 ml phosphate-free DME medium, 2% dialyzed FCS, and then for 2.5 h in 15 ml of the same medium with 2.5 mCi ^{32}P-orthophosphate in the absence or presence of dibutyryl cAMP (0.2 mM), or for the last 30 min of incubation with the Ca^{++} ionophore A23187 (1 µg/ml). (*A*) Untreated cells were lysed by N_2 cavitation, (350 psi) (3) to preserve the integrity of Golgi elements, and the extracts incubated for 30 min at 37°C without (lanes *1* and *2*) or with (lanes *3* and *4*) proteinase K (100 µg/ml), or alkaline phosphatase (100 U/ml) (lanes *5* and *6*), in the absence (*odd lanes*) or presence (*even lanes*) of 0.2% Triton X-100, before immunoprecipitation of $GIMP_c$ with antibody 15C8. Immunoprecipitations performed with antibody 5G6 produced the same results. Immunoprecipitation of $GIMP_t$ with antibody 18B11 revealed no phosphorylation of the protein. Note that the phosphate incorporated into $GIMP_c$ was only protected in the intact Golgi elements from the action of the enzyme. (*B*) Stimulation of $GIMP_c$ phosphorylation by cAMP. ^{32}P-$GIMP_c$ immunoprecipitated from untreated cells (lane *1*) and cells incubated with dibutyryl cAMP (lane *2*) or the calcium ionophore A23187 (lane *3*). Note the stimulation of phosphorylation by the cAMP analog. (*C*) ^{32}P-$GIMP_c$ immunoprecipitated from untreated cells incubated without (lane *1*) and with a mixture of Endo F, neuraminidase, and O-glycanase (lane *2*) (see Materials and Methods). Note that treatment with the glycosidases did not remove the phosphate incorporated into $GIMP_c$. The slight decrease in the ^{32}P incorporated to $GIMP_c$ after incubation of the protein with the glycosidases cannot be ascribed to specific removal of the phosphate by the enzymes, as a similar decrease in ^{35}S incorporated into $GIMP_c$ was also observed after incubation of ^{35}S-$GIMP_c$ with the same enzymes. (*D*) Phosphoaminoacid analysis of $GIMP_c$ phosphorylated in vivo. Areas labeled as P-Ser, P-Thr, and P-Tyr indicate the localization of phosphoaminoacid markers in the chromatogram. P_i corresponds to orthophosphate. Note the exclusive incorporation of phosphate into serine.

in a cytoplasmic domain resistant to proteinase K and alkaline phosphatase. Since phosphorylation of $GIMP_c$ is stimulated by cAMP, localization of the phosphorylation site in the lumenal domain would indicate that cAMP could be a messenger for intracellular signals in addition to extracellular ones (47). With respect to this, it is conceivable that an adenylate cyclase located in the membranes of the Golgi system (15) could respond to signals of cytoplasmic origin differently from those regulating the plasma membrane adenylate cyclase. Therefore cAMP levels in the Golgi system and cytoplasm could be independently regulated. The location of large amounts of cAMP type II kinase on the cytoplasmic surface of the Golgi cisternae (18, 37) suggests that, in addition, membrane Golgi proteins can be phosphorylated in their cytoplasmic domains. cAMP-independent kinases are also found in the Golgi system (14), thus raising the possibility that signals different from cAMP may also induce the phosphorylation of Golgi proteins.

The marked difference in the half-lives of $GIMP_c$ and $GIMP_t$ indicates a different turnover of these two Golgi components. Whether this difference reflects the independent turnover of the *cis*-medial and *trans* compartments housing $GIMP_c$ and $GIMP_t$ will have to wait until the determination of the half-lives of other proteins with the same distributions is made.

Finally, the three anti-GIMP antibodies developed here should be useful markers to study the traffic of proteins through the Golgi system (for a discussion on this topic see 25), and the assembly and disassembly of this organelle occurring in the transition between cellular interphase and mitosis. We have recently prepared sizeable amounts of purified $GIMP_c$ and $GIMP_t$, and developed polyclonal antibodies

against these proteins. These antibodies are intended to be used to purify Golgi elements, with the aim of characterizing the proteins contained in their lumen and membranes and studying the regulation of the phosphorylation of $GIMP_c$. Also, the antibodies and oligonucleotides corresponding to sequence stretches of the proteins will be used to clone the genes coding for the two GIMPs and study their expression and sorting.

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